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PARTICULARLY DANGEROUS INFECTIOUS DISEASES AND INFECTIOUS DISEASES WITH NATURAL FOCALIZATION

/Following is a complete translation of a Russian-language book by N. I. Nikolayev et al entitled Osobo Opasnyye i Prirodnoochagovyye Infektsii (English version above), Moscow, Medgiz, 1962, pp 1-272./

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Foreword

The present collection of articles on particularly dangerous infectious diseases and infectious diseases with natural focalization is designed for scientific workers and practical specialists of the epidemic-control service.

The collection is being published in accordance with the decision of the Ministry of Health USSR by way of an exchange of experience and exchange of scientific achievements. In it the results of research work of plague-control institutions and the departments of particularly dangerous infectious diseases of the republic, kray and oblast sanitary-epidemiological stations are being published.

The trends and contents of the articles being published are determined by problems of epidemiology, epizootology, diagnosis, therapy, microbiology and immunology, pathogenesis, specific and general sanitary prophylaxis of plague, cholera, tularemia, brucellosis, anthrax and problems of improving production of bacterials for these infectious diseases.

In this collection light is also being thrown on the results of the study of other particularly dangerous infectious diseases and infectious diseases with natural focalization and some problems of medical zoology and parasitology.

The publication of the results of research and the latest achievements on these problems will assist epidemic-control institutions of the Soviet Union in their work of solving problems marked out by the decree of the CC CPSU and Council of Ministers USSR dated 14 January 1960, "Concerning Measures for the Further Improvement of Medical Care and Safeguarding the Health of the Population of the USSR."

EPIDEMIOLOGY AND EPIZOOTOLOGY

Plague Epizootics among House Mice in the Lower Reaches of the Ural River in 1958-1959

B. K. Fenyuk, B. Ye. Osolinker (deceased), A. A. Lalazarov, N. L. Gershkovich, L. Yu. Ziskind, M. V. Zubova, R. V. Kovaleva, K. Ya. Lavasidis, A. V. Laskina, Ye. G. Mishchenko, E. V. Molodovskaya, A. V. Popov, G. B. Postnikov, Ye. V. Prokhorova, A. G. Radchenko, A. Ye. Starikov, T. I. Tabunina

(Gur'yev, Saratov, Moscow)

On 10 May 1958, on the left bank of the Ural River, 30 kilometers to the southwest of the settlement of Iskine and 30-35 kilometers to the southeast of Gur'yev 25 house mice (Mus musculus) were caught, from one of which on 12 May a culture of the plague microbe was isolated.

An extensive epizootological investigation made after this showed that on the banks of the Ural River, in its floodplain and delta region there was a marked increase in the census of house mice and that an active plague epizootic was occurring among them over a large territory on the left bank. In the second half of September plague-infected house mice and meadow voles (Microtus arvalis) were also found on the right bank near the Ural River.

The epizootic among mice lasted until May 1959. The last cultures of the plague microbe were isolated from a house mouse caught on 10 April and from fleas caught in the nest of the house mouse on 15 April. However, in the spring of 1959, plague appeared on the right bank in colonies of dwarf susliks (Citellus pygmaeus) in the form of a brief (May-June) local epizootic.

The 1958-1959 epizootic was distinguished by a number of interesting characteristics, in connection with which it deserves detailed description, particularly since relatively few papers have been written on the plague problem in small mouse-like rodents in the literature.

Material and Method

All information about the 1958-1959 epizootic was collected, by and large, during the course of practical activity of the Gur'yev Plague-Control Station, its sections and epidemic-control detachments.

Epizootological investigations of the region of the increased mouse census at different times were made by the following organizations.

1. The central laboratory of the Gur'yev Plague-Control Station (all year round). 2. The Yamanka section of the Gur'yev Station (all year round). 3. The Iskine epidemic-control

detachment of the Central Observation Plague-Control Station (April 1958-June 1959). 4. The Orlik epidemic-control detachment of the Yamanka section (October-December 1958 and April-June 1959). 5. The epidemic-control detachment of the Yamanka section in the settlement of Kurlys (April-June 1959). 6. The railroad car laboratory of the Kazalinsk Railroad Plague-Control Station (April-December 1958 and spring 1959).

The places from which the field material was taken for bacteriological examination were divided territorially as equally as possible. However, the degree to which various sections were investigated was different. Mice and voles were caught with "Hero" traps, using the trap-night method which made it possible to count the animals obtained for the bacteriological laboratories with consideration of their census. For a single test a line of 50 traps set at 5-meter intervals was counted. Such lines were arranged 1-3 kilometers or more from one another. However, on four of the sections with areas of 25 or 50 square kilometers ("Kirpichnyy Zavod," "Bol'shoy Mokryy," "Gogol'skiy," and "Bezymyannyy"), which were investigated more regularly and completely, the spaces between the lines of traps amounted to only 0.5 kilometer. Other rodents (susliks, sand rats and jirds) were caught with leg traps.

The ectoparasites of the mice were obtained by combing them out of the animals caught in the laboratory and by means of digging up the holes for the purpose of taking the nests out, and the ectoparasites of susliks, sand rats and jirds were obtained, in addition to combing, by the "strip" method from the openings of the holes.

Liver and spleen (and sometimes lung) tissue as well as blood were plated out on Hottinger's agar (dry medium) from each animal being investigated. Biological tests were performed on white mice using a group of no more than 15 animals of the same species caught in the same place. Individual biological tests were performed on animals found dead. Depending on the size of the collection from one spot and from rodents of the same species ectoparasites were investigated by means of group or individual cultures on agar. The group included different numbers of fleas, but, as a rule, no more than 50. The cultures isolated were identified in accordance with instructions. The virulence of the plague microbe was determined, by and large, on freshly isolated strains.

From May 1958 to June 1959, 51,190 animals (of these 31,804 were house mice and 2,120 were meadow voles) and 158,134 ectoparasites (including 16,349 fleas of mouse-like rodents) were investigated bacteriologically in the region of the epizootic and adjacent areas.

Conditions under Which the Epizootics Occurred

The 1958-1959 epizootic among house mice occurred in

connection with a marked increase in their census over a large territory of the floodplain and upper part of the delta of the Ural River, which had not been observed in the past 20 years.

The census of house mice in deserts around the lower reaches of the Ural River is very low. Usually their numbers are also small in the floodplain and delta of the Ural River, although these habitats are characterized by distinct features of azonaltiy. At the same time, in different varieties of habitats of the floodplain and delta house mice are always encountered. Among these varieties are: extensive growths of reeds and shore pigweed (Atriplex littoralis) on areas of the sea bottom exposed by regression of the Caspian Sea, places with beggar weed along the dry or, at times, water-filled eriks /shallow channels in the river delta/, the ruins of old buildings, gardens, plantations and similar places. However, even here the population of house mice is not very constant, because they die during the floods of the Ural River, and along the coast of the Caspian Sea they die from the driving winds coming from the sea.

A marked increase in the census of house mice in 1958 was brought about by a number of factors and primarily by the unusually high spring flood of the Ural River in 1957, which exceeded the average level for the previous seven years by more than two times. The high water in the spring went far beyond its usual limits, flooding a tremendous territory in the lower reaches of the river, from Novobogatinsk in the west to Rakusha in the east, and penetrated deeply into the deserts of a number of places. The higher up along the course of the river, the narrower the flood zone became, and at the level of the village of Kulagino practically none of the floods went beyond the floodplain proper (Fig 1).

In 1957, the flood caused the death of rodents en masse. A few mice were preserved only on the unflooded grivy /low elongated elevations from erosion and deposition in river valleys/, islands and other elevations, on trees, and in various buildings, or were displaced to the desert.

Apparently, there was active reproduction of the mice after the flood (no observations were made), because over the entire flooded area after the water receded an abundant plant cover developed with a large quantity of fruit, which furnished the mice with rich food supplies and good shelter from predatory birds. The dry autumn of 1957 and the very mild winter of 1957/58, with a large quantity of food stored until the spring, contributed to good survival of the mice under autumn-winter conditions, and the low flood level in 1958 killed only a very few rodents. Finally, the abundance of precipitation in the spring and beginning of the summer in 1958 were responsible for new good vegetation and fruit-bearing of many plants, as a result of which the food supply for the mice was renewed.

In the final analysis, in May-June 1958 the house mice census reached a high level over a large territory. In calculating it by the method of trap-nights not uncommonly from 25-30 to 60 percent of the traps had mice in them. Naturally, as a result of the mosaic distribution of vegetation groupings, caused by the mesozone topography of the floodplain and the degree of irrigation during the flood, there was also a mosaic distribution of mouse colonies of different densities territorially.

The mouse census was the lowest (0-4 percent trap catch) on the elevations which had not been flooded in 1957 and which preserved their desert appearance in 1958.

Along with the increase in the mouse census in 1958 there was a considerable increase in the number of mouse fleas. The average monthly abundance index of fleas in the hair of house mice in the delta near Gur'yev amounted to 0.64 in May 1958 and 2.0 in September, whereas in previous years it did not exceed 0.3 and frequently it was much less.

The problem of how plague penetrated into the mouse population of the floodplain can be solved only in retrospect. However, the existing indirect data are very convincing.

In the Ural-Emba Desert, which is adjacent to the flood zone on the east, in 1957 and 1958 a plague epizootic occurred among colonies of great sand rats, Rhombomys opimus (see Fig 1). Since the areas with a high mouse census came right up to the desert, it was easy for the mice to establish contact with the great sand rats, particularly on the elevated desert areas which remained like islands in the actual flood zone. On such "islands," sometimes quite large, colonies of the great sand rat, meridional jird (Meriones meridianus) and crested jird (M. tamariscinus) remained, contact between which and the mice increased with the increase of the census of the latter.

In this connection, it should be mentioned that a plague microbe culture was isolated from a great sand rat caught on 20 June 1958 in the flood zone on a griv /see previous note on this/ left unflooded in 1957 at the Sokol Channel near the Gogol'skiy natural landmark (see Fig 1). True, in this case two suppositions are equally probable: either the sand rat was infected from mice in the spring or summer of 1958 or, conversely, in the colonies of sand rats on this griv the plague pathogen had appeared even before the 1957 flood. However, in both cases, this fact speaks for the existence of contact between mice and sand rats. The findings of mouse fleas (Ceratophyllus mokrzeckyi, Leptopsylla taschenbergi) on great sand rats in places where they have colonies together with mice and of great sand rat fleas (Xenopsylla skriabini, X. conformis, Ceratophyllus laeviceps, Rhadinopsylla cedestis) on mice is evidence of close contact between the animals.

The problem of the time that plague penetrated into the mouse population is interesting. Suppositionally, we can mention

three periods: the time of the crest of the flood in the spring of 1957, the autumn-winter season of 1957/58 and the spring of 1958.

It may be assumed that at the first of these times contact between mice and sand rats was very close, since mice which had been displaced by the flood into the desert and onto the islands and which became homeless temporarily formed their colonies directly in the great sand rat holes. However, the plague pathogen could hardly establish itself in the mouse population at that time, because after the water receded their colonies were very much thinned out and, naturally, with a very small number of fleas.

The second and third periods are more probable. The censuses of mice and their fleas at this time increased appreciably, and the more extensive migrations of the mice late in the autumn and early in the spring could have provided the necessary degree of contact with sand rats for the infection. The absence of data in the literature concerning the rate of spread of epizootics in mouse colonies makes difficult the more exact solution of the problem which we have posed.

Development of the Epizootic

The generalized statistical material characterizing the development of the epizootic after it was detected is shown in Table 1 and on Fig 2. It includes data not on all the animals investigated but only on those which were caught on the left bank and thereby only within limits of the southern, largest area of distribution of the epizootic, which we called the "main region of the epizootic." Almost the entire 1957 flood zone was investigated, although to different degrees, but with a negative result over its major portion.

The indices showing infection of mice presented in Table 1 and Fig 2 do not completely depict the natural dynamics of the epizootic for the following reasons.

First of all, as one of the preventive measures against possible infection of people, following the detection of the epizootic, extensive mouse extermination was undertaken with poisoned grain bait (rye containing 8 percent zinc phosphide). From 4 June to 24 December 1958 an area of 418,900 hectares was treated on both sides of the Ural River in the region of the increased mouse census using airplanes. Places with the highest mouse censuses, where quite a large number of rodents remained after the baits had been spread out once as well as the greater portion of the "main region of the epizootic" were treated two-three times during this period. In all, with consideration of the repetitions, 608,900 hectares were treated. The effectiveness of control amounted, on the average, to about 82 percent

Table 1

Degree to Which House Mice and Other Species of Animals were Infected with Plague on the Left Bank in the "Main Region of the Epizootic (1958-1959)

(1) Месяц	(2) Исследовано зверьков и выделено культур чумного микроба (в знаменателе)					(3) Процент зараженности		
	домовые мыши (4)	большие песчанки (5)	„мелкие" песчанки (6)	прочие виды (7)	всего (8)	общий (все виды) (9)	(4) домовых мышей прямой (10)	выравненный (11)
1958 г.								
(12) Апрель	162/0	33/0	13/0	157/0	365/0	0	0	0
(13) Май	639/4	26/1	55/0	38/0	758/5	0,66	0,62	0,27
(14) Июнь	2589/5	163/2	37/0	34/0	2823/7	0,25	0,19	0,71
(15) Июль	1948/26	28/0	2/0	7/0	1985/28	1,41	1,44	0,91
(16) Август	1309/20	41/0	12/0	7/0	1369/20	1,46	1,53	1,36
(17) Сентябрь	710/5	26/0	10/0	7/0	753/5	0,66	0,70	1,18
(18) Октябрь	616/6	10/0	16/0	4/0	656/6	0,92	0,97	1,09
(19) Ноябрь	777/13	3/0	13*/1	19**/1	812/15	1,85	1,67	1,42
(20) Декабрь	1078/16	14/0	2/0	24/0	1118/16	1,43	1,48	1,55
1959 г.								
(21) Январь	528/8	—	2/0	101/0	631/8	1,25	1,51	1,42
(22) Февраль	151/1	—	—	8/0	159/1	0,64	0,66	1,25
(23) Март	37/0	—	—	3/0	40/0	(0)	(0)	0,75
(12) Апрель	78/1	13/0	3/0	44/0	122/1	(0,82)	(1,28)	0,68
(13) Май	31/0	68/0	21/0	184/0	304/0	(0)	(0)	0,43
(14) Июнь	125/0	27/0	58/0	231/0	641/0	0	(0)	0

*The culture was isolated from a crested jird caught in a great sand rat colony in the vicinity of the village of Kamynin.

**The culture was isolated from 11 shrews (Crocidura sp.), caught in the Gogol'skiy Natural Landmark. /Table 1, continued next page/

/Table 1, continued from previous page/

1. month; 2. animals investigated and cultures of plague microbe isolated (in the denominator); 3. percentage of infection; 4. house mice; 5. great sand rats; 6. "small" sand rats /jirds/; 7. other species; 8. total; 9. total of all species; 10. by direct calculation; 11. adjusted; 12. April; 13. May; 14. June; 15. July; 16. August; 17. September; 18. October; 19. November; 20. December; 21. January; 22. February; 23. March.

Fig 2. Change in the percentage infection of mice in the "main region of the epizootic."

1. degree of infection, %.

and varied in different 5,000-10,000-hectare "squares" from 79 to 98 percent. Only in two squares did it amount to just 48.5 and 57.2 percent. Simultaneously, in the region of increased census of the rodents deratization and disinfestation (of fleas) were carried out three times in all inhabited places, and all of the numerous haystacks were treated once by means of scattering poisoned bait over a strip of 1.5-2 meters around each stack. In the final analysis, the mouse census was reduced both in the field (2-4 percent trap catch in October as against 20-30 percent in May-June) and in the inhabited places (1-5 percent as against 10-20 percent). Such a thinning-out of the population density of the mice must have exerted some effect on the natural course of the epizootic. Secondly, a certain influence on the course of the epizootic could have been exerted by the gathering of hay which took place in June-July and which caused a partial remigration of mice from the exposed meadows to the haystacks and places with untouched vegetation.

It is entirely probable that increase in the rate of infection of mice in the summer was caused specifically by the migrations which were brought about by the hay harvesting and that the reduction from August to September was caused by the remote effect of control measures.

The mouse control measures, particularly those accomplished once, could not bring about a rapid extinction of the epizootic, since it was not accompanied by disinfestation of the rodent holes in the field. If we consider that an average of only about 82 percent rodents died as the result of the utilization of poisoned bait and almost all fleas living in the holes were preserved and that the mobility of animals in connection with the hay harvesting was increased, the increase in the rate of infection under these conditions must be considered expected. However, at the beginning of autumn, as a result of repeated mouse extermination, their census began to decrease; and the epizootic temporarily went into a decline.

Late autumn and early spring rises in the rate of infection of mice (November-January) can be explained by partial recovery of the mouse census as the result of their reproduction (from May to September 1958 the average monthly percentage of gravid female house mice ranged from 19 to 34; in October it was equal to 5; in November, 3.5) and autumn migrations caused by a cold spell.

Beginning with January, with the subsequent steady reduction in the rodent census the epizootic steadily went into a decline, chiefly, now, under the influence of natural factors (the relatively severe winter of 1958/59, the presence of predators, etc.)

The conclusion which we drew that extermination of the rodents alone (without disinfestation of their holes) failed to lead to a rapid cessation of the epizootic is well illustrated

by the results of the investigation of a single epizootic area which we called "Kirpichnyy Zavod" (Fig 3). This section, 25 square kilometers in area, was investigated most regularly. In June 1958, plague was first found on it. In July, 27 cultures of the plague microbe were isolated from mice and their fleas caught in this area. On 16-18 July in this section poison bait was scattered; however, in August 21 cultures were again obtained from this section, in connection with which on 22-23 August the section was treated again, which led to a sharp reduction in the mouse census. In all, on the 226 trap lines (4,300 trap-nights) in the next two months only 78 mice were caught, among which none was found to be plague-infected. No plague microbe cultures were isolated in September-October either from the examination of 244 fleas (including 63 fleas from 21 nests). However, the infected fleas were apparently preserved, because after the recovery of the rodent census which began by November plague was activated among them again. In November (from 17 to 30 November) on this section a plague-infected mouse was found, and four cultures were isolated from fleas. On 24-27 November poison bait was scattered about the section the third time, and on 12 December here the last plague-infected mouse was caught. Subsequently, despite regular investigation of this area, it has been impossible to find plague on it.

Additional data on the course of the epizootic can be obtained from Table 2, in which the results of investigation of mouse fleas in the main region are given.

Two facts should be noted which follow from the Table: a) the high percentage of positive plague samples in bacteriological examination of mouse fleas speaks for their great part in the development of the epizootic and b) with increase in the number of fleas in the sample when groups of them are investigated for plague (bottom line on the right) the frequency with which positive results are obtained increases.

Aside from fleas caught on the bodies and in the nests of house mice, a bacteriological investigation was made of fleas from the holes and hair of other rodents as well as ixodial and gamasid ticks and, in very small numbers, lice of the rodents. In all, from May 1958 to June 1959, 1,260 samples of all these ectoparasites were examined from the main region of the epizootic by means of group cultures, and 14 cultures of the plague microbe were isolated. Among them were: three cultures isolated in June 1958 from fleas taken from great sand rats; one in May 1958 from fleas taken from a meridional jird; one in October 1958 from fleas combed from a water vole (Arvicola terrestris); two in November 1958 from fleas taken from shrews (Crocidura sp.); one in June 1958 from ixodial ticks (nymphs of Hyalomma); three in September, October and November 1958 from gamasid ticks taken in all four cases from house mice; and finally, two cultures in June

Table 2

Plague Infection of Fleas from Hair of House Mice and From Their Nests in the Main Region of the Epizootic (1958-1959)

Год и месяц	Исследовано проб с разным числом блох из шерсти и выделено культур (в знаменателе): число блох в пробе 1	2-10	11-30	31-50	всего проб	Процент зараженности: число блох в пробе 1	2-10	11-30	31-50	в среднем	Исследовано проб из гнезда и выделено культур (в знаменателе)
1958 г.											
Май	6/0	28/1	5/2	—	39/3	(0)	(3,6)	40,0	—	(7,7)	—
Июнь	37/0	74/0	40/0	13/4	164/4	(0)	(0)	(0)	(30,8)	2,4	—
Июль	34/0	89/5	39/5	22/7	184/17	(0)	(5,6)	(12,9)	(31,7)	9,2	1/0
Август	355/2	182/5	10/0	3/0	550/7	0,6	2,7	(0)	(0)	1,3	48/0
Сентябрь	88/2	108/1	31/0	1/0	228/3	2,3	0,9	(0)	(0)	1,3	15/0
Октябрь	165/1	83/10	21/3	7/0	276/14	0,6	(12,0)	(14,3)	(0)	5,1	16/1
Ноябрь	156/2	55/6	9/2	3/1	223/11	1,3	(10,9)	(22,2)	(33,3)	4,9	66/3
Декабрь	237/8	49/14	2/2	1/0	289/24	3,4	(28,6)	(100)	(0)	8,9	47/0
1959 г.											
Январь	59/6	5/1	1/1	—	65/8	(10,2)	(20,0)	(100)	—	(12,3)	4/1
Февраль	12/1	3/0	—	—	15/1	(8,3)	(0)	—	—	(6,6)	—
Апрель	23/0	17/2	1/1	—	41/3	(0)	(11,8)	(100)	—	(7,3)	1/1
Итого и в среднем	1172/22	693/45	159/15	50/12	2074/96	1,9	6,5	10,1	34,0	4,6	198/6

1. year and month; 2. samples with different numbers of fleas from hair investigated and cultures isolated (in the denominator); 3. number of fleas in the sample; 4. total samples; 5. rate of infection; 6. average; 7. samples from nests investigated and cultures isolated (in the denominator); 8. May; 9. June; 10. July; 11. Aug.; 12. Sept.; 13. Oct.; 14. Nov.; 15. Dec.; 16. Jan.; 17. Feb.; 18. April; 19. total and average.

of 1959 from fleas caught in the nests of the yellow suslik (Citellus fulvus) and one in the same month from lice taken from the same rodent.

Therefore, the findings of infected ectoparasites on animals of different species, the high percentage of positive samples in the investigations of mouse fleas, the involvement of other mammals in the epizootic--crested jirds, shrews and, in the Chkalovo focus, also meadow voles--attest to the fact that in 1958 the plague epizootic was active among house mice. At the same time, the relatively low rate of infection of animals by months (0.25-1.85) would appear to contradict this conclusion.

This may be explained by the fact that in summarizing the material by months in Table 1, we artificially reduced somewhat the infection rate, because in the number of mice investigated those animals are included which were caught in the main region of the epizootic but in sections of it in which plague could not be detected even once. Therefore, in Table 3, we have presented a random sampling of the results of investigation of mice by day in July and August 1958, when the epizootic was most active prior to the autumn recession. The random sampling was made up from materials collected on the epizootic area "Kirpichnyy Zavod" described above. On the same day only a part of this section was covered, and the mice caught each day may be regarded as a relatively objective sample of the population.

From Table 3 it is seen that the infection of house mice in various population samples not uncommonly is considerably higher than the average for the month for the entire main region and reaches 6-8 percent. Therefore, the conclusion that the epizootic was quite intense among mice in 1958-1959 can be considered correct.

In accordance with the mosaic distribution of mouse colonies the areas included in the epizootic were, to a certain degree, distributed mosaically also. In addition, the epizootic moved, which is seen from a diagram of the investigation of epizootic section "Tyndyk" with an area of 50 square kilometers (Fig 4).

In the investigation of this section from June to November not a single plague microbe culture was found. Since the section was in a region with a high mouse census they were exterminated on it three times (20-22 July, 25-26 August and 23 September 1958). Therefore, to a certain degree the plague epizootic detected on it in December 1958 during the investigation of this section was unexpected; five cultures were isolated from mice and 13 from their fleas. In this section plague was found among the mice until April 1959. Here in June 1959 plague-infected ectoparasites of the yellow suslik were collected, about which we have written above. There is reason to believe that the epizootic on the "Tyndyk" section occurred later than in the adjacent areas.

Table 3

Infection Rate of House Mice in Various Population Samples ("Kirpichnyy Zavod" Section in the Main Region of the Epizoctic, 1958)

Дата (1)	Число исследованных мышей (2)	Выделено культур (3)	Процент зараженности (4)	Исследовано кровопаразитов (5)	Выделено культур (6)
1 июля (7)	12	1	8,3	21	0
3 "	46	3	6,5	53	0
7 "	33	1	3,0	188	0
8 "	82	5	6,1	334	5
9 "	47	0	0	201	1
10 "	98	2	2,0	162	4
17 "	89	5	5,6	46	0
4 августа (8)	52	2	3,8	78	2
5 "	57	1	1,3	166	1
7 "	94	1	1,0	151	2
12 "	45	1	2,2	65	0
14 "	17	1	5,9	36	0
18 "	37	3	8,1	97	0
19 "	94	4	4,2	149	1
28 "	47	1	2,1	67	1

Note. The days on which not a single plague microbe culture was isolated are not included in the Table.
1. date; 2. number of mice investigated; 3. cultures isolated; 4. infection rate; 5. ecotoparasites investigated; 6. cultures isolated; 7. July; 8. August.

Spread of the Epizootic outside the Limits of the Area where it Began

Arising at the junction between the desert and the Ural River Valley, the epizootic among mice spread to the west, into the depths of the floodplain, apparently by means of a relay transmission of the plague pathogen from hole to hole. It is entirely probable that this process was accelerated by the migrations of the rodents themselves. A certain part could also have been played by predators, carrying infected fleas. By this method the epizootic reached the boundaries of its area of distribution on the left bank, going specifically to the Ural River. Of the greatest interest are cases in which small foci of the epizootic were detected far outside the main area of distribution, including the right bank of the river.

There is reason to consider the Ural River an obstacle on the route of advance of plague, preventing the everyday crossing of rodents and therefore plague pathogens from one shore to the

other (on historical or particularly geological time scales such a crossing could have been made repeatedly). This permitted us to regard the Volga-Ural and Transural natural plague foci, including the Ural-Emba section, relatively independent (B. K. Fenyuk, 1951, 1956).

Actually, to this point the epizootic of plague had occurred only in the colonies of its main reservoirs (the dwarf suslik, great sand rat and meridional jird) with the involvement of relatively few secondary reservoirs (jerboas, red-tailed /Meriones erythrourus/ and crested jirds, etc.); not a single case of crossing of the epizootic over the Ural River was noted. Such a crossing was prevented not only by the river itself but also by the broad floodplain in many places, in which colonies of the main plague reservoirs were encountered only sporadically and into which apparently plague did not penetrate.

The occurrence of an epizootic in the floodplain itself in 1958 among colonies of house mice multiplying in large numbers brought plague right up to the Ural River, as a result of which an absolutely unusual situation was created. Under these conditions, it was much easier for the plague pathogen to cross from the left bank of the river to the right and to take root in the right bank area, where the mouse census was also markedly increased.

Plague-infected mice or their fleas could have been carried over the Ural River by large birds (short-eared owls, marsh hawks, sea-gulls, and kites). The possibility that the mice were carried over the river on boats with freight on a ferry at the village of Yamanka or on vessels travelling up and down the river is entirely probable. On the ferry, specifically, motor vehicles loaded with hay were transported from the left to the right bank of the Ural River.

The first local focus of the imported epizootic was found on the right bank of the Ural River at the village of Chkalovo (see Fig 1). The extensive environs of the village began to be investigated in May 1958; however, until September plague was not found here (Table 4). The epizootic detected in September was acute but over a very limited territory. Possibly, the extermination of mice, conducted on this area of the floodplain on 14-16 October, prevented its greater territorial spread. The epizootic was maintained at its starting place until December 1958.

The second such focus was detected in October 1958 in the region of Zelenyy Settlement (see Fig 1). On 12 October here a plague-infected house mouse was caught, and at the end of October a local epizootic was found in the fourth, fifth (the village of Kurlys) and sixth Caucasian villages, located opposite Zelenyy on the left bank, approximately 100 kilometers to the north of the northern boundary of the main region of the epizootic.

Table 4

Results of Investigation of Focus of Epizootic near Settlement of Chkalovo (Right Bank of Ural River) in 1958-1959

Месяц (1)	Исследовано (2): домовых мышей (5)	обыкновенных полевок (6)	проб блох (7)	проб гамазовых клещей (8)	Выделено культур из (3): мышей (5)	полевок (6)	блох (7)	гамазовых клещей (8)	Процент зараженности (4): домовых мышей (5)	обыкновенных полевок (6)	Процент положительных проб блох (9)
1958 г.											
Май (10)	183	—	—	—	0	—	—	—	0		—
Июнь (11)	43	—	—	—	0	—	—	—	0	—	—
Июль (12)	264	2	—	—	0	0	—	—	0	0	—
Сентябрь (13)	534	18	114	10	0	1	1	0	0	(5,6)	0,9
Октябрь (14)	832	44	132	2	4	0	2	0	0,5	0	1,5
Ноябрь (15)	173	36	102	13	1	0	17	1	0,6	0	16,7
Декабрь (16)	133	26	6	—	0	1	1	—	0	(3,8)	(16,7)
1959 г.											
Январь (17)	111	42	—	—	0	0	—	—	0	0	—
Февраль — июнь (18)	81	258	228	29	0	0	0	0	0	0	0

Note. Mainly fleas and gamasid ticks taken from house mice and meadow voles were investigated; in addition, from February to June 1959, an investigation was made of fleas taken from the meridional and crested jirds and from the dwarf suslik.

Key: 1. month; 2. investigated; 3. cultures isolated from; 4. infection rate; 5. house mice; 6. meadow voles; 7. flea specimens; 8. specimens of gamasid ticks; 9. percentage of flea specimens positive; 10. May; 11. June; 12. July; 13. September; 14. October; 15. November; 16. December; 17. January; 18. February-June.

During October-December on the territories of these Caucasian villages 634 rodents and 460 ectoparasites were caught and investigated; from these, 19 cultures of the plague microbe were isolated (seven, from house mice; four, from meadow voles; one, from a crested jird; and seven, from mouse fleas). As the result of the investigation during these months in the settlement of Zelenyy and its environs a negative result was obtained on the right bank (767 rodents and 340 ectoparasites).

The last plague microbe culture was isolated in the left bank region of this focus on 12 December 1958. In the spring-summer season of 1959 here a bacteriological investigation of 608 rodents and 1,223 ectoparasites on the right bank and 333

rodents and 135 ectoparasites on the left bank (where there had been almost no mice in 1959) was made with a negative result.

The origin of the focus in the settlement of Zelenyy is not so clear as that in the settlement of Chkalovo. The left bank floodplain in the area between the northern epizootic points in the main region and the focus opposite the settlement of Zelenyy were not so completely investigated as to guarantee the impossibility of missing local epizootics if they were present there. However, a diffuse epizootic could not have been missed, and the possibility of a relay transmission of plague from the main region of the epizootic to the settlement of Zelenyy must be rejected. There is practically no basis for the belief that this focus was of local origin. Therefore, it remains for us to conclude that it was created as the result of distant importation of plague from the south, most likely along the river.

The only plague-infected mouse caught in the settlement of Zelenyy could have appeared there as the result of importation from the south or, most likely, from an epizootic focus on the opposite bank of the Ural River.

A focus of a local epizootic among dwarf susliks on the right bank of the Ural River, on the shore of the Chernaya Rivulet, detected in 1959 (Fig 5), deserves great attention. After the detection of the first plague-infected suslik on 18 May, this place was subjected to a careful investigation. In May-July 1959 1,882 rodents were caught here (including 1,087 dwarf susliks), 3,751 fleas and 3,049 ixodial and gamasid ticks. In all, 18 plague microbe cultures were isolated.

The epizootic was recorded only in May-June (the last culture was on 15 June), although in July 153 rodents, 243 fleas and 392 ticks were investigated. The locality was extensively studied, but it was possible to detect an epizootic on only a localized territory. The species variety of ectoparasites infected with plague during this epizootic was characteristic.

In 1959, on the right bank of the Ural River plague-infected mice were not found; however, it may be assumed that the epizootic among susliks on the Chernaya Rivulet occurred as the result of passage of plague to them from mice, most likely from the Chkalovo focus of 1958.

Suslik colonies of different population densities are found on the entire territory between the Volga-Ural Sands and the Ural River, with the exception of the southernmost part. In 1940, in this region (opposite Yamanka) a diffuse plague epizootic smouldered among the susliks (P. Ye. Nayden, 1959). However, there is no reason to believe that the local 1959 epizootic on the Chernaya Rivulet was simply the end of an independent epizootic among dwarf susliks in 1958 in more northerly regions. This entire region had been studied by the Yamanka Plague-Control

Section in the spring-summer season of 1958 and quite attentively in previous years, but constantly with a negative result.

Finally, it must be mentioned that on 23 May 1959 the only plague microbe culture from a meridional jird, caught 18 May at the Karabatyr natural landmark, located approximately 140 kilometers from the Ural River, that is, at a great distance from the epizootic among the mice and susliks (see Fig 5), was isolated through a biological test (on a house mouse).

We do not have the opportunity here to go into detail about this case, although it is exceedingly interesting, since no signs of plague had been recorded in the Volga-Ural Sands since 1953. We should like to note only that we have not found sufficient arguments for confidently considering plague in the Karabatyr imported from the region of the epizootic among mice nor for regarding it unreservedly as a sporadic case of plague among jirds in non-epizootic years, that is, for assuming the hypothesis that the plague microbe culture isolated from the jird was of local origin. Possibly, further investigation of the Volga-Ural Sands will throw light on this problem.

Additional Information

During the entire period of the epizootic 293 plague microbe cultures were isolated in its main region and in the foci in the settlement of Chkalovo, opposite the settlement of Zelenyy and on Chernaya Rivulet. The distribution of these cultures according to the objects from which they were isolated and according to foci is shown in Table 5.

From Table 5 it is seen that interspecies contact during this epizootic was intense, which is explained by the involvement of several species of animals in it. The following findings are most interesting: a) infected mouse fleas Ceratophyllus mokrzeckyi in the holes of the dwarf suslik, on shrews, and on the water vole; b) plague-infected suslik ticks, Rhipicephalus schulzei, on jerboas (Allactagulus acontion); c) infected jerboa fleas, Mesopsylla tuschkan, in the holes of the dwarf susliks; d) plague-infected fleas in the holes of the yellow suslik and plague-infected lice in the hair of this rodent.

During the 1958-1959 epizootic for the first time plague microbe cultures were isolated from mouse fleas, Leptopsylla taschenbergi, and gamasid ticks, Laelaps algericus and Eulaelaps kolpakovae. The first two findings have already been described in the literature (R. V. Kovaleva and N. L. Gershkovich, 1959; I. V. Rumyantseva and M. R. Netsengevich, 1960).

It should be noted that 11 plague microbe cultures were isolated from mice caught in human dwellings (one in the settlement of Zelenyy; two, in the village of Kurlys; one, in the sixth village; the others, in solitary structures in the floodplain). The presence of plague-infected house mice in settlements as well as in haystacks (five cultures of mice and one from voles)

Table 5

Distribution of Plague Microbe Cultures on its Reservoirs and Vectors in Various Foci of the 1958-1959 Epizootic

Виды животных (1)	Места обычн. животных (2)	Выделено культур (3)			
		основная зона (4)	чкаловский очаг (5)	зеленовский очаг (6)	очажок на Черной речке (7)
Млекопитающие (8)					
Домовая мышь (9)	Поле скирды и постройки (15)	107	5	8	--
Обыкновенная полевка (10)	Поле и скирды (16)	3	2	4	
Большая песчанка (11)	Поле (17)	1	—	—	
Гребенщиковая песчанка (12)	"		—	1	
Малый суслик (13)	"		--	--	4
Землеройка (14)	"	1	—	--	
Блохи (18)					
Ceratophyllus mokrzeckyi	Шерсть домовых мышей (19)	87	19	7	--
" "	" землероек (20)	2	-	--	--
" "	" водяной полевки (21)	1		--	--
" "	Гнезда домовых мышей (22)	6	—	—	—
" "	Норы малого суслика (23)	—	--	—	1
Leptopsylla taschenbergi	Шерсть домовых мышей (24)	8	2	—	—
Xenopsylla skrjabini	" больших песчанок (25)	2	—	—	—
" conformis	" " " (26)	1	—	—	—
" "	" полуденных песчанок (27)	1	--	—	—
Ceratophyllus gesquorum	Норы малого суслика (28)	—	--	—	2
Neopsylla setosa	" " "	—	—	—	2
Neopsylla setosa	Шерсть малого суслика (29)	—	—	—	1
" "	Норы желтого суслика (30)	1	—	—	—
Oropsylla ilovaiskii	" " " (31)	1	—	—	—
" "	Норы малого суслика (26)	—	—	—	1
Mesopsylla tuschkan	" " "	—	—	—	1
Клещи (29)					
Hyalomma sp. (нимфы)	Шерсть домовых мышей (19)	1	-	--	-
Rhipicephalus schulzei	" малого суслика (23)	-		--	1
" "	" тарбаганчика (30)	-		—	2
Eulaelaps kolpakovae	" "	—	--	—	1
Laelaps algericus	" домовых мышей (19)	1	—	--	—
Gamasidae gen. sp.	" " "	2	1	—	—
" "	" тарбаганчика (32)	—	--	—	1
" "	Норы малого суслика (23)	—	—	—	1
Вши (31)					
(32) Вши грызунов	Шерсть желтого суслика (34)	1		—	—
	(33) Всего . . .	227	28	20	18

[Table 5, continued next page]

/Table 5, continued from previous page/

1. species of animals; 2. places the animals were caught; 3. cultures isolated; 4. main region; 5. Chkalovo focus; 6. Zelenyy focus; 7. focus from Chernaya Rivulet; 8. mammals; 9. house mouse; 10. meadow vole; 11. great sand rat; 12. crested jird; 13. dwarf suslik; 14. shrew; 15. field, stacks and structures; 16. field and stacks; 17. field; 18. fleas; 19. hair of house mice; 20. hair of shrews; 21. hair of the water vole; 22. nests of house mice; 23. holes of dwarf susliks; 24. hair of great sand rats; 25. hair of meridional jirds; 26. holes of dwarf susliks; 27. hair of dwarf susliks; 28. holes of yellow susliks; 29. ticks; 30. hair of jerboa; 31. lice; 32. rodent lice; 33. total; 34. hair of yellow susliks.

contributed to increasing the endemicity of the epizootic. However, prophylactic measures taken by the Gur'yev Plague-Control Station and chiefly the regular disinfestation and deratization of various structures, vaccination of the population and extensive sanitary propaganda as well as rodent extermination in the field, which lowered the autumn concentration of mice in buildings, made it possible to prevent cases of plague among people.

Plague microbe cultures isolated during the epizootic described were fully typical with respect to their morphological, cultural and biochemical characteristics and were glycerin-positive varieties. All the cultures were lysed by plague bacteriophage.

Study of the virulence of 30 strains isolated during various periods of the epizootic from mice (17), meadow voles (1), crested jirds (2), fleas (8) and gamasid ticks (2 strains) showed that they were all highly virulent. The MLD for white mice and guinea pigs was equal to 10 microbes in the great majority of strains. There were only two exceptions: a) in strain 252 isolated 17 July 1958 from a house mouse the MLD was 100 microbes for white mice, and b) in strain 7073 isolated from a house mouse 18 August 1959 the MLD for guinea pig was set arbitrarily at 100,000 microbes because a dose of 100 microbes did not cause death of a single guinea pig, but doses of 1000 and 10,000 microbes were not tested.

Characteristics of the 1958-1959 Epizootic

The most characteristic feature of the 1958-1959 epizootic was the fact that it reached a high degree of development as early as the spring-summer season, whereas all epizootics described in the literature among small mouse-like rodents were found and possibly also began in the autumn, that is, during the period when the censuses of these rodents usually reach their yearly maximum;

in the spring and summer infected mice and voles were encountered only sporadically, and thereby rarely, during epizootics among the main reservoirs of plague (V. N. Fedorov, 1944; B. K. Fenyuk, 1948; V. N. Fedorov and others, 1955; Yu. M. Rall', 1958).

This example shows that intense and diffuse epizootics of plague among small mouse-like rodents can occur in the natural focus in any season of the year if the census of animals and their fleas reaches a high level and if their mobility is sufficiently great to assure the necessary contact with the main reservoirs.

The second characteristic can be considered the development of the epizootic in azonal habitats: the floodplain and delta of the Ural River. Although they lie in the region of natural plague foci they cannot in any way be considered enzootic for this infectious disease. A combination of conditions was needed, such as a marked increase in the census of mice and their fleas and the movement of the epizootic among great sand rats right up to the floodplain so that the epizootic penetrate into the mouse population and assume the nature of an independent phenomenon.

In this characteristic the epizootic of 1958-1959 imitated the characteristics of epizootics among mice and voles in 1937-1938 in the Volga-Akhtubinsk floodplain as well as in 1946 among the reeds of the delta portion of the Volga in Dengizskiy Rayon of Gur'yevskaya Oblast (B. K. Fenyuk and others, 1959).

Very characteristic of the 1958-1959 epizootic was the fact that it went far outside of its main area of distribution and overcame such an obstacle as the Ural River. This fact confirms the relative nature of such obstacles, although it does not deny their significance in those cases where the epizootics do not go outside their biotopes, colonized by the main plague reservoirs.

The 1958-1959 epizootic showed the fact that plague could pass from a population of such secondary reservoirs as mice and voles to a population of the main reservoirs (susliks), and thereby into an inactive part of the natural focus at that time.

Experience in the study of the epizootic among mice in 1958-1959 as well as the 1937-1938 epizootic mentioned above permits us to emphasize the need for a more careful epizootological investigation of mice and voles in azonal habitats and oases in the desert and semidesert region in those cases where the census of these rodents is increased.

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The Problem of Variation of the Plague Microbe in the Course of an Epizootic

N. Ya. Yeremitskiy and N. A. Yeremitskaya

Aral'sk

The factual material for the present report was constituted by the results of a study of plague microbe cultures isolated on the territory of the central part of the Aral Sea region of Kara Kumy in 1956-1959. One hundred thirty-one strains of plague microbe were studied; 47 from great sand rats; four, from meridional jirds [Meriones meridionalis]; one from crested jirds [Meriones tamariscinus]; one, from jerboas [Scirtopoda telum]; one, from gray hamsters [Cricetulus migratorius]; and 77 from fleas. The work was done at the central laboratory of the Aral Sea Plague-Control Station (1956-1957) and the Shaken epidemic-control detachment (1958-1959).

The Epizootic Circumstances

In making up the brief epizootological characterization of the territory studied, the material of the phyicians A. Kh. Arslanova, A. I. Volosivets and O. Ye. Tatsiy was used in addition to our own.

In the central part of the Aral Sea region of Kara Kumy the plague microbe was isolated for the first time in 1953 from rodents and their ectoparasites. Beginning with this year, plague epizootics were recorded in this region annually. In 1956, four small local foci of epizootics were found, in which five epizootic points were demonstrated: the Kaydaul and Bes-Tyube Mountain. From these points 16 plague microbe strains were isolated: seven from great sand rats; one, from a meridional jird; eight, from fleas.

In 1957, epizootics were recorded in five separate local areas, in which 18 epizootic points were demonstrated and 64 cultures of plague microbe isolated, including 37 from great sand rats, one from crested jirds and 26 cultures from fleas. The epizootics were noted throughout the warm period of the year, from April to November. The course of the plague infection in rodents was distinguished by considerable acuteness: in 26.5 percent of the great sand rats sick with plague bacteriemia was noted; a culture was isolated from the rodents in the majority of cases by means of direct seeding of the organs on agar plates; the growth of the plague microbe was profuse in 50 percent of the cases.

In 1958, epizootics covered a large territory, as a result of which a confluence of certain separate epizootic regions occurred. During the year 25 epizootic points were demonstrated and 88 plague microbe cultures isolated: 60 from great sand rats; five from meridional jirds; one from crested jirds; two from jerboas; one from the Siberian polecat [Mustela eversmanni]; one from house mice; and 18 from fleas. Despite the more extensive spread of the epizootics in 1958 than in 1957 the plague microbe was found in the blood of great sand rats sick with plague in only two cases; great sand rats were encountered with signs of chronic plague.

In 1959, the epizootic assumed an even more diffuse nature. On a

smaller territory 18 epizootic points were demonstrated, from which 107 plague microbe cultures were obtained: 31 from great sand rats; five from meridional jirds; one from gray hamsters; 70 cultures from fleas. The nature of the disease in the great sand rats in 1959 was relatively benign; not a single case of bacteriemia was recorded; the plague microbe was obtained from 16 sand rats only by a biological test; from 15 sand rats the culture was isolated from only one organ, liver or spleen.

In autumn 1959 10 cultures of the plague microbe were isolated which were affected by bacteriophage: five from rodents and five from fleas. From all the cultures plague bacteriophage was obtained without difficulty.

In conclusion it should be pointed out that in 1956-1959 in the central part of the Aral Sea region of Kara Kumy every year two seasonal rises in plague epizootic activity were noted among rodents: in May-June and in September-October. As a whole, the epizootic activity of the part of the natural plague focus studied increased beginning with 1956 and reached the maximum in 1959.

Microbiological Studies

In the study of plague microbe cultures the main attention was given to the morphology of growth on solid and liquid nutrient media, the relationship to plague bacteriophage, as well as biochemical and virulent properties.

The growth morphology was studied on agar and Hottinger's bouillon at a pH of 7.1-7.2. As a growth stimulant hemolyzed blood was added to the agar in a proportion of 1:1000. Considerable variegation of the colony morphology was noted, but at the same time in the majority of strains it was typical of the R variant of the plague microbe. Medium-sized and small dark brown colonies were observed with elevated centers, granular, somewhat dryish, with an uneven festooned margin; many with a delicate peripheral zone. Trabeculated colonies were encountered in the form of a "daisy" or "sunflower" [the text actually reads "sunflower seeds" which is apparently an error], colonies with an indented center and a ridge along the periphery were encountered. In other cases signs characteristic of OR and OS variants of the plague microbe were noted: colonies with a bright yellow color, with slight granulation, with a flat surface, thinned-out smoothed margins, and, in addition, absolutely achromogenic colonies, flat, smooth, with thinned-out wavy or smooth margins.

In the majority of cases the growth of the strains studied on Hottinger's bouillon was typical: the bouillon remained clear; on the bottom a flocculent precipitate was noted. Growth was also noted on the side of the test-tube and clumps were seen suspended in the bouillon. Some strains gave a slight turbidification of the bouillon and a flocculent precipitate on the bottom. It should be noted that dissociative changes of the strains studied were distinguished by instability, as a rule, and after two or three subcultures the strains acquired the morphology characteristic of the R variant.

Strains of the plague microbe with signs of dissociation began to be isolated in 1958-1959. In 1956-1957 no such strains were found. In 1958, five dissociative strains were isolated; in 1959, seven; they were obtained from great sand rats and meridional jirds as well as from fleas.

Two strains (103 and 434) with signs of dissociation, isolated in 1959, were very much affected by bacteriophage. Strain 103 was isolated in September from Ceratophyllus laeviceps fleas. In the native cultures of the fleas three medium-sized colonies grew out which were of mucoid consistency, achromogenic, with a smooth surface and even margin; later, they were completely lysed. The culture was preserved through a subculture made on solid medium containing antiphage serum. Strain 434 was isolated in October from a meridional jird. In native cultures it gave a profuse growth of medium-sized yellowish colonies with a delicate smooth granulation, without a peripheral zone. Eight cultures affected by plague bacteriophage, isolated at the same time, showed the growth morphology typical of the R variant. From all 10 cultures involved by bacteriophage, as stated above, plague bacteriophage was obtained.

All the cultures studied, not excepting those involved by phage, were readily lysed by plague and pseudotuberculosos bacteriophage.

Fermentative activity with respect to glucose, lactose, sucrose, maltose, mannitol, glycerin and rhamnose was studied in 131 strains of the plague microbe. For this purpose, media with the carbohydrates and polyatomic alcohols mentioned were utilized which were prepared in semiliquid agar. The strains studied possessed the fermentative activity typical of the plague microbe; on the second-third day they split glycerin, glucose, maltose and manntol to acid but did not ferment sucrose, lactose or rhamnose. All the strains studied were glycerin-positive varieties of the plague microbe. Various cells of the same strains broke down glycerin at different rates; 16 cells of 10 cultures did not decompose it for as long as 15 days (maximum observation period). None of the 15 strains studied showed positive nitrification or denitrification tests.

The virulent characteristics were studied in 57 strains. Strains with typical morphology were studied by random selection; strains with signs of dissociation were all checked. The virulence was also checked in all strains involved by bacteriophage. The virulent characteristics were studied on white mice, which were injected subcutaneously with 10, 100 and 1,000,000 microbes (three-four mice for each dose).

The cultures in which the virulent characteristics were studied were isolated in different seasons--from May to November 1956-1959--from great sand rats, meridional and crested jirds as well as from Xenopsylla skriabini, Ceratophyllus laeviceps, Coptopsylla lamellifer, Rhadinopsylla cedestis fleas. In rodents various forms of the course of plague infection were noted, from a disseminated acute plague to chronic plague, in which the plague microbe was isolated only from the contents of dense encapsulated abscesses.

The results of the experiment showed that all of the strains studied possessed a high degree of virulence. On subcutaneous injection of 100 microbes, white mice died on the third-seventh day. At autopsy pathological changes were noted characteristic of the disseminated form of plague. Cultures of the parenchymatous organs and blood on nutrient media showed the profuse growth of the plague microbe.

Therefore, no differences were noted in the virulent characteristics of the cultures isolated in different periods of the epizootic activity of the focus from rodents with different courses of the plague infection, from fleas or cultures with signs of dissociation and markedly affected by bacteriophage.

For the purpose of clarifying the minimum lethal dose of the cultures studied white mice were infected subcutaneously with 10 microbes, utilizing three-four mice for each culture. The experiments showed that the great majority of cultures caused the death of all experimental white mice in this dose. In the animals which died a characteristic pathological picture was observed, and cultures from their organs and blood showed the profuse growth of the plague microbe. However, when mice were infected with 10 cultures isolated in 1958-1959 the dose of 10 microbes did not kill all the animals: one or two mice in each group survived; cultures of the organs of the mice which died showed the growth of the plague microbe but not in all cases, and, as a rule, this growth was meager. Therefore, in this respect we were able to establish a certain difference between the virulent properties of the various strains.

Among 10 strains in which a certain reduction of virulence was noted, three were very much affected by bacteriophage and one strain dissociated; the other six were morphologically the same as the other strains studied which showed a higher degree of virulence. It should be noted that in the seven strains affected by bacteriophage no reduction of virulence was noted, and the minimum lethal dose of these strains for white mice was equal to 10 microbes.

Discussion of the Results Obtained

The results of the studies showed that plague microbe cultures isolated from nature on the territory of the central part of the Aral Sea region of Kara Kumy in 1956-1959 possessed marked growth polymorphism on agar. Similar results were obtained by V. M. Tumanskiy (1943), G. N. Lenskaya (1946) M. N. Sokolova (1959) M. F. Bondarenko (1959), L. M. Osadchaya (1960) and others. This fact is of great importance for the diagnosis of the plague microbe, and it should be taken into consideration in the practice of investigation work.

The polymorphism of plague microbe colonies in a number of cases depends on the dose of the culture used for streaking and the quality of the nutrient medium. However, frequently, on the same series of agar and with the same profuseness of growth the freshly isolated strains are very much different from one another in their colony morphology. The variability of the colonies of freshly isolated cultures is apparently caused by the extensive variety of conditions under which the plague microbe exists in nature. Thus, under conditions of an active epizootic, the plague microbe, entering the bodies of various species of rodents and ectoparasites, must adapt itself to changing habitat conditions. In cultivating a plague microbe isolated from an animal on nutrient media, the conditions of its habitat change even more, and it again must adapt to them, and since the original condition of the cultures varies, this adaptation occurs differently, having an effect on the fine structure of the colonies. Standardization of the living conditions in the subsequent subcultures on synthetic nutrient media leads to a relative uniformity of the colony morphology.

Despite the pronounced polymorphism of the colonies the majority of the cultures studied in 1956-1959 can be considered typical strains of the plague microbe. They all showed the growth morphology on nutrient media which

was typical of the R variant, were readily lysed by plague and pseudotuberculosis bacteriophages, had the typical biochemical activity of the plague microbe and possessed a high degree of virulence. However, in 1958-1959 more than 20 strains of the plague microbe with signs of variation were isolated. The majority of them dissociated; 10 strains were affected by bacteriophage (which was isolated from them subsequently); some strains showed reduced virulence.

The experience of studying natural plague foci in the USSR shows that at various stages of development of epizootics the virulence of the plague microbe changes. As a rule, during the period of the course of active epizootics various cultures with reduced virulence begin to be suppressed. Subsequently, during the period of reduction of epizootic activity the number of these cultures increases. Such data are presented by Ya. Ye. Punskiy (1958-1960), A. A. Levina and B. K. Fenyuk (1959), N. M. Sokolova (1959), M. F. Bondarenko (1959), and L. M. Osadchaya (1960).

In the study of plague epizootics in the Aral Sea region of Kara Kumy for four years no plague microbe cultures with relatively low virulence were isolated. However, this material shows only an apparent contradiction to the data in the literature, because the work was done in a period coinciding with an increased epizootic activity of the focus. In this respect, in the Aral region it was apparently possible to detect only the beginning of the process of reduction of the virulence of the plague microbe in nature, which was observed in 1958-1959. Seasonal variations in the intensity of the epizootics, according to data obtained in the Aral region, do not reflect on the virulence of the plague microbe. The same results were obtained by R. V. Kovaleva, A. V. Rumyantseva, T. N. Ponomareva, and others (1959) in the northeast Caspian region.

In this connection the study of the virulence of the plague microbe in the bodies of animals sick with chronic plague is of great significance. In the literature there is mentioned (V. N. Lobanov and V. N. Fedorov, 1938; N. M. Sokolova, 1959 and others) that in the body of the rodents sick with chronic plague the plague microbe shows a reduction of its virulent characteristics. Reduction of the virulence of the plague microbe in the bodies of animals with species resistance to plague has been observed experimentally by Ya. L. Semenova (1958). In 1958-1959 in the Aral region four rodents were found sick with chronic plague with dense encapsulated abscesses. However, the virulence of all cultures isolated thereby was very high: the CLD for white mice was equal to 10 microbes. The same results were obtained by N. A. Pletneva (1958), R. V. Kovaleva, A. V. Rumyantseva and others (1959) under natural conditions and by L. B. Adimov (1959) under experimental conditions.

The fact deserves attention that the 10 cultures affected by bacteriophage which were isolated in the Aral region showed a high degree of virulence: the CLD for white mice was equal to 10-100 microbes.

Therefore, in the Aral Sea region of Kara Kumy in 1956-1959 signs of variation of the plague microbe were observed under natural conditions. The variation was superficial, because no marked changes were noted in the main hereditary characteristics. By 1959 the epizootic had become less acute and, in all probability, had gone into a decline along a declining curve. This indicates the frequency with which cultures definitely affected by bacteriophage were isolated, which on culture showed plaques; it also indicates the

record of pathological changes characteristic of plague in animals cultures from which showed no growth.

Conclusions

1. On the territory of the Aral Sea region of Kara Kumy a continental race of the plague microbe which ferments glycerin is widespread.

2. Strains of the plague microbe isolated in 1956-1959 in the Aral region were distinguished by marked polymorphism of the colonies in the native cultures on agar plates.

3. The great majority of the plague microbe strains isolated in 1956-1959 in the Aral region of Kara Kumy show great virulence. Their CLD for white mice is equal to 10 microbes.

4. Under the natural conditions of the Aral region in 1958-1959 signs of variation of the plague microbe were observed, expressed in a dissociation in the native cultures, involvement by bacteriophage and a relative reduction of virulence. Dissociation of the cultures was not always accompanied by a reduction of virulence, just as reduction of virulence was not always associated with dissociation.

5. In the bodies of rodents sick with chronic plague, the plague microbe did not lose its high degree of virulence. Seasonal variations in the activity of the plague epizootics had no effect on the virulent characteristics of the plague microbe. Some bacteriophage-affected cultures also maintained a very high degree of virulence.

6. The cell composition of plague microbe cultures isolated in 1959 from nature in the Aral region of Kara Kumy was inhomogeneous with respect to glycerin. Various cells of the same strains decomposed glycerin at different rates; some strains did not break down glycerin for as long as 15 days (the observation period).

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*"Srednyaya" means "middle" or "central" but "Srednyaya Aziya" does not mean "Central Asia"; it is equivalent to our term "Middle East," but since the latter, as ordinarily used, does not include Russia the term is translated "Southwest Asia." Central Asia, the area around Mongolia and Lake Baikal in Siberia, is rendered in Russian by "Tsentral'naya Aziya."

Activity of Plague Transmission by Certain Species of Fleas

A. A. Flegontova and L. S. Malafeyeva
(Saratov)

Study of the comparative activities of different species of fleas in their transmission of plague is of considerable epizootological interest. As is well known, in rodents--plague reservoirs--usually several species of fleas are parasitic simultaneously. Thereby, on the species of rodents in different localities, now one species, now others are more numerous. The fact that there is a seasonal replacement of some species of fleas by others is also well known. Undoubtedly, data on the comparative activities of different species of fleas as vectors of plague will assist in explaining various problems of the dynamics of the epizootic process.

The idea that different species of fleas are different in their activity in transmission of plague has been expressed by investigators repeatedly, almost since the fact was established that fleas are the main vectors of plague from a sick rodent to a healthy one.

In the study of the present problem the determination of the criteria which would make it possible to carry out a comparative evaluation of the activities of different species of fleas as plague vectors is very important.

One of the authors of the present article (A. A. Flegontova, 1951), after confirming the observations of Bacot and Martin (1914) on considerable material to the effect that infection of a warm-blooded animal with plague can be accomplished only by a flea in whose gizzard a "block" of plague microbes has been formed, concluded that the "ability of fleas of a certain species to be active plague vectors is determined by the duration of preservation of plague microbes in their bodies, the actual frequency with which the "block" is created in them, the period for which it is maintained, and the lifespans of the "fleas with the blocks."

We used mainly these criteria for characterizing the flea species which we studied as plague vectors.

The aim of our work was the study of the activity of three species of fleas of the genus Ceratophyllus in the transmission of plague: C. fasciatus (rat flea), C. tesquorum (suslik flea) and C. laeviceps (sand rat flea). In addition, for the purpose of comparison with the species of fleas indicated above we made a study of the activity of the rat flea Xenopsylla cheopis and the suslik flea Neopsylla setosa in the transmission of plague.

In the literature the information on the activity of fleas of the genus Ceratophyllus in the transmission of plague is quite limited.

In the present work, we made an attempt to study the infectious power of the fleas when they are kept under different temperature conditions.

During the course of the investigation it was made clear that the genus to which the fleas belong does not determine their activity as plague vectors. In carrying out work of this kind a study should be made of the comparative infectious capacities of different species of fleas parasitic on the same species of rodents.

Method of Work

The method of our experiments amounted to the following. We infected the fleas by having them feed on plague-infected agonal white mice during the period of intense bacteriemia (no less than 60 microbes in every field of the blood smear). After the infective feeding fleas which had sucked until their stomachs were completely filled (determined by means of microscopic examination) were used in the experiment. Fleas which were not completely satiated were not used.

Groups of fleas infected by this method were kept at temperatures of 27°, 20°, 10° and 5° and a relative humidity of 80-90 percent.

Regularly, after every two-three days the fleas were fed up on healthy white mice. After each feeding the fleas were examined under the microscope, thereby continuously observing the conditions of their digestive tracts. Fleas in which the "plague block" had been created in the gizzard were separated from the general mass of fleas and kept at the same temperature. Death of a white mouse after a group of fleas in which gizzard "blocks" had been found on microscopic examination had fed on it constituted a partial check on the actual occurrence of the block.

All fleas which died and were killed during the experiment were subjected to bacteriological examination by means of an individual culture on agar plates. All the mice which died were also studied bacteriologically.

Results of the Experiments and Discussion of Them

The results of the work done are shown in Tables 1 and 2. In Table 1 the criteria are given which we used for the evaluation of different species of fleas as plague vectors. In Table 2 the period needed for the formation of the plague "block" in different species of fleas is shown.

Rat Fleas X. Cheopis and C. Fasciatus

As is seen from Table 1, X. cheopis kept at 20° eliminated the plague microbe in only a very small number of cases (8 percent of the total number of infected fleas). In a considerable group of the fleas which had not eliminated the plague microbe a block of plague microbes was formed in the gizzard. We observed the formation of the "block" in 60-70 percent and, in various experiments, 80 percent of the total number of infected fleas.

The process of "blocking" in X. cheopis proceeds at a rapid rate. From Table 2 it is seen that in various flea individuals of the group of plague microbes the block was created in the gizzard as early as the third day after the infective feeding. It was formed in a month in 5/6 of all the infected fleas, and only in 1/6 did the "blocking" process drag out for longer periods. Thus, we were able to observe the formation of the "block" in the X. cheopis on the 72nd day after infection, and the maximum time the plague microbe was preserved in the body of this species of flea in our experiments was equal to 85 days.

The data presented are evidence to the effect that a considerable number

Table 1

Vector Efficiency of Different Species of Fleas in Plague as a Function of the Temperature at which They are Kept

(1) Вид блохи	(2) Температура, °	(3) Количество зараженных блох	(4) Количество блокированных блох (5) число	(4) Количество блокированных блох %	(6) Максимальная продолжительность жизни „блокированных" блох, дни	(7) Максимальный срок сохранения чумного микроба в блохах, дни	(8) Блохи, освободившиеся от чумного микроба, %	(9) Максимальный срок выживания блох, дни
C. fasciatus	27	45	—	—	—	—	100	17
	20	781	80	11,5	12	103	74	103
	10	124	—	—	—	87	72	87
	5	121	1	0,8	4	87	90	87
X. cheopis	20	400	268	67,0	18	85	8	85
C. tesquorum	27	200	—	—	—	69	86	75
	20	444	46	10,3	15	95	38	95
	10	690	70	10,1	46	236	41	236
	5	296	55	18,6	33	192	2	192
N. setosa	27	25	2	8,0	4	33	44	33
	20	259	91	35,1	20	85	46	97
	10	75	1	1,3	10	54	13	58
	5	105	17	16,2	10	81	21	81
C. laeviceps	20	600	96	16,0	14	65	42	65
	10	353	77	16,0	15	84	36	84
	5	42	4	9,5	10	49	5	50

1. species of flea; 2. temperature; 3. number of infected fleas; 4. number of fleas with "blocks"; 5. number; 6. maximum lifespans of "blocked" fleas, days; 7. maximum period of preservation of plague microbes in fleas, days; 8. fleas which have eliminated plague microbe, %; 9. maximum survival of fleas, days.

of X. cheopis fleas becomes capable of transmitting plague to a healthy animal in quite short a time after the infection. In our experiments all mice on which the "blocked" fleas fed died of disseminated plague. Therefore, what has been presented above permits us to put the flea X. cheopis in the group of very efficient plague vectors.

Different activity indices in plague transmission were obtained for another species of rat flea, C. fasciatus. The majority of fleas of this species readily eliminates the plague microbe at all ambient temperatures, even a temperature as low as 5°. It should stated that a temperature of 27° was very unfavorable for both the fleas themselves and for the existence of the plague microbe in their bodies. At this temperature C. fasciatus lived a maximum of 17 days, during which all fleas used in the experiment eliminated the plague microbe. At a lower temperature (20°, 10° or 5°) the lifespans of the fleas were prolonged to 87-103 days, but the number of fleas

Table 2

Time Needed for Formation of "Block" in Different Species of Fleas as a Function of the Maintenance Temperature

(1) Вид блохи	(2) Температура, °	(3) Количество «блокированных» блох	(4) Число блох, у которых «блок» образовался в следующие сроки от заражения, дни																				(5) 203 дня	Срок образования «блока», дни (6)	
			3—5	6—10	11—20	21—30	31—40	41—60	51—60	61—70	71—80	81—90	91—100	101—110	111—120	121—130	131—140	141—150	151—160	161—170	171—180			минимум (7)	максимум (8)
C. fasciatus	20	80	2	7	33	25	9	1	—	2			—	—	—	—	—	—	—	—	—	—	—	3	64
	10	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	-	-
	5	1	—	—	—	—	—	—	—	—	—	1	—	—	—	—	—	—	—	—	—	—	—	78	
X. cheopis	20	268	6	18	129	41	64	6	-	—	1		—	—	—	—	—	—	—	—	—	—	—	3	72
C. tesquorum	20	46	—	2	8	5	7	6	3	—	15	—	—	—	—	—	—	—	—	—	—	—	—	10	79
	10	70	—	—	—	2	1	3	6	6	11	12	8	5	3	3	3	2	3	—	2	1		22	203
	5	55	—	—	—	—	—	3	3	12	5	11	4	1	3	2	2	2	1	3	—	—		44	162
N. setosa	27	2	—	—	2	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	15	-
	20	91	—	15	26	26	14	9	—	—	—	1	—	—	—		—	—	—	—	—	-		7	81
	10	1	—	—	—	—	1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—		40	
	5	17	—	—	2	3	8	3	1	—	—	—	—	—		—	—	—	—	—	—	-		18	51
C. laeviceps	20	96	—	25	42	15	9	2	3	—	—	—	—	—	-	—	—	—	—		—	—		8	53
	10	77	—	—	11	21	10	21	11	1	2	—	—	—	—	—	—	—	—	—	—	—		12	76
	5	4	—	—	—	3	1	—	—	—	—	—	-	—	—	—		—	—	—	—	—		25	35

1. species of flea; 2. temperature; 3. number of "blocked" fleas; 4. number of fleas in which th "block" was formed in the following period after the infection, days: 5. days; 6. time of "block" formation, days; 7. minimum; 8. maximum.

which eliminated the plague microbe was high, as before (72-90 percent).

We observed the formation of the "block" in C. fasciatus only at 20° and then in a small number of infected fleas (11.5 percent). At 27 and 10° the "block" did not form in any case, and at 5° a "block" was formed on the 78th day after infective feeding in only one out of 121 infected fleas but the flea was unable to produce infection in a white mouse.

These figures indicate that in the population of C. fasciatus there cannot be simultaneously a large number of fleas capable of infecting their hosts with plague as exists in X. cheopis. Therefore, if for any reason X. cheopis is excluded from the epizootic process and only C. fasciatus remains the epizootic must assume a more sluggish course.

In contrast to the previous views presented above, M. Balthazard and coauthors (1958) showed that in India there are no independent foci of rat plague but the natural focalization of this disease is provided for by one of the species of large gerbils, Tatera indica. The plague penetrates secondarily into the rat population. In places where the plague microbe encounters an active spreader of the infection, the flea X. cheopis, after entering the rat population, very active epizootics break out among the rats. If plague penetrates into a rat population where the main species of flea parasitic on rats is C. fasciatus such active epizootics cannot develop.

The inability of C. fasciatus fleas to form a plague "block" under low temperature conditions and the inability of a considerable number of the fleas to eliminate the plague microbe brings to naught the role of this flea as a plague vector in temperate latitudes.

Suslik Fleas C. Tesquorum and N. Setosa

In experiments on the study of the infectious activity of C. tesquorum the effect of the ambient temperature was very clearly demonstrated on the process of elimination of the plague microbe by infected fleas. While at 27° in our experiments more than 85 percent of the infected fleas eliminated the plague microbes, within limits of 10-20° the number dropped to half (about 40 percent), and at 5°, a total of only 2 percent of the fleas. These figures are evidence to the effect that under conditions of low temperature the fleas of this species eliminate the plague microbe much more slowly.

As far as the formation of a "block" in C. tesquorum is concerned, here an exceedingly interesting detail was demonstrated: in fleas of this species the "block" occurs when they are kept under low temperature conditions (5 and 10°). As is seen from Table 1, the formation of the "block" at 5° was twice as frequent as at 20°. We are inclined to explain this phenomenon by the fact that the lifespan of infected C. tesquorum fleas was twice as great at 5° as at 20°.

The ability of the fleas of this species to form "blocks" of plague microbes at low temperatures in combination with the long-term survival of the infected fleas at such temperatures gives C. tesquorum importance in the long-term preservation of the plague microbe pathogen in nature. This is evidenced also by cases in which the "block" was formed in them five-seven months after the infective feeding and cases in which the animals on which

these "blocked" fleas fed died of plague.

Although the frequency with which the "block" is formed in C. tesquorum in our experiments was comparatively low (from 14.3 to 23.4 percent depending on the ambient temperature), the lifespans of the "block" fleas were considerable, from 15 to 46 days, whereby they did not depend on the temperature conditions. It should also be noted that the plague microbe survived in the bodies of fleas as long as 236 days at 10°; 192 days at 5°. We do not consider these periods the maximum.

It should be noted that the indices of these experiments alone, without consideration of ecological characteristics of the fleas, cannot completely characterize the infective power of one species or another of fleas. L. Kartman (1957) believes that for the purpose of characterizing one species or another of fleas as a plague vector it is necessary to take into consideration the experimental data on the infectibility of the fleas as well as the data of field observations: the total flea census and infection rate with the plague microbe in nature.

If we compare the experimental data which we obtained on the infective capacity of two species of suslik fleas, it is seen that the percentage of "block" formation in N. setosa at 20° is three times greater than in C. tesquorum (see Table 1). However, this does not mean that the flea C. tesquorum must be put in the category of inefficient vectors of the plague microbe (see, for example, C. fasciatus).

Epizootological data make it possible for us to believe that both of these species of suslik fleas are active vectors of the plague microbe, but in each of these species, because of its ecological characteristics, participation in the epizootic process in nature is manifested in different seasons.

The relationship between the intensity of summer epizootics of plague in susliks and the index of abundance of fleas on these rodents is well known (V. I. Kuzenkov, 1940). N. S. Novokreshchenova (1960) points out that the index of abundance of C. tesquorum in suslik holes in epizootic areas is appreciably greater than in non-epizootic areas. C. tesquorum fleas can, during the season in which they occur in large numbers, account for the active transmission of the plague microbe to the population of dwarf susliks. At the same time, the ability of fleas of this species for maintaining the plague microbe in their bodies for a long time and forming a "block" in remote periods after the infection gives us the basis for ascribing a place of no little importance to C. tesquorum in "carrying" the infectious disease from one epizootic season of the year to the next.

N. setosa fleas are the main and predominant species of fleas inhabiting suslik nests (70-90 percent). The maximum number of these fleas is observed in the spring and then in the autumn-winter (I. G. Ioff, 1941). However, during the summer period these fleas remain the predominant ones in the suslik nests (A. A. Flegontova, 1937).

The formation of the "plague block" in N. setosa kept at 27° is curious; it is not observed at this temperature either in C. tesquorum or other species of the genus Ceratophyllus (C. fasciatus, C. laeviceps). The frequency of cases in which the "block" is formed in N. setosa was the greatest at 20°. At this temperature, 46 percent of the total number of infected fleas eliminates the plague microbe. At low temperature (5°) the number of cases in which the fleas eliminate the plague microbe is cut in half (21 percent).

The greatest census of N. setosa is observed early in the spring. During this period they can provide for the spread of the so called early spring epizootics among the susliks. With increase in the census of C. tesquorum the role of vector of the plague microbe begins to be played by fleas of this species. In the experiments of A. A. Flegontova (1951) the plague microbe was preserved in the body of N. setosa as long as 142 days (not the maximum period), and "blocked" fleas of this species lived as long as 23 days. In the present experiments we were also able to observe the formation of a block in N. setosa in such remote periods after the infection as 51 days (at 5°) and 84 days (at 20°). The very fact that the block is formed in N. setosa at low temperatures, which correspond approximately to the temperature of the nest in the winter period, gives us the basis for considering the participation of N. setosa fleas proved in the long-term preservation of the plague microbe in nature.

The ability of C. tesquorum and N. setosa for feeding on hibernating susliks (I. G. Ioff, 1941; A. A. Flegontova, 1951) creates conditions for the reproduction and preservation of the plague microbe in the bodies of fleas in the winter. With the delayed process of digestion in fleas under low temperature conditions (5-10°) observed in the winter in suslik nests, the fleas do not require frequent feeding, and the flea thereby survives much longer than at high temperatures. All this creates the possibility of slow reproduction of the plague microbe and the formation of a "block" in the fleas in the remote periods after the infection.

Therefore, suslik fleas C. tesquorum and N. setosa can preserve the plague pathogen in their bodies for a long time under experimental conditions at low ambient temperatures and can cause the death of rodents susceptible to plague in the remote periods after their becoming infected and, therefore, can be responsible for the "carriage" of plague infection in nature from one epizootic season to the next. The conclusions from our experiments are corroborated by the data of epizootological investigation. Thus, V. M. Tumanskiy and I. M. Polyak (1931) found plague-infected N. setosa and Ctenophthalmus pollex fleas in a suslik's nest in November, five months after an epizootic among susliks had stopped. I. S. Tinker and P. N. Stupnitskiy (1932) found plague-infected N. setosa in a suslik's nest in February, that is, eight months after the past year's epizootic among susliks and on the eve of their awakening from hibernation and the beginning of a new epizootic season.

What has been stated above on the two species of suslik fleas, C. tesquorum and N. setosa, confirms the fact that for the purpose of evaluating the epizootological activity of fleas experimental data alone are inadequate. It is necessary to take into consideration also a number of factors associated with the ecological characteristics of the vectors themselves and those of their hosts. With respect to the frequency with which the block is formed under experimental conditions C. tesquorum fleas showed themselves to be less active plague vectors than did N. setosa. However, if we consider the ecological characteristics of these two species as well as the epizootological data, great epizootological significance should be ascribed to both.

C. laeviceps Fleas

In our experiments the frequency with which the block was formed in infected C. laeviceps at temperatures of 10 and 20° was the same. At 5° the number of "blocked" fleas decreased by more than 1½ times compared with their number at 10 and 20°. Evaluating these figures, consideration should be given to the biological characteristics of C. laeviceps and their hosts.

The main and, in a number of cases, the only hosts of the flea C. laeviceps are non-hibernating rodents, various species of sand rats, for which spring-summer and autumn-winter epizootics are characteristics. While during the season of the spring-summer epizootics various species of the fleas of the genus Xenopsylla (conformis, gerbilli, skriabini, minax and others) predominate, during the season of the autumn-winter epizootics, in a number of places the predominate species is the flea C. laeviceps. In addition, fleas of the genus Xenopsylla are inactive during the autumn-winter season in a number of cases.

According to the data of A. A. Flegontova (1940) and S. A. Kolpakova (1944), seasonal variations of the census in the case of C. laeviceps are much less pronounced than in X. conformis. Thereby, in the summer months the C. laeviceps census is the least for the year; in the winter months, the greatest. S. A. Kolpakova noted a quite active migration of C. laeviceps fleas from their holes, even in the very cold season.

The parasitism of C. laeviceps fleas during the entire year and increase in their census in the autumn-winter as well as the year-round activity of the host to this species of flea are the ecological factors which are responsible for the participation of these fleas in the spread of infection during autumn-winter epizootics.

Since experimentally C. laeviceps is capable of forming the "block" with an ambient temperature of 10° or even 5°, it is entirely possible that this flea plays the exclusive part in the autumn-winter epizootics among some species of sand rats. Cases in which the "block" is formed 2½ months after an infective feeding attest to the considerable part played by C. laeviceps in the winter.

Therefore, on the basis of epizootological observations it may be concluded that the spring-summer plague epizootics among sand rats [this includes the great sand rat and the jirds] are brought about by various species of fleas of the genus Xenopsylla, a sharp rise in the census of which is noted specifically during this period.

Autumn-winter epizootics are conditioned by C. laeviceps fleas. They apparently participate in maintaining the infection during the winter and account for various sporadic cases among rodents at this time.

Here, it is fitting to point out that aside from C. laeviceps, fleas of the genus Coptopsylla which experimentally are very active plague vectors (A. A. Flegontova, 1957), participate in the autumn-winter epizootics among sand rats (great sand rats, meridional jirds and others).

In conclusion, one very important fact, in our opinion, should be noted. The long stay of the plague microbe in the organism of the flea under laboratory conditions did not at all change the virulence of the strain, which remained stable.

During the course of the present work the virulence of the original plague microbe culture of the 708 strain and then of the same culture which had been in the bodies of C. tesquorum fleas for seven months at temperatures of 5 and 10° and in the bodies of N. setosa for 3½ months at 5° was checked on guinea pigs. In checking this virulence infecting doses of from 1 to 10,000 microbes were used. As the result of titration of these cultures it was determined that a subculture of this strain, passaged through the bodies of fleas, remained virulent. Guinea pigs infected with this subculture died in a period of four to six days (1 CLD of the subculture of this strain is equal to 10 microbes).

Conclusions

The most complete and most accurate idea of the role of fleas in the epizootology of plague can be gained only with consideration of the results of experimental study of the infectious power of fleas of a given species and field study of their ecology. This principle is readily confirmed by the results which we obtained for two species of rat fleas (X. cheopis and C. fasciatus) and two species of suslik fleas (C. tesquorum and N. setosa).

The rat flea C. fasciatus is a much less active plague vector than X. cheopis. This fact, along with the ecological characteristics of C. fasciatus fleas, leads to the fact that they are incapable of maintaining acute active epizootics among rats in temperate latitudes.

Of the two species of suslik fleas, C. tesquorum and N. setosa, the former species proved to be less effective as a plague vector than the latter under experimental conditions. The data of epizootology in combination with the ecological characteristics of these fleas permit us to consider both C. tesquorum and N. setosa active vectors of the plague microbe during acute epizootics.

C. tesquorum fleas, at the low temperatures of the suslik nest during the cold season (5-10°), are capable of preserving plague microbes in their bodies for a long time (236 days), forming a plague "block" at such temperatures and causing the death of animals from plague in remote periods after they themselves have been infected (203 days).

The capacity of long preservation of the plague pathogen and the "block" formation at low temperatures is also shown by N. setosa (84 days).

On the basis of these data, it may be considered that suslik fleas C. tesquorum and N. setosa can "carry" the infection from one epizootic season to the next.

C. laeviceps fleas, parasitic on non-hibernating rodents and, in a number of places, the predominant species in the autumn-winter, experimentally showed themselves to be quite effective plague vectors, capable of forming a "block" at low ambient temperatures (5-10°). Considering this fact, it is possible to ascribe an exclusive role in autumn-winter epizootics to a flea of this species in various cases in a fully substantiated manner. Cases in which the "block" is formed 2½ months after the infective feeding of fleas permit us to suppose that C. laeviceps participates also in maintaining the infection in the winter and is responsible for sporadic cases among rodents at this time.

The long stay of the plague microbe in the bodies of C. tesquorum fleas

(seven months at temperatures of 5 and 10°) and the bodies of N. setosa (3½ months at a temperature of 5°) kept under laboratory conditions did not alter the virulence of the plague microbe.

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Changes in the Landscapes and Reasons for the Shift in the Boundaries of the Plague Epizootic in the Northeast and East Caspian Regions

A. A. Lavrovskiy
(Saratov)

Extensive material published at present in the Soviet and foreign presses on plague epizootology shows in all evidence that this infectious disease with natural focalization is characterized by periods of certain rises and falls in its activity, occurring both in time and space. This is responsible for the need for making further studies in the field of territorial differentiation of the less well studied plague foci.

Among such relatively poorly studied territories in this respect is the extensive northeastern portion of the Caspian Plain, lying within limits of Makatskiy and Zhilokosinskiy Rayons of Gur'yevskaya Oblast (the Embinsk and Transembinsk Plains).

It is not by chance that in recent papers of B. K. Fenyuk (1958) and Yu. M. Rall' (1958) the indistinctness of the relations between the Transural natural plague focus and the plain southwest Asian plague focus is emphasized. Undoubtedly, this erasure of boundaries and difficulty of differentiation of these foci is caused by the absence of natural obstacles to the dispersal and settlement of the main reservoirs of the plague microbe in the territory, which was particularly well confirmed in recent decades, when a shift occurred in the boundaries of the plague epizootic in the northeast and east Caspian regions against the background of a change in the areas of distribution of certain species of rodents.

We shall dwell specifically on this interesting and practically important process in our communication, attempting to detect the rules and regulations underlying the shift in the boundaries of epizootics in a number of maritime regions of Gur'yevskaya Oblast.

Modern Regression of the Caspian Sea and the Penetration of Rodents into Areas of Dry Land Exposed by the Receding Sea

Periodic variations in the level of the Caspian Sea occurring over many years and centuries and the phenomena of new formation or disappearance of dry land associated with it constitute characteristic features of this salt lake-sea, the largest on earth.

An extensive literature has accumulated on this problem, in which the reasons and the degree of variation of the Caspian Sea are analyzed in detail from geological and historical aspects, not excluding the very recent period.

Without being able to dwell on these problems in detail and referring those interested to the special literature, we should like to note only some characteristics of the modern regression of the Caspian Sea.

Shortly after the last peak of the so called neocaspian transgression (the end of the 18th century-beginning of the 19th century) a long period of regression of the sea occurred, which is continuing even now. However, this most recent history of the regressing Caspian Sea has been interrupted repeatedly by phases of stabilization or even temporary rises, noted by

many investigators even in the past century. Therefore, the rhythm of the Caspian Sea during the present-day regression can be depicted graphically in the form of a gradually declining curve with many peaks (A. V. Shnitnikov, 1956). A particularly sharp drop in this curve was noted from 1929 to 1957 when the level of the Caspian Sea dropped by more than 2.4 meters literally before our eyes, being at the lowest levels for the past 400 years (28 meters below sea level). The result of this regression was a reduction of the water table of the Caspian Sea by 30,000 square kilometers and a reduction of the extent of its coastal strip by more than 600 kilometers.

There was an appreciable reduction in the size of the shallow water area in the northern and northeastern parts of the Caspian Sea, where in the shortest time it regressed from its previous shores a distance of tens of kilometers, exposing the broadest areas of dry land (Fig 1).

The vegetation formed on these young territories has undergone different stages of development in accordance with the local conditions. Thus, on the north shore, where the freshening influence of the Volga, Ural and, to some degree the Emba Rivers, is expressed, on the dry strip (which not uncommonly reaches widths as of 30-40 kilometers) mixed vegetation groupings of the hairgrass-Russian thistle and of the Russian thistle type were formed with the predominance of annuals, in places forming thick areas of weeds. At the same time, bushes, represented by various species of the genus Tamarix, have become widespread here.

An inseparable element in the landscape of this area of the coast are Baer's hillocks [sandy ridges, well developed on the north shore of the Caspian Sea; the widths of these ridges are from 200 to 400 meters; their lengths, from several 100's of meters to 7-8 kilometers], many of which even recently have been represented by the most complicated archipelagos of sea islands or have risen like caps amid the inaccessible maritime marshes. In this respect, the extensive region of Baer's hillocks, called the Tentyak-Sor, between the Ural and Emba Rivers is particularly noteworthy in this respect. This is what A. L. Polenov and T. Deminskiy (1907) wrote about these unique places: "In going from Zhilaya Kosa to Gur'yev it is necessary to travel sometimes 300-400 versts [a verst is about a km] in order to circumvent the extensive quag areas. These latter, located along the sea and sticking out to the north like tongues, are impassable in either spring or summer. It is necessary to go around them far to the north almost to the Uil and then again drop down to the south in the direction of Gur'yev. In these impassable places which are profusely overgrown with bulrushes noma rest on the shore in the winter."

Later, with the regression of the Caspian, the Tentyak-Sor natural landmark lost its connection with the sea and, gradually drying up, was converted into a very complicated labyrinth from the alternation of Baer's hillocks and detritus-covered depressions, from time to time filled with water from the Sagiz River, and then only in the springtime. The result of these processes was the active accumulation of salt in the former bay-depressions, the extinction of the reed associations and the vigorous incorporation of halophytes, which developed particularly luxuriously on the cumulative shell-sand alluvia rimming the bases of the hillocks like a border.

The evolution of the landscapes on the areas of dry land from which

the sea had receded, lying to the south of the Emba, proceeded by somewhat different routes. The absence of a river drainage in this part of the coast and the high degree of salt in the exposed bottom bring about a partial or complete elimination not only of mairgrass-reed combinations but also transitional plant groupings (of meso- and xerohalophytic types), so characteristic of dried-up territories in the northern part of the Caspian Sea, from the spectrum of phytocoenoses created. For this reason phytocoenoses of the halophytic type with a predominance of perennial bushes and subshrubs occupied the leading position on the east coast. Here, Halocnemium strobilanum has become particularly widespread, being located along the margins of the numerous small depressions and along the periphery of dried-up bays (Mertvyy Kultuk, Kaydak); niter bushes, characteristics of accumulations of gypseous gray sand along the seashore, have also become common.

Changes occurring in the appearance of the maritime landscapes have predetermined the possibility of active settlement of various animals in the maritime region. However, in various areas along the coast processes of dispersal and settlement of rodents have not occurred at the same rate and have been qualitatively different, which has been determined, on the the one hand, by the species spectrum of animals from the original shore, the place from where the immigration started, and, on the other hand, by the type of vegetation created on areas of dry land exposed by regression of the sea. Thus, for example, in the area between the Ural and Emba Rivers, where there has been an appreciable influence from the freshening effect of the Ural River and, as a result of this, where mixed plant groupings of annuals have developed well on a broad coastal strip, the following rules and regulations for the distribution of rodents are observed.

The first strip from the sea (width of 1 to 4 kilometers), occupied by hygrophytic vegetation and periodically flooded even when there are light driving winds, cannot, for practical purposes, be inhabited by rodents, because the damp oozy bottom of the coast and the proximity of subsoil water are limiting factors even for the moisture-loving forms of life.

Three-four kilometers from the seacoast or 30 kilometers from the main shore (the second strip with vegetation of the hygrohalophytic type), on areas of dry land which are much less often flooded and in the canebrakes the gray vole and the house mouse appeared, first in very small numbers and then in appreciable numbers, whereby, as has been noted by I. I. Khudyakov, P. Ye. Nayden and R. V. Gintlis, the first mouse-holes were found on those areas where the drop in the level of ground water approaches 100 centimeters. In the winter time, in this strip, not uncommonly the gray hare /Lepus europaeus/ and, of the group of carnivorous animals, the Siberian polecat /Mustela eversmanni/, the fox and tatar-fox, are found along this strip.

Five-six kilometers from the sea-coast, or 25 kilometers from the main shore, with the appearance of the glasswort, seepweed and alkaligrass among the plant groupings (third strip), colonies of crested jirds and then meridional jirds are encountered sporadically; the colonies of these animals occur here exclusively on elevated components of the microterrain, which entirely recently consisted of sand bars in the sea, islands or shelves.

Even farther from the water's edge, on a broad strip occupied by mixed groupings of saltworts, pribrezhnitsa *, tamarisks and sea lavenders, not

* transliterated from Russian

uncommonly forming a closely knit bush-grass covering, the population density of the rodents increases considerably. This part of the dry land (fourth strip), exceedingly rarely flooded (only with the strongest driving winds or stormy overflows of the Ural River), can be called the optimum zone for colonies of house mice, meridional and crested jirds. We noted a particularly high census of these species along the edges of drainage canals, which in many places broke through the maritime lowlands, near winter camps abandoned by shepherds as well as in old ostozh'ye* surrounded by low embankments and overgrown with dense weeds. The constant abundance of rodents in such places attracts predators here. As the main shore is approached, with the appearance of Halocnemium in the plant groupings (fifth strip) with vegetation of the xerohalophytic type, the colonies of the gray vole disappear and there is an appreciable reduction in the census of house mice; however, the total species spectrum of rodents is full because of the inclusion of new representatives of steppe and desert fauna in it: the dwarf and, less often, yellow susliks, jerboas and sometimes mole voles [Ellobius talpinus].

On the main shore, corresponding to the level at which the waters of the Caspian Sea were in 1930, colonies of the great sand rat appear together with fully formed phytocoenoses of the desert type (at the Gogol'skiy landmark, Rakusha and Zhilaya Kosa), and the groupings of the animals now include a complete set of species characteristic of desert landscapes.

Therefore, in the area between the Ural and Emba rivers the broad coastal strip of dry land which emerged from under the water can be regarded as an optimum zone for the dispersal and settlement of house mice and crested jirds; here, in the presence of good food and protective conditions almost every year a relatively high and stable census of these species is noted (A. A. Lavrovskiy, 1959).

We observed a somewhat different picture of dispersal and settlement of rodents on the east bank of the Caspian Sea (from the lower reaches of the Emba River to the coastal strip of Buzachi and Mangyshlak inclusive), where on the extensive territories of recently exposed sea bottom a solonchak desert has formed with the characteristic vegetation of a halophytic type on it. On these places on the coast mainly the great sand rat appears, which penetrates from the main shore toward the receding sea for tens of kilometers in places. Thus, on the section of the coastline between Karaton and Prorva, solitary colonies of the great sand rat were found at a distance of 10-15 kilometers from the main shore in an extremely uniform locality with very occasional Halocnemium bushes and annual Russian thistles. Moreover, in the region of the Mertvyy Kultuk natural landmark this sand rat settled the former islands of Buinskiye, Lebyazh'i and Pustynnye, at present located in the center of a tremendous salina a distance of more than 30 kilometers from the main shore.

After the great sand rat and sometimes at the same time, the red-tailed jird penetrates into the new areas of dry land, whereby the colonies of the great sand rat not uncommonly are unique outposts for the colonization of other rodents in these places. As we have ascertained, the dispersal and settlement of sand rats in this territory occurs actively and rapidly; however, the animals do not always succeed in taking a firm hold in the new places. It is sufficient to present the following characteristic example. In the spring

[*transliterated from Russian; possibly means hayrick]

of 1957 we had the opportunity of investigating a small island (with an area of about 1.5 hectare), which had recently appeared in the northwestern part of the Buzachi Peninsula. Separated from the Burunchuk Sand Bar by an area of 5 kilometers of almost barren salina and a small shallow-water narrow, this island was found to be already inhabited by the house mouse and the red-tailed jird. Fresh mouse-holes were located under clumps of very occasional tamarisk plants, and an inhabited colony of red-tailed jirds occupied the central highest part of the island with holes going down into the layer of finely broken shells, permeated by cane rhizomes and the abundant Turneforcia here. It is perfectly obvious that the rodents were able to penetrate here only from Burunchukskaya Kosa /sand bar/ during the time when the outgoing tidal winds were drying the shallow water narrow. The next year, the colony of red-tailed jirds was not found on this island.

The meridional jird, yellow suslik and sometimes the house mouse follow the great sand rat and red-tailed jirds into the solonchak region along the coast; we found these rodents for the first time at a distance of 8-10 kilometers from the main shore in places where ashen /this may be an error for eolovyye, which means wind deposits/ deposits of dust-like sands are observed with a more heterogeneous vegetation, represented by Atriplex verrucifera and solonchak wormwood shrubs in addition to the main plants, Halocnemium strobilanum and annual Russian thistles.

At a distance of 4-5 kilometers from the main shore we found dwarf susliks (the southern boundary of distribution of the dwarf suslik on the east coast of the Caspian Sea goes 15 kilometers to the south of the Karaton natural landmark. Then, the boundary of the area of distribution of this species turns to the east and ends within the limits of Ustyurt) and solitary specimens of jerboas, and in the transitional zone before the main shore (on a strip 1-1.5 kilometer wide), cut through by a multitude of beds of dried-up channels with strips of stunted cane preserved in some places (very recently in these areas of the coast the water vole lived, which was pointed out by A. M. Kolosov in 1935, and which was confirmed by our findings of the skulls of this vole near the village of Karaton), house mice were found in considerable numbers.

We found particularly high population densities in colonies of sand rats and susliks as well as the most complete species spectrum rodents on the elevated areas corresponding to the previous coastal strip and the islands of Boyardy, with the ruins of Kazakh earthen huts and fishing villages scattered everywhere.

Comparative data on the census and disposition of the rodents in various places along the coast, including areas of dry land which recently emerged from under the water, are given in Table 1.

These in their most general outlines represent the picture of colonization of different areas of the Caspian Sea-Coast by rodents. An essential influence on these processes is exerted by winds which occur periodically on the Caspian Sea, "moryany" /driving winds from the sea/, in which the water, gradually coming onto the lowland shore, displaces the rodents from the areas of dry land which they colonized. During strong, stormy moryany, when the tide water comes in precipitously, not only a displacement but also a mass extermination of the rodents in the flooded zone is observed.

Table 1

Disposition and Census of Small Animals in Various Parts of the Coast of the Caspian Sea (Data of 1957-1958 Investigation)

Характеристика места учета и расстояние его от коренного берега и от уреза воды (в скобках) (1)	(2) Добыто зверьков за сутки на каждые 100 ловушек и 50 капканов														
	(3) больших песчанок	(4) красохвостых песчанок	(5) полудневных песчанок	(6) гребенщиковых песчанок	(7) малых сусликов	(8) желтых сусликов	(9) больших тушканчиков	(10) малых тушканчиков	(11) земляных зайчиков	(12) домовых мышей	(13) обыкновенных полевок	(14) серых хомячков	(15) ушастых ежей	(16) хомячок	(17) всего
(18) *Участок побережья между Уралом и Эмбой*															
(19) Затопляемые сырые места побережья; 25—30 км (0—3 км)	—	—	—	—	—	—	—	—	—	—	—	—	—	--	0
(20) Тростниковая; 20 км (10 км)	--	—	—	—	—	—	—	—	—	2	1	—	—	--	3
(21) Тростниково-солеросовая; 15 км (15 км)	—	—	—	1	—	—	—	—	—	20	2	—	—	—	23
(22) Гребенщиково-кермековая; 8—10 км (20—22 км)	—	—	5	13	—	—	—	—	—	36	4	—	—	—	58
(23) Солянково-сарсазановая; 3—4 км (26—27 км)	—	—	2	2	1	—	—	—	1	6	—	—	—	--	12
(24) Коренной берег, полынно-солянковая (30 км)	7	—	5	2	3	—	1	—	1	15	—	—	1	-	35
(25) *Участок побережья между Эмбой и сором Мертвый Култук*															
(26) Пухлый солончак, 25—30 км (5 км)	—	—	—	—	—	—	—	—	—	—	—	—	—	-	0
(27) Сарсазановая; 15 км (20 км)	2	—	—	—	—	—	—	—	—	—	—	—	—	—	2
(28) Сарсазаново-солянковая; 10 км (25 км)	5	1	1	—	—	—	—	—	—	2	—	—	—	--	9
(29) Солянково-сарсазаново-лебедовая; 4 км (29 км)	5	3	4	—	3	1	—	1	—	2	—	—	--	—	19
(30) Тростниково-солянковая, вдоль русла протоки; 1—1,5 км (30—30,5 км)	—	1	1	—	—	—	—	—	—	27	—	—	1	--	30
(31) Коренной берег, полынно-сарсазановая с эфемеровой синузией (35 км)	16	3	8	—	6	1	3	1	1	5	—	—	1	—	45

1. characteristics of the place under consideration and distance of it from the main shore and from the water's edge (in parentheses); 2. number of animals caught per day for every 100 mouse traps and 50 leg traps; 3. great sand rats; 4. red-tailed jirds; 5. meridional jirds; 6. crested jirds; 7. dwarf susliks; 8. yellow susliks; 9. alactagas /Allactaga /Table 1, continued next page/

Характеристика места учета и расстояние его от коренного берега и от уреза воды (в скобках) (1)	(2) Добыто зверьков за сутки на 100 давилок и 50 капканов															
	большая песчанка (3)	красохвостая песчанка (4)	полуденная песчанка (5)	гребенщиковая песчанка (6)	малый суслик (7)	желтый суслик (8)	большая тушканчик-лопатонос (9)	малый тушканчик (10)	земляной заяц (11)	домовая мышь (12)	обыкновенная полевка (13)	серый хомячок (14)	ушастый еж (15)	землеройка	всего (16)
(32) Побережье Бузачинского полуострова (мыс Бурунчук)															
(33) Островок с тростником и солянками; 20 км (0 км)	—	9	—	—	—	—	—	—	—	4	—	—	—	—	13
(34) Сор, лишенный растительности; 18 км (2 км)	—	—	—	—	—	—	—	—	—	—	—	—	—	—	0
(35) Песчано-ракушечная гряда (бывшие острова); 15 км (5 км)	2	5	2	-	—	13	—	—	—	4	—	—	1	1	28
(36) Сор зарастающий; сарсазановая; 7 км (13 км)	9	—	—	—	—	—	-	1	—	—	—	—	—	-	10
(37) Окраина сора и коренной берег; полынно-сарсазановая (20 км)	23	2	—	-	—	2	—	1	-	-	—	1	—	—	29
(38) Побережье южной части Мангышлака (урочище Саржа)															
(39) Дюнные пески вдоль берега моря; 3 км (0 км)	—	—	12	—	—	—	—	—	—	2	—	1	—	—	15
(40) Окраина сора; гребенщик, песчаная полынь, сарсазан; 1,5 км (1,5 км)	15	1	14	—	—	—	—	—	—	—	—	—	—	—	30
(41) Граница сора и коренного берега; гребенщиково-сарсазановая (2,5 км)	5	2	5	—	—	—	—	—	—	—	-	2	—	—	14
(42) Коренной берег, бугристый ландшафт (3 км)	5	3	—	—	—	—	—	2	3	—	—	—	2	—	15

/Table 1, continued from previous page/ jaculus/; 10. jerboas /Allactaga elater/; 11. jerboas /Allactagulus acontion/; 12. house mice; 13. meadow voles; 14. gray hamsters /Cricetulus migratorius/; 15. long-eared hedgehogs /Erinaceus auritus/; 16. shrews; 17. total; 18. section of the coast between the Ural and Emba Rivers; 19. damp flooded places on the coast; 20. cane; 21. cane-glasswort; 22. tamarisk-sea lavender; 23. Russian thistle-Halocnemum; 24. main shore, wormwood-Russian thistle; 25. section of the coast between the Emba River and the Mertvyy Kultuk salina; 26. puffic solonchak; 27. Halocnemium; 28. Halocnemium-Russian thistle; /Table 1, continued next page/

/Table 1, continued from previous page/ 29. Russian thistle-Halocnemium-pigweed; 30. cane-Russian thistle, along the channel bed; 31. main shore, wormwood-Halocnemium with ephemeral synusia; 32. coast of Buzachi Peninsula (Cape Burunchuk); 33. islets with cane and Russian thistle; 34. salina without vegetation; 35. sand-seashell ridge (former islands); 36. weed-overgrown salina; Halocnemium; 37. the edge of the salina and main shore; wormwood-Halocnemium; 38. coast of the southern part of Mangyshlak (Sarzha natural landmark); 39. sand dunes along the sea-shore; 40. edge of salina; tamarisk wild sage, Halocnemium; 41. boundary between the salina and main shore; tamarisk-Halocnemium; 42. main shore, hilly landscape.

As an example we can point to a spring moryan in 1946, as a result of which on the north shore of the Caspian Sea the water came right up to the fishing villages of Bol'shoy Mokriy, Gogol'skiy, Rakusha, and Zhilaya Kosa which had been abandoned many years ago, that is, it flooded a strip of 20-25 kilometers which had previously been perfectly dry. The autumn moryan of 1952 is particularly memorable; at that time, under the force of a southwesterly hurricane wind, which raged for several days, tremendous masses of water were dashed against the shore and flooded extensive areas of the desert.

A similar effect on coastal fauna is exerted by the stormy spring overflows of rivers feeding the Caspian Sea. Thus, for example, in 1942, the flood water of the Ural River coming from the shores flooded a tremendous area in the lower reaches of this river, causing the death of the rodents living there (I. M. Mamontov, 1957). The same thing was repeated in the spring of 1957, at which time extensive lowland areas of the sea-coast which prior to that time had been solidly colonized by the house mouse, meridional and crested jirds, were covered with water from the overflow of the Ural and Emba Rivers. Any elevation, small islet, and sometimes haystacks or the ruins of Kazakh mud huts served as shelter for the animals trying to save themselves from the oncoming water. Naturally, under these conditions the concentration of rodents on localized areas of dry land, just as at the junction between the main shore and the flood zone, was unusually great, whereby in parallel with the increase in the population density of the rodents there was an increase in the degree of interspecies and intraspecies contact among their populations.

After the water recedes on territories which had been flooded, the plant cover, as a rule, develops particularly luxuriously, and the excellent food advantages of these areas attract mice and sand rats here from nearby unflooded places. The new influx of the immigrant rodents and intense multiplication of them under optimum conditions lead to the fact that at the end of the year, or at most, two years, the biocoenoses are fully restored here.

Dispersal and Settlement of Certain Rodents and Change of their Areas of Distribution in the Northeast Caspian Region

Analyzing the history of formation of the fauna of the Caspian lowlands, we automatically direct attention to the close connection existing between the occurrence of "waves of migration" among the animals and the .

time at which marked changes occur in the natural landscapes.

It is easy to see that such "waves" were inevitably synchronized with the repeatedly occurring variations of the Caspian Sea and served as the cause of a change in the boundaries of the areas of distribution of many species of animals.

Many investigators have directed attention to the leading part played by variations in the sea level in the evolution of areas of distribution of animals. Thus, N. K. Vereshchagin and I. M. Gromov (1952) believe that during the periods of regressions of the Pleistocene Caspian and in the subsequent xerothermal phase of the Holocene the active migration of desert faunal combinations, probably including such rodents as the yellow suslik and the great sand rat, occurred from east to west. D. M. Shteynberg (1954), analyzing the reasons for the discontinuity of the areas of distribution of a number of animals in the area between the Volga and Ural Rivers, connected these characteristics with the direct influence of variations in the level of the Caspian Sea in the late Tertiary. However, the method of retrospective analysis, to which not uncommonly investigators resort, is not always capable of bringing us closer to the understanding of all the various, complicated and not uncommonly controversial processes occurring in the Caspian Plain. At the present time, when we are the witnesses of the next deep regression of the Caspian Sea, it would be particularly unforgivable to overlook this noteworthy "experiment of nature," which puts the most interesting factual material into the hands of investigators.

Undoubtedly, many modern species of animals, including rodents of interest to us, possess great potentiality for dispersal and settlement which, however, is realized only when certain favorable conditions occur. An infinite number of examples confirming this conclusion could be given: the vigorous dispersal and settlement of the dwarf suslik in the Precaucasus; the advance of the gray hare into western regions of Siberia and Kazakhstan; the expansion of the area of distribution of the Dagestan hamster into the Don and Stavropol' steppes; periodic penetrations of the tarbagan and hoofed jird /Meriones unguiculatus/ into the Transbaikal from the Mongolian People's Republic, and others.

Specifically such favorable conditions for the dispersal and settlement of desert forms of rodents were created in recent decades in the Caspian Lowlands, where following the drop in the level of the sea new extensive areas of dry land of the arid type appeared, and marked changes occurred in the maritime landscapes in the direction of desert formation.

In the northeast and east Caspian the tendency toward dispersal and settlement was demonstrated in the most clearcut manner among the group of sand rats. A number of factors contributed to the successful migrations of these animals: 1) the sufficiently high degree of mobility of the sand rats and the broad range of their adaptations; 2) the disappearance of previously existing obstacles to dispersal and settlement as the result of drying of rivers and lakes, gulfs and coastal marshes with the gradual transformation of them into a multitude of saline depressions; 3) the appearance of new optimum habitats for the rodents--the exposed light-textured bottoms of sea littorals (alluvial sand, shell-covered shoals), the luxurious development of Russian thistle-psammophyte vegetation near the salinas and in the sand

dunes; 4) farming activity of man, who after exploring the commercial stocks of the most valuable types of raw material along the sea, constructed irrigation channels, oil pipelines and dams here, laid down dirt roads and narrow-gauge railroad lines.

Of particularly great zoogeographic and epizootological interest is the process of restoration of the previous area of distribution of the great sand rat, being observed at the present time in the area between the Emba and Ural rivers. In this connection, the original studies of paleozoologists working at Mangyshlak (I. M. Gromov and V. A. Fokanov, 1959) and in the lower reaches of the Ural River (N. K. Vereshchagin and I. M. Gromov, 1952; I. M. Gromov, 1957) should be noted; according to them, the regression of the Khvalynsk Sea, which marks the beginning of drying of the climate and desert formation in the landscapes of the Caspian region, serves as the cause of migration of desert forms of rodents (great sand rat, meridional jird, jerboa [Scirtopoda telum]) to the north of present-day boundaries (Fig 2). Later, apparently in connection with the change in the landscape in the direction of a certain mesophyllic nature of the vegetation, the Pleistocene expansions were replaced by a period of dying-out and recession of these species toward the southeast. Undoubtedly, such variations in the areas of distribution could have occurred in the Holocene also, against the background of change in the climate and repeated variations of the Caspian Sea.

We find interesting data on historical changes in the area of distribution of the great sand rat in the work of A. N. Formozov (1938), who, after analyzing in detail the problem of the reasons for the extinction of certain rodents in Kazakhstan (the yellow lemming, great sand rat, mole vole, steppe cony), concluded that the reduction of the areas of distribution in species of central Asiatic origin is associated with a deterioration of conditions for their existence as the result of a change in climatic factors. In his work, A. N. Formozov, referring to A. M. Kolosov (1935a, 1935b), pointed out that the most northwesterly point at which the great sand rat was found was the Koschagyl natural landmark, located on the left bank of the Emba River, 100 kilometers from the sea. (A. M. Kolosov, who made his investigations in the lower reaches of the Emba River in 1935, points out that he found hair and teeth of a great sand rat in the Dzhil'dybay natural landmark (near Makat), one case, and in the Uzungach natural landmark, one case; in addition he mentions the Bek-Beke Sands, where apparently the great sand rat was encountered at that time. At the same time, in contradiction with these data, A. M. Kolosov writes in his conclusion that the northern boundary of the area of distribution of this species probably passes along the left bank of the Emba River, in the region of Koschagyl, where a single great sand rat was caught).

Further observations in the change of the distribution of the great sand rat showed that in recent decades a process of restoration of the area of distribution of this species is being realized in the northwest Caspian. While N. A. Bobrinskiy and others (1944), like A. N. Formozov, drew the northwestern boundary of the area of distribution of the great sand rat along the left bank of the Emba River, B. S. Vinogradov and I. M. Gromov (1952) noted that at this time the great sand rat was being encountered in

the environs of the settlement of Rakusha, which is 30 kilometers to the west of the Emba River.

The boundary of the area of distribution of the great sand rat was studied in greater detail by zoologists of the Gur'yev Station. Thus, I. I. Khudyakov, Ye. Ye. Nayden and R. V. Gintlis, studying the maritime section of the area between the Ural and Emba rivers in 1951, found its colonies 15-20 kilometers to the west of Rakusha Settlement, and in the last seven-eight years the great sand rat was found in the environs of Gur'yev, 8-10 kilometers to the east of the Ural Floodplain, opposite the settlement of Yamankhalinka, 18 kilometers to the north of Dossor Settlement, 6 kilometers to the south of the Makat Railroad Station; in the Sagizmeshita Natural Landmark on the right bank of the Sagiz River and, finally, in the Kaynar Natural Landmark according to reports and personal communications of the zoologists of the Gur'yev Station.

Therefore, the northern boundary of distribution of the great sand rat in the area between the Ural and Emba rivers at the present time is approximately at a latitude of 47° 60'.

According to the material of Ye. P. Bondar' (1956), A. S. Burdelov and M. N. Leont'yeva (1956), S. N. Varshavskiy and M. N. Shilov (1956), M. I. Ismagilov (1957), M. N. Shilov (1957), in the eastern part of the area of distribution, including the Betpak-Dalu Desert and the northern Balkhash area, the great sand rat, as a rule, does not cross 47° north latitude anywhere; only in the region of Zharkamys, Chelkar and Irgiz (the northern Aral region) is an extensive projection delineated which almost approaches the 48th parallel.

At the present time, apparently, it may be considered that the great sand rat in the area between the Ural and Emba rivers has reached or almost reached its rubicon, which coincides with the landscape boundary of this species, the northern margin of the desert zone.

Let us concentrate on certain details in the dispersal and settlement of the great sand rat in the northeast and east Caspian region. Of particular interest in this respect is the Tentyak-Sor Natural Landmark. As has already been mentioned, 40-50 years ago this extensive natural landmark with an area of more than 300,000 hectares represented a very complicated system of gulfs of the Caspian Sea with an infinite number of islands rising above the marshy impassable plain. Not a single naturalist visited these places previously, and we do not know what rodents lived on Baer's hillocks in Tentyak-Sor at that time; we can judge this only indirectly from the current composition of the rodent fauna on the territory of interest to us. (On Baer's hillocks in Tentyak-Sor we caught several species of rodents: the great sand rat and meridional jird, dwarf suslik, jerboa /Allactagulus acontion/, Scirtopoda telum, small alactaga /Allactaga elater/, mole vole, and house mouse. Therefore, at the present time, the basic rodent fauna living here is represented by desert forms. Unfortunately, we could not collect bony remains of extinct animals in Tentyak-Sor with the exception of fragments of a hair seal's skull found in the marine deposits). After the gulfs dried and changed to a system of salinas, immigrant rodents from adjacent desert areas, including the great sand rat, could enter here easily.

The Tentyak-Sor natural landmark, which we visited twice (1955 and 1958), was constantly densely inhabited by great sand rats. It was hard to find any Baer's hillocks from which this rodent was absent; they clearly preferred settling on sand-shell alluvia at bases of the hillocks, where Russian thistle grew particularly well. The inhabited colonies fairly often fused into solid chains

rimming the salinas and were sometimes found on the slopes and even peaks of the hillocks with numerous jerboa and dwarf suslik holes.

Therefore, after penetrating into the Tentyak-Sor region, the great sand r undoubtedly entered an area of optimum conditions. (The possibility has not been ruled out that during the period when the Caspian was at a higher level, when the depressions between the hillocks in this natural landmark were filled with water, the great sand rat could have survived on some of the hillocks of Tentyak-Sor. However, even with this supposition the general ideas about the restoration of the area of distribution and rapid increase in the census of this species in the area between the Ural and Emba rivers are not substantially changed.) Represented here by a very vigorous and actively multiplying population, it colonized its territory rapidly, and in the last two years, after settling the maritime region for a distance of 6-8 kilometers from the main shore, came into very close contact with populations of house mice, meridional and crested jirds.

We have studied in detail the process of dispersal and settlement of the great sand rat in the direction of the receding sea in the Zhilaya Kosa and Atrau natural landmarks (Fig 3). Thereby, it should be noted that 1958 was a year of active multiplication of this species of sand rat and was characterized by a particularly high degree of mobility of the great sand rats not only in the area between the Emba and Ural rivers but also in other parts of the area of distribution, which, for example, was well shown by S. N. Marin (1959) for the territory of the Aral Sea region of Kara Kumy.

The change in the area of distribution of the red-tailed jird in the Caspian region is of the same great interest. However, it should not be overlooked that data published in the literature on the northwest boundary of distribution of the red-tailed jird in the Caspian region suffer from great inaccuracies. Even in such a capital work as the classifier of mammals of the USSR (Bobrinskiy and coauthors, 1944), we find a statement that the red-tailed jird lives in the lower reaches of the Ural River, which was clearly erroneous for that time; the same error was unfortunately repeated in the summary by A. V. Afanas'yev and coauthors (1953).

As a matter of fact, according to material of the Gur'yev Plague-Control Station, the northern boundary of the area of distribution of the red-tailed jird quite recently went along the northern fringe of western Ustyurt and the Mertviy Kultuk salina. Nevertheless, I. I. Khudyakov, P. K. Nayden and R. V. Gintlis caught this rodent (and in no small numbers) in the vicinity of the village of Karaton (30 kilometers to the south of the Emba River) and in the adjacent areas along the sea-coast. In 1957, red-tailed jirds were caught directly on the left bank of the Emba River in the Baybet natural landmark, and in 1958 the red-tailed jirds not uncommonly were caught in the traps on the right bank of this river, opposite the Baybet landmark just mentioned (R. V. Kovaleva and coauthors, 1959; A. A. Lavrovskiy, 1959). Finally, which is particularly important, two red-tailed jirds were caught in colonies of great sand rats in the region of the Korsak Settlement, located 15 kilometers to the west of the Emba River.

Therefore, the tendency toward dispersal and settlement of this desert species in the north along the coast of the Caspian Sea is beyond doubt. The latter fact is confirmed particularly by the studies of A. M. Kolosov (1935), who was unable to find the red-tailed jird in the lower reaches of the Emba River either on the left or right banks of this river. (A. M. Kolosov examined more than 2000 fox and tatar-fox excretions collected in the Emba and

Transemba Deserts. In no case were bony remnants of the red-tailed jird found).

We do not have the opportunity here to go into details about the dispersal and settlement of other rodents in the northeast and east Caspian region (the yellow and dwarf susliks, meridional and crested jirds, jerboas and house mice) particularly since this has already been mentioned in general outlines in the previous chapter. It should be noted only that among the species listed, in contrast to the great sand rat and red-tailed jird, the processes of dispersal and settlement in this part of the Caspian Plain did not substantially change the boundaries of the areas of distribution. (It should be noted that while the regression of the Caspian Sea constituted an impetus for dispersal and settlement of desert forms of rodents, the steppe and more moisture-loving forms, conversely, showed reductions in their numbers and in a number of places disappeared under the conditions of increase in the dryness of the climate in the maritime strip. For example, the common hamster [Cricetus cricetus] and the steppe lemming [Lagurus lagurus] stopped being found in the lower reaches of the Emba River; the Norway brown rat and water vole, stopped being found in the section of the coast between the Ural and the Emba River, etc.).

Characteristics of the Shift of the Boundaries of Plague Epizootics in the Northeast and East Caspian Regions

In the last decade plague has penetrated into regions where previously it was entirely unknown (for example, the southern regions of the area between the Emba and Ural rivers, the Tentyak-Sor Natural Landmark and the vicinity of Gur'yev) in the northeast and east Caspian regions against the background of general activation of plague epizootics. In a number of cases active epizootics occurred on the territories which as far as we remember were under the waters of the Caspian Sea or even "yesterday" represented a system of shoals, sand bars or islands in the sea (see Fig 2). Such a rapid spread of the plague infection into areas of dry land recently formed can be correctly understood in the light of those changes in the landscapes and dispersals and settlements of the number of animals in the Caspian region which they caused, about which we have spoken in detail in the first sections of this article.

As an illustration of this thesis we are analyzing data on the dynamics of the plague epizootics in certain maritime regions of Gur'yevskaya Oblast.

First of all, we should like to dwell on events occurring in the Buzachi Peninsula. As is well known, plague epizootics among great sand rats and other rodents in this part of the Caspian Plain were first established in 1926 (A. K. Ignat'yev, 1927; S. M. Nikanorov, 1927). It is important to note that during those years the area and outlines of the coastal strip of Buzachi were absolutely different: extensive shallow-water areas of the gulfs at that time separated a group of islands of the Kirel' Archipelago, covered by cane along the shores. Naturally, the sand rats could not get onto these islands at that time.

The second cycle in the epizootic wave, demonstrated on Buzachi Peninsula, was in 1948, when on the background of a marked increase in the census of great sand rats here an acute diffuse epizootic was noted with the

involvement of a large group of animals in it: great sand rats and red-tailed jirds, house mice, foxes and camels (N. P. Arkhangel'skaya and co-authors, 1957: reports of the Gur'yev Station). In the 22 years which have elapsed since the first events on Buzachi, the maritime landscapes of the peninsula have changed beyond recognition: extensive sea straits have dried up, canebrakes have disappeared, and numerous islands included in the Kirel' Archipelago (Dolginskiye, Orta-Espinskiye, Burunchukskiye) have joined the continent. This entire territory, at the time of the occurrence of epizootics in Buzachi, was already colonized by rodents and fleas which had come there from continental territory. Thereby, as was shown, the population density of the sand rats, like the flea census in the holes and on the rodents themselves in these new territories, was unusually high (the average population density of great sand rats per hectare was 23: the maximum was 70; the average flea index per rodent was 60).

The first cultures of the plague microbe from great sand rats and red-tailed jirds in the maritime regions (the Put'k Kommunizmu Kolkhoz of the Orta-Espinskiy Rural Soviet) were obtained 17 and 22 June 1948. Subsequently, over the course of a year on this territory alone 20 cultures were isolated (including one from a camel in the Karazhan-Bas Natural Landmark). As on the old continental areas of Buzachi Peninsula, the epizootic here was very active, so that the need arose for conducting extermination operations with repeated baiting of sand rats on the same areas on which the epizootics had remained in the most stable manner. The work was done mainly along the coastal strip (from the former Cape Dolgiy to the Burunchuk Sand Bar, a distance of more than 50 kilometers), where on the marine sand-shell alluvia a particularly high rodent and flea census was noted.

The epizootics on Buzachi, including the regions along the sea, of which we have just spoken (the Orta-Espinskiye "focus"), lasted through 1951. However, by the autumn of that year they took on a very focal nature and gradually subsided (P. I. Shamanek, 1959).

In Zhilokosinskiy Rayon of Gur'yevskaya Oblast (the Transemba Plain), the epizootic among great sand rats was demonstrated for the first time also in the spring of 1948 (Amangel'dinskiy Village Soviet) 70-80 kilometers from the sea-shore. In 1950, the plague epizootic was also found on the left bank of the Emba River (the sand area of Kara Kumy, vicinity of the Koschagyl, Kul'sary and Tyulsi Natural Landmarks) a considerable distance from the coastal strip. However, in 1951 the epizootic was activated here and spread in northerly and northwesterly directions (N. P. Arkhangel'skaya and others, 1957). It occurred particularly acutely in the vicinity of the village of Teren-Ozek, located in the region of the receding Caspian (the former islands of Boyardy) and on the right bank of the Emba River in the Korsak region (a section bordering on the Tentyak-Sor natural boundary). In the latter case, the epizootic occurred on young colonies of the great sand rat, which, as has already been mentioned, probably penetrated into this region after the regression of the Caspian and the drying of the mouth of the Emba.

In subsequent years, 1952-1954, epizootics were recorded only in the left-bank regions of Zhilokosinskiy Rayon and showed a "tendency toward moving" toward the east and northeast in the direction of Aktyubinskaya Oblast. However, in the autumn of 1956 the plague microbe cultures were

again obtained from great sand rats and jirds in the area between the Emba and Ural rivers. As shown by investigations of epidemic-control detachments of the Gur'yev Station, epizootics occurred in the vicinity of the Komsomol'sk, Bek-Beke, Sagiz, Koshkar oil fields, that is, they penetrated into the Tentyak-Sor Natural Landmark, densely inhabited by great sand rats (it may be supposed that the epizootic process in these places had been occurring continously since 1951, when a culture of the plague microbe was first obtained from great sand rats caught in the vicinity of Korsak on the right bank of the Emba River).

In 1957, the boundaries of the epizootics in the area between the Emba and Ural rivers were appreciably expanded. Aside from the old "points" cultures were also isolated in the central portion of this natural landmark (the Tentyak-Sor oil exploration area), whereby, aside from great sand rats meridional jirds were involved in the epizootic process.

The epizootic situation became particularly complicated in the area between the Ural and Emba rivers in the spring-summer of 1958. An acute diffuse plague epizootic was recorded over tremendous territory, including Gur'yevskiy, Makatskiy and Zhilokosinskiy rayons. By comparison with 1957, the center of the epizootic apparently shifted to the southwestern portion of Tentyak-Sor, from where it spread rapidly in the direction of Gur'yev, reaching its nearest environs, while in the south it dipped toward the maritime plain, represented by young landscapes formed at the site of the receding Caspian. In the zone along the sea an epizootic was recorded over a broad strip inhabited by sand rats and house mice; the cultures here were isolated on a section of the coast from the village of Rakusha in the east to the Bol'shoy Mokryy and Kamynin landmarks in the west.

A characteristic feature of the 1958 epizootic in the area between the Ural and Emba rivers was the mass involvement of house mice in it (R. V. Kovaleva and others, 1959a: B. K. Fenyuk and others, 1959). Infection of other rodents, not counting great sand rats, was thereby very slight (solitary meridional jirds and one Scirtopoda telum). The absence of specific disease among the colonies of the dwarf suslik, despite the high census and close contacts between these animals and great sand rats, attracted particular attention; this applied also to places where the epizootics were occurring very actively.

As far as the penetration of plague epizootics into the maritime plain was concerned, this process was not a chance phenomenon against the background of the 1957-1958 events: it was prepared for by the entire previous history and particularly by such a qualitative change in the maritime faunal combinations as the appearance of a new component in them, the great sand rat.

The immediate impetus which put into movement the epizootic process in the region along the sea consisted of stormy spring overflows of the Ural and Emba rivers in 1957. As the result of flooding of large territories along the sea, the rodents displaced from these places in colossal numbers concentrated on the elevated components of the terrain: along the fringe of the main shore with its ubiquitous Kazakh mud huts, on islands, on sand-shell alluvia and along the edges of canals, specifically in these places the closest contact was made between house mice, meridional and crested jirds, on the one hand, and great sand rats on the other. As early as after the recession of

the flood wave and appreciable thinning-out of the rodent population we found a mass of food remains, an infinite number of tracks and whole heaps of droppings left by house mice and sand rats in the colonies of the great sand rats. Naturally, under these conditions, interspecies and intraspecies contacts, just like the mutual exchange of parasites, was unusually extensive among the rodents. Thus, for example, in house mice, caught in large numbers, not only in the mouse traps but also in the leg traps set at the inhabited and empty holes of the great sand rats, not uncommonly specific great sand rat fleas (Xenopsylla scrisbini) were found. We have no doubt that specifically at the junction of the main shore and the lowland areas along the sea (the region of the ruins of Kazakh mud huts) in the summer of 1956 the plague pathogen penetrated into the population of house mice from the great sand rats.

As the result of deaths from floods and probably as a result of epizootics the census of meridional and crested jirds (previously quite high) dropped to a minimum by 1958 in the region along the sea. The great sand rats, which had settled only on the elevated parts of the terrain and were more resistant to plague, were preserved in these places in appreciable numbers: in 1958, they multiplied actively and vigorously dispersed and settled in the direction of the receding sea, whereby in the new colonies arising on the sand-shell alluvia, a large number of fleas was observed.

House mice which survived the floods on islands and on the main shore rapidly multiplied under the optimum conditions of the post-flood period, and in 1958 they again colonized the biotopes along the sea. The high census of them with considerable "infection" by the specific fleas Ceratophyllus mokrzeckyi and Leptopsylla taschenbergi contributed to extensive dissemination of the plague infection on the territory between the Ural and Emba rivers (R. V. Kovaleva and N. L. Gershkovich, 1960).

Conclusions

In most general outlines we have analyzed the rules and regulations of the evolution of maritime landscapes and the reasons for the displacement of the boundaries of plague epizootics in the northeast and east Caspian regions, where the recession of the sea occurred very quickly, as a result of which new extensive areas of dry land of the arid type appeared, on which processes of the formation of biocoenoses occurred according to the general schema described above.

On the east coast of the Caspian Sea in recent decades a tendency has been defined toward dispersal and settlement of some of the desert animals (chiefly the great sand rat and red-tailed jird) in the north and northwest. After dispersing and settling in the area between the Ural and Emba rivers, the great sand rat made great changes in the structure of the biocoenoses of this part of the Caspian Plain and essentially determined the rules and regulations of occurrence and of the course of the plague epizootics here.

Now, we can consider with adequate basis that the boundaries of the southwest Asian desert focus and its northwestern part have been extended here appreciably in recent decades. In other words, regions unfavorable for plague within the limits of the Ural and Emba rivers (to the north

approximately to the 48th parallel) should apparently be regarded as a part of this extensive plain focus.

As far as the epi-cotological prognosis for the territory of interest to us is concerned, at the present day it is being delineated from an unfavorable aspect. In this connection, the need is rising for further careful study and planning of extensive investigations not only on the territory between the Ural and Emba rivers but also in the maritime regions lying to the west of the mouth of the Ural River, where in recent years a high census of house mice, meridional and crested jirds has also been noted.

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Material on the Ecology of Three Species of Great Sand Rat Fleas from a Comparative Aspect

N. S. Novokreshchenova
(Saratov)

Among the fleas parasitic on the great sand rat, the most numerous are representatives of the genus Xenopsylla. The main part in the transmission of plague among great sand rat populations is ascribed to fleas of this genus (M. A. Mikulin, 1951; N. F. Darskaya, 1955 and others).

In connection with the epizootological significance of these fleas the study of their ecology is particularly essential. A number of the ecological characteristics of great sand rat fleas were demonstrated in their investigations by O. A. Fedina and P. I. Shiranovich (1950), M. A. Mikulin and P. I. Shiranovich (1950), M. A. Mikulin (1951), N. F. Darskaya (1955), and A. F. Dudnikova (1960), but the ecology of these parasites has been inadequately studied, and there is very little information in the literature on some species, like, for example, X. skriabini.

In the present work we are presenting material on the ecology of three species of great sand rat fleas of the genus Xenopsylla: X. skriabini, X. gerbilli gerbilli and X. hirtipes.

We set before ourselves the task of presenting our data from a comparative aspect, utilizing the comparative parasitological method (V. N. Beklemishev, 1945). According to the basic principle of this method, it may be concluded that underlying the ecological differences between blood-sucking arthropods are their different degrees of attachment to the bodies of their hosts, which in turn are based on different characters of their food relations.

Depending on the degree of attachment to the host, we compared various species of fleas with one another as well as sand rat fleas of the genus Xenopsylla as a whole with fleas of the dwarf suslik, which are very much different in their ecology from great sand rat fleas.

We attempted to establish the degree of relationship between various species of fleas and their hosts quantitatively, using as the main criterion the date of the distribution of fleas between the host and its hole.

It should be noted that in essence I. G. Ioff (1941) gave us the idea for the comparative parasitological approach to the study of flea ecology, proposing a division of them into "hair fleas" and "nest fleas" and others.

In working out his classification for Xenopsylla on great sand rats, I. G. Ioff limited himself to the idea that they apparently belong to the category of "hair fleas."

Material and Method

We made the study of the ecology of X. skriabini fleas in 1953-1954 while working at the Gur'yev Plague Control Station of Zhilokosinskiy Rayon in Gur'yevskaya Oblast. In our collections from this region X. skriabini amounted to 83-99 percent of all great sand rat fleas.

We collected material on the ecology of X. gerbilli gerbilli and X.

hirtipes in 1956-1957 during work in the comprehensive expedition of the "Mikrob" Institute and the Turkmen Plague-Control Station. The scientific directors of the expedition were B. K. Fenyuk and A. A. Flegontova. The place of our work was a comparatively small area of large sand dunes. Here X. gerbilli and X. hirtipes were encountered together and constituted 87-99 percent of the fleas which we collected from the great sand rats and their holes.

Usually, the collections of fleas from hosts and from their holes were conducted in the spring (March-May) and autumn (September-November). Only in 1954 were the observations made in the summer period as well.

During this work an investigation was made of more than 3000 sand rats and more than 125,000 of their holes. Thereby, about 94,000 fleas of the genus Xenopsylla were collected. Parasitological investigation was conducted simultaneously with a count of the census of the hosts and the density of their holes.

The great sand rat holes were investigated mainly by the strip method, that is, the fleas were collected from the entrance /that is, until the hole bends/ of the holes. Subsequently, for convenience, we shall call the entrance to the holes simply "holes." We were able to dig up the holes in only 18 colonies.

The indices of abundance on a single object and the "indices of abundance per hectare" constituted the indices of the flea censuses on the sand rats and in their holes. We obtained the latter index by multiplying the index of abundance of the fleas on the sand rats or in the holes by the density of the sand rats or density of the holes. Utilizing data obtained from digging up the holes, we were able in various cases to calculate the index of the "total abundance per hectare" (Ioff, 1949), which represents the total number of fleas in the holes and on the sand rats. Part of this total ("integral") index, the part which applied to sand rats, characterizes the degree of relationship between the fleas of this species and the hosts.

In the study of the feeding activity of fleas we determined the degree to which their intestines were filled and calculated the percentage of individuals which had blood-filled stomachs (Ioff's alimentary index of vector activity, 1949).

The multiplication of fleas was characterized by three indices: 1) the number of females with readily visible eggs; 2) the number of young which had just come out of the flea cocoons with very clear chitinous integument; 3) a relative increase in the number of males in the population as an index of the existence of mass hatching (I. F. Zhovtyy and B. I. Peshkov, 1958; N. S. Novokreshchenova, 1960).

Distribution of Fleas between Sand Rats and Holes

Our material, confirming the conclusions of N. F. Darskaya (1955), show that a decisive influence on the distribution of fleas between the sand rats and their holes is exerted by the census and activity of the sand rats, which is directly expressed in the number of inhabited and uninhabited colonies.

In comparing the average numbers of X. skrjabini on sand rats and in their holes (recalculating the numbers for one hectare) in May 1953 it is seen

that during this period approximately the same number of fleas were counted on the sand rats as in the holes (Table 1). In 1954, these relationships were different: in the holes two-three times more fleas were found than on the sand rats. We can associate this relative increase in the number of fleas in the holes in the spring only with the mass deaths of sand rats in the winter of 1953-1954 and early spring of 1954 and the large number (up to 80 percent) of uninhabited colonies resulting from this with numerous fleas at the openings of the holes, which migrated here in search of a host. The number of fleas in these colonies in the spring was 10-15 times greater than in the uninhabited holes. We also observed a large number of fleas in the entrances of the holes in the empty colonies in areas from which the sand rats had been eliminated by means of poisoned bait. The indices of abundance in the holes here in June, July and August were two, eight and 14 times greater than the corresponding monthly indices for the territory where no rodent extermination was conducted and where inhabited colonies predominated. We noted a high census (index of abundance amounted to five per hole) in the entrances of the holes of uninhabited colonies even at the beginning of November, when the frosts begin, and the index of abundance in the holes of inhabited colonies amounted to a total of 0.03. It should be noted that the highest flea census in the uninhabited colonies was observed in the first few months after death of the rodents. In the colonies in which there were no rodents for more than a year we did not find any fleas at all.

We made a study of the distribution of X. gerbilli and X. hirtipes between the sand rats and holes on territory with a relatively high and stable census of sand rats, where practically no empty colonies were encountered. Here, the absence of uninhabited colonies also had a high degree of influence on the nature of the flea distribution: in the holes (their entrances) three-seven times fewer fleas were encountered than on the sand rats (Table 2).

Based on these data, it is difficult to agree with M. A. Mikulin's opinion (1951) that the distribution of fleas is in no way connected with the census of the hosts and is conditioned by entirely different factors, mainly by temperature changes.

In the nests of the great sand rats in the spring and summer we found, as did O. A. Fedina and P. I. Shiranovich (1950), very few fleas. Thus, of the 10 inhabitable colonies dug up in May 1953, we found nests in only five, whereby in three nests there were no fleas at all, and in two, three X. skriabini individuals were found.

Among the food plants which we found by digging up these 10 colonies we discovered appreciably more X. skriabini fleas than in the nests. However, in the summer their number was also low and did not exceed seven individuals per food compartment.

In May 1953, through digging up the colonies we were able to calculate the index of total abundance for X. skriabini; this figure represented the total number of X. skriabini on sand rats, in the food compartments of the holes and in the nests when recalculated per hectare. Its value amounted to 256, whereby 44.6 percent of this number applied to sand rats themselves. Assuming that the number of fleas present per host gives an arbitrary idea of the total time which the fleas of a given species spend on the host, it may

Table 1

Distribution of X. skriabini Fleas between Sand Rats and Their Holes (Gur'yevskaya Oblast, Zhilokosinskiy Rayon)

Дата обследования	Обследовано (2)		Индекс обилия блох (3)		(4) Индекс обилия блох на 1 га[1]	
	песчанок (5)	нор (6)	на 1 песчанку (7)	в 1 норе (8)	на песчанках (9)	в норах (10)
1953 г.						
Май (11)	147	2 184	12,8	1,1	130	99
Июнь (12)	120	1 689	7,3	0,8	79	69
Сентябрь (13)	642	3 388	4,6	0,4	45	36
Октябрь (14)	1382	4 658	9,4	0,25	105	24
Ноябрь (15)	169	1 762	5,0	0,03	—	—
1954 г.						
Май (11)	82	4 838	18,0	3,7	75	252
Июнь (12)	165	12 306	5,2	2,2	80	154
Июль (16)	120	10 083	12,4	1,0	101	72
Август (17)	71	7 854	12,9	0,4	104	30

[1] In the investigation of the holes the fleas were collected from the entrance. The index of abundance of the fleas per hectare on the sand rats or in the holes was obtained by multiplying the indices of abundance on rodents or in the holes by the density of the rodents or holes.

Key: 1. date of investigation; 2. number examined; 3. indices of abundance of fleas; 4. index of abundance of fleas per hectare[1]; 5. sand rats; 6. holes; 7. per sand rat; 8. in one hole; 9. on the sand rats; 10. in the holes; 11. May; 12. June; 13. September; 14. October; 15. November; 16. July; 17. August.

be stated that X. skriabini fleas in the spring (in May) spend about half of their time on the great sand rat.

For X. gerbilli and X. hirtipes we have at our disposal similar data for the autumn period. In October 1957 we dug up eight colonies, without finding a nest thereby. The fleas were concentrated in the supplies of food plants, which filled extensive passages of the holes in the center of the colonies. The index of total abundance for X. gerbilli amounted to 203 and the part of it which applied to sand rats amounted to 54.1 percent. For X. hirtipes these indices were equal respectively, to 122 and 41.8 percent. Hence, it follows that X. gerbilli in the autumn (in October) spends approximately half of its time on its host; X. hirtipes, somewhat less, showing a lesser degree of attachment to the host than the first species.

In accordance with the comparative parasitological method, we compared this material with similar data which we had obtained from fleas of the dwarf

Table 2

Distribution of Fleas between Sand Rats and Holes
(Iolotanskiy Rayon of Maryyskaya Oblast)

Дата обследования (1)	Обследовано (2)		X. gerbilli				X. hirtipes			
	песчанок (5)	нор (6)	индексы обилия блох (3) на песчанку (7)	в норе (8)	индексы обилия блох на 1 га (4) на песчаниках (9)	в норах (11)	индексы обилия блох (3) на песчанку (7)	в норе (8)	индексы обилия блох (4) на 1 га на песчаниках (9)	в норах (10)
1956 г.										
(13) Март	--	--	—	--	--	—	—	—	—	—
(14) Апрель	-	2977	-	0,27	—	40	--	0,08	—	9
(15) Май	—	2258	—	0,21	—	31	—	0,1	—	15
(16) Сентябрь	7	2320	4,1	0,19	57	28	0,4	0,15	5	22
(17) Октябрь	25	3340	5,2	0,23	72	34	4,0	0,19	56	28
(18) Ноябрь	28	2240	3,3	0,08	46	12	0,21	0,10	3	15
1957 г.										
(13) Март	25	2400	6,8	0,19	123	28	5,9	0,11	82	16
(14) Апрель	32	2800	11,9	0,26	166	39	5,3	0,15	74	22
(15) Май	44	2540	9,5	0,18	133	27	3,3	0,10	48	15
(16) Сентябрь	8	4234	6,9	0,04	96	6	3,1	0,03	43	4
(17) Октябрь	28	5192	7,9	0,10	110	15	3,7	0,08	51	12
(18) Ноябрь	—	1680	--	0,02	—	3	—	0,02	—	3

1-10. Same as for Table 1. 13. March; 14. April; 15. May; 16. September; 17. October; 18. November.

suslik (N. S. Novokreshchenova, 1960). Thereby, the much greater degree of attachment of X. skriabini, X. gerbilli and X. hirtipes to their hosts was demonstrated not only by comparison with a typical nest flea Neopsylla setosa but also by comparison with Ceratophyllus tesquorum fleas which are connected with the suslik to a great degree. During the period of its active existence the dwarf susliks had on their bodies from 3 to 4 percent of the total number of N. setosa and from 12 to 20 percent of C. tesquorum. Therefore, the degree of connection of sand rat fleas of the genus Xenopsylla with their host is approximately two-three times greater than that for C. tesquorum and 10-12 times greater than for N. setosa.

Differences in the distribution of male and female rodent fleas between their hosts and holes were noted by I. G. Ioff (1941) and M. A. Mikulin (1951). I. G. Ioff wrote that in the hair of male rodents there are fewer fleas, as a rule, than in the hole. M. A. Mikulin, based on his own data on the distribution of fleas of great sand rats, believed that in rodent fleas these relationships were the opposite. At the same time, both authors considered the distribution characteristics of fleas of different sexes common to all rodent fleas rather than a species characteristic.

The distribution of male and female fleas has species specificity and depends on the degree of attachment of the given species to the host (Table 3).

Table 3

Number of Male Fleas on Hosts and in their Holes

Вид блохи (1)	Количество блох (2)	Год обследования (3)	Объекты обследования (4)	(5) Процент самцов по месяцам по отношению к общему количеству блох								
				III	IV	V	VI	VII	VIII	IX	X	XI
N. setosa	3 598	1949	(6) Суслики		41,3	25,0	16,6	22,2	--	—	--	-
. .	1 779	1949	(7) Норы		40,0	33,0	40,0	26,0	-		-	
. .	5 325	1950	Суслики		36,6	37,5	25,0	16,6	-		--	--
. .	12 681	1950	Норы	-	40,0	50,0	33,3	33,3	--			--
C. tesquorum	7 157	1949	Суслики	--	42,8	35,7	41,1	37,5		-		-
. .	2 341	1949	Норы	-	50,0	37,5	50,0	33,3		--		
. .	4 063	1950	Суслики		47,3	33,3	41,6	41,4	-	-	-	-
. .	15 276	1950	Норы		30,5	33,0	36,8	33,5	-	-		-
X. skrjabini	13 425	1953	(8) Песчанки		--	40,9	47,2	—	—	41,3	45,7	-
. .	6 158	1953	Норы		—	20,0	14,1	—	—	35,1	39,1	—
. .	4 747	1954	Песчанки		—	49,0	40,4	51,0	43,0	--		--
. .	59 916	1954	Норы	-	-	28,6	22,7	33,3	25,0	—		--
X gerbilli	254	1956	Песчанки		--	—	—	--	—	68,6	76,9	40,0
. .	2 690	1956	Норы	--	18,3	15,1	-	-		21,0	29,1	21,0
. .	1 290	1957	Песчанки	58,7	59,3	52,9	-	-	-	49,2	56,9	—
. .	2 133	1957	Норы	26,3	23,3	20,9			-	20,0	24,0	14,2
X hirtipes	109	1956	Песчанки	—	—	—		-		68,1	30,0	—
. .	1 578	1956	Норы	—	19,3	11,1	-	--	--	20,0	16,6	—
. .	593	1957	Песчанки	—	41,5	42,9	-	--	--	32,2	35,5	—
. .	1 723	1957	Норы	--	20,0	17,0			-	13,6	21,9	21,0

1. species of flea; 2. number of fleas; 3. year of investigation; 4. objects examined; 5. percentage of males by months with respect to the total number of fleas; 6. susliks; 7. holes; 8. sand rats.

As is evident from Table 3, with increase in the degree of attachment to the host there is an increase in the relative number of male fleas encountered on the body surfaces of the rodents compared with their numbers in the holes. For example, in June 1949 male N. setosa fleas were present in the collections from the susliks to the extent of 16 percent; from the holes, 40 percent; In the sand rat flea X. hirtipes, distinguished by much greater attachment to the host, males amounted to 30-68 percent of the collections from the sand rats, and from 11 to 22 percent in the collections from the holes.

Feeding and Reproductive Characteristics of Sand Rat Fleas

The high degree of feeding activity of great sand rat fleas in nature was demonstrated through a study of their "alimentary index." On the average, according to our data, in sand rat flea populations individuals with blood-filled stomachs were encountered in a smaller percentage of cases than among dwarf suslik fleas. This is in agreement with the greater attachment of the sand rat fleas to the body of the host, as a result of which, as was shown above, they spend comparatively more time on the host, being subject to a greater degree to the effect of the body temperature of the host, which is elevated by comparison with the environment. Because of this, the great sand rat fleas are distinguished by more frequent feeding and more rapid digestion of blood (N. F. Darskaya, 1955; K. K. Murzakhmetova, 1958).

The great degree of attachment of sand rat fleas to their hosts is also expressed in the distribution of fleas with blood-filled stomachs. While among suslik fleas such individuals are encountered in the holes and on the hosts in approximately the same numbers (N. S. Novokreshchenova, 1960), in the sand rat fleas individuals with blood-filled stomachs are encountered in much higher percentages on the hosts than in their holes (Tables 4 and 5).

The feeding characteristics of fleas which we noted are closely connected with the nature of their reproduction. In accordance with the greater connection with the host and with greater feeding activity, great sand rat flea females with eggs are encountered on the body of the host in larger numbers than in the holes. In September 1956 all X. gerbilli females taken from the sand rats had eggs. In the spring and autumn of 1957 the percentage of females with eggs of this species on the sand rats amounted to 38-86 percent, whereas in the holes (in the entrance) the percentage was only 0-16. For dwarf suslik fleas these relationships were the opposite: the main mass of fleas with eggs were encountered in the holes.

As is seen from Table 4, in Gur'yevskaya Oblast in the autumn of 1953, in the X. skriabini population there were no females with eggs. This was in agreement with their lesser feeding activity during this period, because individuals with blood-filled stomachs owing to the delayed rate of digestion of blood were considerably more numerous than in the spring and summer. There was a predominance of young fleas with very much developed fat bodies. Therefore, the autumn population of X. skriabini fed and reproduced less actively than the summer population, showing characteristics of adaptation to the winter period. Similar differences between spring and autumn populations of X. conformis have been noted in Dagestan by N. F. Darskaya and others

Table 4

Дата обследования	Собрано блох		% блох с заполненным кровью желудком		% самок с яйцами	
	с песчанок	из нор	на песчанках	в норах	на песчанках	в норах
1953 г.						
Май	1884	2 424	22,1	15,3	16,0	7,5
Июнь	873	1 307	19,0	14,2	21,3	5,0
Сентябрь	2238	1 283	43,0	21,8	0	0
Октябрь	7576	1 081	28,8	24,0	0	0
Ноябрь	854	63	29,8	19,0	0	0
1954 г.						
Май	1477	17 931	7,9	3,0	22,2	2,6
Июнь	871	27 619	6,3	1,0	16,6	0,6
Июль	1497	10 555	5,6	1,2	1,6	0,07
Август	922	3 841	3,9	2,5	9,6	0,06

1. date of examination; 2. fleas collected; 3. percent of fleas with blood-filled stomachs; 4. percent of females with eggs; 5. from the sand rats; 6. from the holes; 7. on the sand rats; 8. in the holes; 9. May; 10. June; 11. September; 12. October; 13. November; 14. July; 15. August.

(1957).

Different relationships exist between the spring and autumn populations of X. gerbilli and X. hirtipes fleas in Maryyskaya Oblast of TuSSR. The feeding activity of these species in the spring and autumn is approximately the same (Table 5). In accordance with this, the spring and autumn populations reproduced actively and females with eggs were encountered in the same numbers in the spring and autumn.

In observations of the reproduction of X. skriabini we determined that two periods of mass hatching occurred in them: in June-July and September-November (Table 6). As is seen from Table 6, in these months the basic hatching index (the number of young which had just come out of the flea cocoons and which had very clear chitin) showed the greatest value.

In addition, we made special observations of the times and intensity of summer hatching of X. skriabini on four groups of colonies located in four places. Hatching occurred in all colonies. However, the largest number of young fleas was observed in different groups of colonies at different times, from 20 June to 26 July, 1954. The number of young fleas during the period of summer hatching increased in various groups of colonies by 5.5-37 times.

Mass summer hatching is apparently characteristic of the majority of representatives of the genus Xenopsylla, which are parasitic on great sand rats (N. F. Darskaya, 1955; A. F. Dudnikova, 1960).

We did not make any summer observations of X. gerbilli or X. hirtipes fleas and recorded only the autumn (October-November) hatching of these fleas

Table 5

Indices of Feeding and Reproductive Activity of Fleas (Iolotanskiy Rayon in Maryyskaya Oblast)

(1) Дата обследования	X. gerbilli gerbilli						X. hirtipes					
	(2) собрано блох		(3) % блох с заполненным кровью желудком		(4) % самок с яйцами		(2) собрано блох		(3) % блох с заполненным кровью желудком		(4) % самок с яйцами	
	с песчанок (5)	из нор (6)	на песчанках (7)	в норах (8)	на песчанках (9)	в норах (10)	с песчанок (5)	из нор (6)	на песчанках (7)	в норах (8)	на песчанках (9)	в норах (10)
1956 г.												
Апрель (11)	--	806	--	15,1	--	20,0	--	189	--	11,3	-	18,0
Май (12)	—	485	—	6,9	—	5,0	—	211	—	7,7	-	16,2
Сентябрь (13)	30	445	0	1,8	100	4,0	3	311	-	1,1	—	4,1
Октябрь (14)	131	786	4,5	0,05	55,8	1,1	100	631	2	2,5	64,3	0
Ноябрь (15)	93	168	12,8	1,1	57,0	0	6	203	—	1,7	—	0
1957 г.												
Март (16)	220	467	3,3	9,1	38,9	12,1	148	268	18,0	6,7	53,8	16,3
Апрель (9)	380	738	18,9	4,4	64,4	10,0	170	429	32,1	3,3	77,3	9,1
Май (10)	416	445	20,0	3,1	75,0	16,4	147	256	28,4	3,0	81,0	9,6
Сентябрь (11)	55	176	5,1	1,0	85,7	0	25	128	3,8	0,9	52,3	0
Октябрь (12)	219	707	17,2	2,4	45,4	6,6	103	642	20,3	6,1	70,0	3,1

1-8. Same as for Table 4; 9. April; 10. May; 11. September; 12. October; 13. November; 14. March.

Table 6

Intensity of Hatching of Fleas in Holes of Great Sand Rats

Месяц (1)	Число недавно выплодившихся блох (в %) ко всем блохам в норах по годам (2)					
	(3) Гурьевская область			(4) Марийская область		
	X. skrjabini		X. gerbilli		X. hirtipes	
	1953	1954	1956	1957	1956	1957
Март (5)	—		—	1,0	—	1,7
Апрель (6)	—	—	0	0,1	0	0
Май (7)	0,6	0,3	1,9	1,7	0	1,0
Июнь (8)	—	2,7	—	—	—	—
Июль (9)	—	6,0	—	—	—	—
Август (10)	—	2,5	—	—	—	—
Сентябрь (11)	5,0	—	15,7	5,0	8,6	4,6
Октябрь (12)	3,5	—	46,5	21,1	33,6	5,7
Ноябрь (13)	5,0	—	53,0	26,6	27,7	20,0

1. month; 2. number of recently hatched fleas in % of all the fleas in the holes by years; 3. Gur'yevskaya Oblast; 4. Mariyskaya Oblast; 5. March; 6. April; 7. May; 8. June; 9. July; 10. August; 11. September; 12. October; 13. November.

(see Table 6).

We judged the presence of mass hatching, in addition, by the relative increase in the number of males. As is seen from Table 3 and 6, it accompanied the increase in the number of young fleas.

Characteristics of Change in the Census of Great Sand Rat Fleas

Previously, we showed that the main factor influencing the change in the dwarf suslik flea census is the census of their hosts (N. S. Novokreshchenova, 1960).

Our material on great sand rat fleas also show these rules and regulations.

In the great sand rats themselves the range of variation in the census is greater than among suslika. In various years, the early spring depressions in the census of sand rats are particularly great, giving rise to great spring migrations of fleas to the holes of the nests.

As we have already mentioned, in the spring of 1954, in Zhilokosinskiy Rayon of Gur'yevskaya Oblast specifically these conditions were created. Thereby, great differences were found in the nature of the changes in the indices of abundance per object and the indices of abundance of the fleas per hectare in connection with the fact that the sand rat census increased sharply from the spring to the summer. The indices of abundance of X. skriabini on the sand rats and in the holes per hectare in May and June 1954 were approximately the same, whereas the index of abundance of fleas per sand rat

or hole in June was 3½ times less than the index in May as the result of the marked increase in the number of sand rats in June over the number in May. Therefore, on the average, there came to be fewer fleas per sand rat.

The degree of change in the indices of abundance of the fleas on the great sand rat per hectare to a great degree fails to correspond to changes in the indices of abundance per object, whereby this does occur for fleas of the dwarf suslik. In other words, changes in the host census have a much greater influence on the great sand rat flea census which are more attached to their hosts than the dwarf suslik fleas.

The great degree of connection between Xenopsylla fleas and their hosts is demonstrated also through a comparison of the monthly indices of the censuses of these fleas on rodents and in the holes. For example, despite the fact that in June-July 1954 a mass hatching of X. skriabini occurred, the number of fleas in the holes did not increase from the spring to the summer, whereas this index on the sand rats themselves increased by 2½ times in July from the figure for June. This occurred because the newly hatched fleas, which need frequent feeding, very rapidly migrated to the sand rats, which at this time had inhabited the majority of vacant colonies. Nevertheless, in the suslik C. tesquorum fleas mass summer hatching leads to an increase in the figures for the June census not only on the susliks but in their holes and nests, which is entirely natural if we consider the comparatively lesser degree of relationship between these fleas and the hosts.

Conclusions

Study of the ecology of three species of fleas of the great sand rat, X. skriabini, X. gerbilli gerbilli and X. hirtipes, by means of the comparative parasitological method showed that the degree of their relationship with the host exerts a determinative effect on the nature of the basic ecological characteristics of each species. In other words, the ecological characteristics of distribution, feeding activity, reproduction, change in census studied demonstrate the specific degree of attachment to the host for each species of flea.

With increase in the degree of attachment of the fleas to the host's body there is an increase in the fraction found on the host in the total index of total abundance per hectare. The male fleas, like females with eggs and individuals with blood-filled stomachs, are encountered in larger numbers in the hair of the rodents than in the entrances to the holes.

Sand rat fleas are attached to their hosts to a greater degree than suslik fleas. This is conditioned by different modes of life of their hosts. The dwarf suslik spends most of the time in its nest, where the fleas are given the constant opportunity of encountering the host. The great sand rats are more mobile and spend considerably less time in the nest. In accordance with this, great sand rat fleas are more attached to their hosts, feed more often, their migratory activity is greater, and changes in the host census have a greater effect on them than on dwarf suslik fleas.

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Oxygen Consumption by Suslik Fleas as a Physiological Test of Their Vitality

K. I. Kondrashkina and A. F. Dubnikova
(Saratov)

Study of the range of adaptive properties of fleas to environmental conditions is necessary to our increasing our knowledge of the ecology of these insects, the vectors of the pathogens of arthropod-borne diseases.

Among the physical conditions of the environment the temperature is one of the most important factors determining the direction and level of vital processes (I. V. Kozhanchikov, 1937; N. I. Kalabukhov, 1933, 1956; R. S. Ushatinskaya, 1957; Smith, 1958).

Change in the rate of oxygen consumption depending on the environmental temperature is a general principle for insects. However, it is well known that the level of gas exchange at the same ambient temperature in insects living under various conditions is different. This is brought about chiefly by the fact that various species of insects have adapted themselves differently to survival under unfavorable conditions during the year. Thus, in insects adapted to living under conditions of reduced temperature usually a depression of multiplication is observed in the summer and relatively low oxygen consumption is characteristic of them. Other insects have, during the course of evolution, worked out a certain capacity of existence within a broad temperature range. Such animals are characterized by a relatively high level of gas exchange, even when cooled.

Our task included the study of the effect of changes in the ambient temperature conditions on the viability of adult suslik fleas Ceratophyllus tesquorum and Neopsylla setosa. As an index of the metabolic rate in the fleas at different temperatures we selected the rate with which they consumed oxygen, being guided by the well known principle that species of insects adapted to living at a relatively low temperature possess certain gas exchange characteristics (I. V. Kozhanchikov, 1939; R. S. Ushatinskaya, 1957).

Material and Methods

We obtained the fleas for the experiments from the nursery of the parasitology laboratory of the "Mikrob" Institute. They all were the progeny of fleas bred in the laboratory from individuals caught in the Volga-Ural Sands. In the fleas the degree of oxygen consumption was determined by a method proposed by E. Nielsen (1938) in the V. G. Shakhbazov modification (1952).

The fleas were put into a bag made of silk mesh (1 x 125 centimeters) and then enclosed in glass containers--microrespirometers with a volume of 200 to 220 microliters (Fig 1). The open end of the microrespirometer cover was connected with a rubber tube to a calibrated micropipet, which was partially filled with colored apricot oil. A piece of cotton moistened with 5 percent potassium hydroxide solution for absorption of the carbon dioxide given off by the fleas was placed on the bottom of the expanded portion of the microrespirometer. For better sealing of the apparatus the microrespirometer cover

and the end of the rubber tube were smeared with vaseline. The hooks of the main part of the microrespirometer and of the cover were connected by means of rubber bands.

For the purpose of assuring stability of the ambient temperature in the microrespirometers they were immersed in a water bath under the control of two thermometers.

From five to 50 satiated or unfed fleas of a certain species and sex were put into one microrespirometer. Thereby, we did not find any appreciable difference in the rate of oxygen consumption per milligram of body weight depending on the number of fleas used in the experiment. However, for the purpose of obtaining more accurate experimental data the majority of experiments were performed with 20 fleas in each. The carbon dioxide given off by the fleas was absorbed with potassium hydroxide. The quantity of oxygen consumed by the fleas was recorded by the movement (drawing in) of the oil column in the calibrated pipet. Each experiment, performed at temperatures from -5 to 30° lasted usually three hours. At higher temperatures we were forced to reduce the duration of the experiment to one-two hours and even to 30 minutes because of the increase in gas exchange of the insects at a high temperature and the limited volume of the micropipet. Before the experiment the fleas placed in the bag were weighed on a torsion balance. The quantity of oxygen consumed was calculated in microliters (1 microliter = 0.001 cc) per milligram of body weight with the use of a correction factor for the barometric pressure (reduction to 760 millimeters of mercury and 0°). In these calculations a correction was also introduced for the change in the oxygen volume in the control container, which was kept under the same conditions but without fleas. At different temperatures 248 experiments with 9,495 fleas of the two species studied were performed.

Results of the Investigation

In setting about the present studies, we started with the premise that the greatest parasitic activity of C. tesquorum and N. setosa in nature comes about at different periods. Thus, N. setosa fleas, parasitic on susliks, are found en masse in the spring. By summer their census falls off sharply, and then by autumn, with reduction of the ambient temperature, their census in the holes increases again. In connection with this, N. setosa fleas are customarily called winter fleas or, more accurately, early spring and autumn-winter fleas (I. G. Ioff, 1941). C. tesquorum fleas are parasitic on susliks from the time they awaken from their winter sleep to the time they go into hibernation, which constituted the basis for calling them summer or spring-summer fleas (I. G. Ioff, 1941).

Considering that the nature of gas exchange can constitute an index of the degree of adaptation of the insects to life under certain temperature conditions, we hoped that a study of oxygen consumption by fleas under changing ambient temperature conditions would assist in understanding the physiological nature of this adaptation.

The rate of oxygen consumption by fleas, C. tesquorum and N. setosa, increases with the increase in the temperature to a certain degree (see the Table).

Rate of Oxygen Consumption by C. tesquorum and N. setosa Fleas under different Ambient Temperatures

(1) Пол	(2) Физиологическое состояние блох	(3) Потребление кислорода блохами (в µл на 1 мг веса в 1 час) при температуре							
		5	0	10	20°	25	4	35	40
		Ceratophyllus tesquorum							
(4) Самки	Накормленные (6)	0,137	0,159	1,047	1,153	1,425	2,875	3,320	0,488
	Голодные (7)	0,083	0,275	0,738	1,474	1,517	1,519	2,196	0,432
(5) Самцы	Накормленные	0,100	0,243	1,049	2,083	2,121	4,592	4,318	1,410
	Голодные	0,082	0,313	0,710	1,833	1,833	1,954	3,348	1,189
		Neopsylla setosa							
(4) Самки	Накормленные	0,054	0,351	0,625	0,929	1,037	1,789	1,926	0,270
	Голодные	0,082	0,341	0,594	0,734	0,398	0,436	1,591	—
(5) Самцы	Накормленные	0,066	0,352	0,967	1,267	1,107	0,808	3,180	1,297
	Голодные	0,098	0,330	0,654	1,103	1,058	2,859	2,122	—

Note. A generally accepted correction (reduction of the gas volume in the container to 0° and 760 millimeters of mercury) was made in the calculations for the oxygen consumed. The average weight of a satiated C. tesquorum female was 0.859 milligram; of a satiated male, 0.463 milligram; of an unfed female, 0.496 milligram; of an unfed male, 0.443 milligram; of a satiated N. setosa female, 0.715 milligram; of a satiated male, 0.465 milligram; of an unfed female, 0.491 milligram; of an unfed male, 0.375 milligram.

Key: 1. sex; 2. physiological condition of the flea; 3. oxygen consumption by fleas (in microliters per milligram of weight in one hour) at a temperature of; 4. females; 5. males; 6. satiated; 7. unfed.

These rules and regulations were expressed distinctly in both females and males, in satiated as well as unfed individuals. However, increase in the gas exchange activity and in the relationship to the temperature was different in both species of fleas. First of all, a greater range of variation in the rate of oxygen consumption is noted in C. tesquorum fleas than in N. setosa. With increase in the temperature from 0 to 35° the rate of oxygen consumption in satiated C. tesquorum females increases from 0.159 to 3.320 microliters; in satiated N. setosa females, only from 3.61 to 1.926 microliters; in satiated C. tesquorum males, from 0.243 to 4.318 microliters; in N. setosa males, from 0.352 to 3.180 microliters (Fig 2). Greater changes in the gas exchange level are noted in unfed C. tesquorum fleas.

In unfed N. setosa fleas (particularly females) the reaction to elevation of the temperature is less distinctly expressed, or, more accurately, it is of a different nature.

If we compare the figures for the rate of oxygen consumption by fleas at each of the temperatures tested, they are considerably higher, as a rule, in C. tesquorum fleas. At a temperature of -5° the fleas of both species are in a torpid state. Under these conditions, apparently only the basal metabolism is maintained, conditioned by the functioning of internal organs. The basal metabolism in C. tesquorum fleas is, as a rule, somewhat higher

than in N. setosa.

With elevation of the temperature to 0° the rate of oxygen consumption in N. setosa fleas increases by 5-5.6 times whereas in C. tesquorum this increase is only by 1.1-3.8 times. N. setosa fleas, more cold-loving, become active to a certain degree even at this relatively low temperature, whereas in C. tesquorum fleas the gas exchange at 0° in a number of cases remains at the same level at which it was at -5°. In connection with this, the rate of oxygen consumption at 0° in N. setosa fleas is much greater than in C. tesquorum.

Approximately the same degree of jump in the rate of gas exchange in C. tesquorum fleas was observed with increase in the temperature to 10°. This jump was particularly appreciable in satiated fleas (see Fig 2). Thus, oxygen consumption at 10° in satiated C. tesquorum females is 6.5 times greater than at 0°; in satiated males, only 4.3 times greater. In N. setosa fleas it increases, respectively by only 1.7 and 2.7 times.

At a temperature of 20° the activity of metabolic processes increases in C. tesquorum and N. setosa; however, once again, more actively in the former. Subsequently, the figure for oxygen consumption in C. tesquorum increases steadily, even with subsequent rise of the temperature to 35°. Only at 40° does it fall off sharply with signs of respiratory paralysis and shock. In N. setosa fleas in various groups of experiments (unfed females and satiated males), even at 25-30° a certain depression of metabolism is observed; at 35° excitation, as it were, recurs and oxygen consumption is increased. However, after an hour's stay at 35 and 40° the majority of N. setosa fleas dies.

Discussion of the Investigation Material

On the basis of the data which we have obtained concerning the rate of oxygen consumption by C. tesquorum and N. setosa fleas under different temperature conditions the conclusion may be drawn that the temperature optimum for these two species of fleas is different. N. setosa fleas adapted themselves to living at a relatively low temperature. For this species of flea a temperature over 20° is unfavorable. Such a conclusion is in complete agreement with the previously established idea that this species is an autumn-winter and early-spring species, and with the principle stated by N. I. Kalabukhov (1933) and I. V. Kozhanchikov (1939) that the metabolic level is more stable with cooling and, conversely, that it is suppressed with elevation of the temperature in insects adapted to living under low temperature conditions. Our conclusions are in complete agreement also with the material of N. S. Novokreshchenova, who observed a depression of the reproduction of N. setosa fleas in the summer, and, finally with the data of many other investigators (V. I. Kuzenkov, 1929; M. M. Tikhomirova, M. V. Zagorskaya and B. V. Il'in, 1935; S. A. Kolpakova, N. P. Lippert, 1937; K. I. Kondrashkina, 1959 and others) attesting to a sharp reduction in the census of fleas of this species during the summer. However, it should be noted that the cause of the hole mode of life and in a number of cases by virtue of particularly favorable conditions (in years with a humid cool summer) a certain part of the population of adult N. setosa fleas can survive throughout the summer.

The high level of oxygen consumption by C. tesquorum fleas and the steady increase in its rate as a function of the elevation of temperature from

-5 to 35° characterizes this species of flea as adapted to existence under conditions of considerable temperature variations. The results of our experiments are also confirmed by data which have been obtained in nature (V. I. Kuzenkov, 1929; M. M. Tikhomirova, M. V. Zagorskaya and B. V. Il'in, 1935; S. A. Kolpakova and N. P. Lippert, 1937, and others). These investigators found C. tesquorum fleas in the nests of dwarf susliks, not only in the summer but also in February as well as in the late autumn, in October-November.

Similar data were also obtained by N. F. Lapina and A. D. Luk'yanova, who showed that C. tesquorum fleas survive better than N. setosa fleas under conditions of considerable temperature variations. In their experiments C. tesquorum fleas lived much longer than N. setosa fleas not only at high temperatures but also at low temperatures.

The resistance of adult C. tesquorum fleas to low temperatures is emphasized even more by their capacity of reproducing after being kept for a long time at low temperature. In our experiments C. tesquorum fleas kept at -5° for a month multiplied after this just as successfully as N. setosa fleas.

Therefore, the idea that C. tesquorum is a spring-summer flea or particularly a summer species is erroneous, in our opinion. The fact that adult C. tesquorum fleas were found in nests in February as well as in October-November cannot be regarded as a chance phenomenon as is indicated by I. G. Ioff (1941). An analysis of data in the literature on this subject, our experimental data as well as new supplementary material presented in the work of N. S. Novokreshchenova (1960) and by N. F. Lapina and A. D. Luk'yanova permit us to consider this phenomenon a regular one.

The relationship between the metabolic rate and the sex of a flea is of some interest. In almost all experiments males consumed much more oxygen per unit of body weight than the females. More active oxygen consumption by males is apparently associated with their greater activity, chiefly with their greater movement. At a temperature of -5°, when the fleas lose the power of movement and the metabolic level is determined only by its basal portion, these differences in the respiratory rates of males and females are slight.

The more active oxygen consumption by satiated than unfed C. tesquorum fleas noted in experiments with high temperatures is associated with an increased energy expenditure under these conditions for digestive processes.

Conclusions

1. The ambient temperature is an essential factor in determining the level of oxygen consumption in C. tesquorum and N. setosa fleas. The higher the ambient temperature the more actively oxygen consumption occurs in them.

2. A lower level of oxygen consumption at temperatures above 20° in N. setosa fleas than in C. tesquorum is evidence of the adaptation of this species of flea to life under conditions with a relatively low temperature. These data are in complete agreement with the recently established idea that these insects are early-spring and autumn-winter species.

3. Under the same temperature conditions C. tesquorum fleas consumed much more oxygen, as a rule, than N. setosa fleas. Thereby, a steady

increase in the rate of oxygen consumption by C. tesquorum fleas was observed when the temperature changed within limits of from -5 to 35°. These data permit us to conclude that adult C. tesquorum fleas adapted themselves to life not only under conditions of high summer temperatures but also under conditions of low winter temperatures.

4. Oxygen consumption in the males of both species of fleas is higher than in females, which is apparently associated with their greater movement.

5. At high temperatures satiated C. tesquorum fleas consume more oxygen than unfed fleas. The increased energy expenditure in satiated fleas at high temperatures is apparently associated with an increase in the digestive process under these conditions.

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TREATMENT AND PATHOGENESIS

Treatment and Prophylaxis of Experimental Plague with Monomycin, Neomycin and Antibiotics of the Tetracycline Series

G. N. Lenskaya, Ye. I. Smirnova, N. R. Ivanov, A. P. Yermilov, M. V. Zubova, S. K. Gizzatullina, A. F. Mel'nikova, G. K. Kovalev, N. P. Arkhangel'skaya (Saratov)

In 1954-1955, D. G. Savostin, G. N. Lenskaya and others at the "Mikrob" Institute determined the high degree of therapeutic effectiveness of colimycin and mycerin for the treatment of bubonic and pneumonic plague in guinea pigs (D. G. Savostin and others, see the present collection). The results of the experiments were reported in December 1955 at the interinstitute scientific conference of plague-control institutions at Saratov. The high degree of therapeutic activity of colimycin and mycerin for the treatment of bubonic and pneumonic plague made it possible for the authors to recommend these preparations, equal in their effectiveness to streptomycin, for the treatment of human plague. Similar results were obtained experimentally by G. A. Zakharova (1960) for colimycin in the treatment of bubonic and pneumonic plague in guinea pigs and by Ye. N. Aleshina, L. N. Makarovskaya, I. S. Tinker and Z. D. Khakhina (1959) for colimycin and mycerin in the treatment of experimental bubonic plague. However, when treatment with colimycin and mycerin in daily doses which when recalculated came close to being the doses prescribed for man in the treatment of pneumonic plague, was given late the percentage of animals which recovered decreased, according to the data of the "Mikrob" Institute.

As far as antibiotics of the tetracycline series are concerned, the good therapeutic effect of which for experimental plague was pointed out by L. N. Makarovskaya (1955), L. N. Makarovskaya and others (1959, 1960) as well as some foreign authors (Sokhey and Habbu, 1952 and others), it is hard to share the opinion of these specialists. The situation is that for the creation of a therapeutic concentration of the preparations in the bodies of white mice L. N. Makarovskaya and coauthors used biomycin [aureomycin], terramycin and tetracycline in daily doses of as much as 20 milligrams for oral administration and as much as 2 milligrams for intramuscular injections. Only such tremendous doses of the preparations assured a good therapeutic effect for the treatment of white mice infected subcutaneously with the plague pathogen; smaller doses were not very effective or were entirely ineffective. In recalculating these doses for a person's weight this amounts to as much as 60 grams a day when the preparations are given by mouth and as much as 6 grams a day for subcutaneous injection. It goes without saying that such doses are absolutely inacceptable for man.

According to the data of Sokhey and Habbu, for the successful treatment of plague in white mice with the use of aureomycin it is necessary to prescribe it in a dose of 6 milligrams per day by mouth, which when converted to a person's weight amounts to 18 grams a day and is also an impractical dose. Finally, we determined the fact (see below) that a considerable percentage of

white mice infected with bubonic or pneumonic plague can be cured by subcutaneous injection of the soluble preparations of aureomycin and terramycin only when doses of no less than 1-1.5 milligram a day are prescribed. Converting this to a person's weight, it amounts to 3-4.5 grams of the preparation a day by subcutaneous injection, whereas, according to pharmacopoieal data, the highest daily dose of aureomycin or terramycin for man is 2 grams a day. The treatment of white mice with doses satisfying these requirements was ineffective.

Therefore, according to these data, preparations of the tetracycline series are hard to recommend as substitutes for streptomycin in the treatment of plague in people. True, K. Ramachasdran (1952) and G. Girard (1953) tried to treat bubonic plague in people with aureomycin and terramycin and noted a positive result with a dose of 2.5-7.5 grams a day. However, their observations are not particularly numerous. In addition, they said nothing about the toxic side effects of the preparations themselves on the human body, which undoubtedly did occur.

Of the group of other antibiotics Sokhey and Habbu used chloromycetin for the emergency prophylaxis of plague in experiments on white mice. The treatment was effective when such doses were prescribed which when converted to a person's weight were equal to 144 grams a day (336 milligrams for a seven-day course of treatment of a white mouse), which certainly cannot be applicable to treatment of people. In our experiments the use of therapeutic doses of chloromycetin was absolutely ineffective.

As far as the observations of L. N. Makarovskaya, Ye. N. Aleshina and Ye. N. Lazareva (1960) concerning the high degree of therapeutic effectiveness of the dibenzylethylenediamine salt of chlorotetracycline are concerned, these data require clarification and chiefly with respect to the dosage and treatment plans. The authors injected 3 milligrams of the preparation into white mice intramuscularly in a single dose, repeating this injection three-four times at two-day intervals. Therefore, it would be necessary to administer 9 grams of the preparation in a single dose to a person in accordance with his weight for the purpose of creating the necessary therapeutic concentration. The authors do not mention whether such dosage satisfies pharmacopoieal requirements, failing to explain also why they consider it possible, in spite of generally accepted treatment principles with antibiotics, to use a two-day interval between the separate injections. Is a large depot created from the injection of the dibenzylethylenediamine salt of chlorotetracycline, or are the positive treatment results conditioned by the exceptional tolerance of white mice to preparations of the tetracycline series and do these doses assure a therapeutic concentration of it in the blood for two days? In addition, death of the control (infected but untreated) animals 5½ days after infection is evidence of the inadequate acuteness of the experiment, which undoubtedly leads to an overestimation in the results.

Therefore, at the present time there is only one effective and completely tested agent for the treatment of plague in people, streptomycin, and there are colimycin and mycerin, which are equivalent to it in therapeutic effect and in the degreeof toxicity experimentally. All this causes us to continue our search for new effective and less toxic antibiotics.

With this aim in view, for the treatment of experimental plague in

guniea pigs we tested the Soviet antibiotic monomycin which has been completely characterized in the literature, both with respect to the cultures producing it and the technology of production of the preparation and with respect to the determination of its interrelationship with antibiotics of the neomycin-kanamycin group as well as its pharmacological characteristics, including toxicity and prophylactic and therapeutic properties for a number of infectious diseases experimentally and clinically [monomycin is produced by Actinomyces circulatus var. monomycini] (Gauze and others, 1960; V. A. Shorin and others, 1960; S. D. Yudintsev and I. A. Kunrat, 1960; V. A. Shorin and others, 1960; V. S. Muraveyskaya, 1960; M. G. Brazhnikova and others, 1960; I. P. Belova, 1960; N. S. Pevzner, 1960; S. P. Shapovalova, 1960; M. G. Rufanov and others, 1960; I. A. Kunrat, 1961; L. P. Ivanitskaya, 1961; V. A. Shorin and others, 1961; T. P. Vertogradova, 1961; Ye. I. Tsvetkova, 1961).

The foreign antibiotic neomycin, which was given a positive appraisal by Meyer (1952), was also tried out.

In this work data are being presented on the testing of antibiotics of the tetracycline series.

The Testing of Monomycin

Monomycin was tested on guinea pigs. Streptomycin, which is the standard for the experimental treatment of bubonic and pneumonic plague in laboratory animals, served as the control of the therapeutic effectiveness.

The experiments were performed under acute conditions with the use of a massive infecting dose of a virulent strain of the plague microbe (1000 CLD). The guinea pigs were infected endotracheally with this dose, as a result of which they developed primary pneumonic plague.

Treatment of various groups of animals was begun eight, 24, 48 and 56 hours after infection, that is, in the incubation period (eight hours), in the initial stage of the disease (24 hours), and then in the phase of dissemination of the infectious process with clearly expressed bacteriemia and foci of inflammation in the liver and spleen (48 hours) and, finally, in the preagonal state, the phase of acute sepsis (56 hours).

Each series of experiments was accompanied by an appropriate control. The control (infected but untreated) guinea pigs in all experiments died in 100 percent of the cases with average lifespans from 3.4 to 3.8 days, that is, in the usual period for endotracheal infection with such a massive dose of a virulent strain of plague microbe.

The treatment of various groups of infected animals was conducted with different daily doses of monomycin and streptomycin: 12, 18 and 24 milligrams a day. The course of treatment was equal to seven days; the intervals between injections were eight hours. It should be noted that the comparative treatment of pneumonic plague with monomycin and streptomycin in guinea pigs was conducted four separate times. Each time the same results were noted, which made it possible to combine the data obtained from the treatment of 480 guinea pigs into Table 1.

As is seen from Table 1, monomycin proved to be a very effective preparation, similar in its therapeutic effectiveness to streptomycin and, therefore, to colimycin and mycerin (the bactericidal activity of these antibiotics

as well as their therapeutic effectiveness are practically equivalent). Even with dosages of 12 milligrams a day the percentage of guinea pigs cured with this antibiotic was not much different from the percentage cured by streptomycin. At the same time, the following attracts attention.

In a daily dose of 12 milligrams monomycin is somewhat inferior therapeutically to streptomycin. Thus, while at the beginning of treatment with both antibiotics practically the same results were obtained eight hours after the infection, in experiments in which treatment was given 24, 48 and 56 hours after the infection, that is, during the period in which dissemination of the infection developed and during the phase of sepsis, the number of animals cured with monomycin was 20-33.3 percent less than the number cured from the use of streptomycin.

With increase of the daily dose to 18 milligrams the difference between the therapeutic effects of monomycin and streptomycin was practically erased. For prophylaxis this dose proved to be equally effective for both antibiotics. In groups in which treatment was begun late, 48 and 56 hours after the infection, the therapeutic effectiveness of monomycin was inferior to that of streptomycin by a total of 6.7-8.0 percent. Therefore, with increase of the daily dose to 18 milligrams the effect of these antibiotics can be considered the same.

Wtth a daily dose of 24 milligrams, both under prophylactic conditions and during treatment begun 24 hours after infection, monomycin and streptomycin also gave the same or almost the same therapeutic effect. In groups in which treatment was begun in the phase of acute dissemination and sepsis (48 and 56 hours after the infection), the advantage of monomycin over streptomycin came out clearly. Thus, in treatment during the phase of acute dissemination with the use of monomycin 26.7 percent more guinea pigs were cured than with streptomycin; during the phase of sepsis, 13.3 percent more.

Such results were brought about by the lower degree of toxicity of monomycin than of streptomycin, which in a daily dosage of 24 milligrams reduces the percentage of guinea pigs cured, as has been pointed out above. The toxic effect of this dose of streptomycin, superimposed on the toxicosis produced in the guinea pigs by dissemination of the plague infection, causes a deterioration in the condition of the animals, as the result of which the percentage of guinea pigs which recover is reduced (D. G. Savostin, G. N. Lenskaya and others, 1961). The therapeutic effect of monomycin used in this dose is increased. Therefore, the lower toxicity of monomycin permits increasing its daily dose for the treatment of pneumonic plague in guinea pigs to 24 milligrams, and, by the same token, makes it possible to increase its therapeutic effect considerably.

Similar results in the treatment of bubonic plague in guinea pigs with monomycin were obtained by G. A. Zakharova (see the present collection). In these experiments, just as in the treatment of pneumonic plague, the advantage of monomycin was shown particularly when treatment was begun late (72-96 hours after infection).

The results obtained permit us to consider monomycin a full-scale substitute for streptomycin, particularly under conditions where the treatment of plague has been begun late.

Table 1

Comparative Therapeutic Effectiveness of Monomycin and Streptomycin in Pneumonic Plague in Guinea Pigs

(1) Препарат	(2) Число выживших свинок (в %) при начале лечения через			
	(3) 8 часов	24 часа	48 часов	86 часов
	(4) *Суточная доза 12 мг*			
(5) Стрептомицин	93,3	93,3	80,0	66,6
(6) Мономицин	86,6	60,0	60,0	46,6
	(7) *Суточная доза 18 мг*			
Стрептомицин	93,3	86,6	92,0	73,3
Мономицин	93,3	68,0	84,0	66,6
	(8) *Суточная доза 24 мг*			
Стрептомицин	76,6	93,3	73,3	60,0
Мономицин	86,6	86,6	100,0	73,3

1. preparation; 2. number of guinea pigs surviving (in %) when treatment was begun after: 3. hours; 4 daily dose of 12 milligrams; 5. streptomycin; 6. monomycin; 7. daily dose of 18 milligrams; 8. daily dose of 24 milligrams.

Testing of Neomycin

The therapeutic and prophylactic effectiveness of neomycin was also tried out in experiments on guinea pigs. They were infected by the endotracheal method--in one series of experiments with a dose of 400 CLD of a virulent strain of the plague microbe; in another series, with 1000 CLD. In both the first and second series of experiments death of the control untreated animals occurred in 100 percent of the cases from 3.3 to 3.9 days after infection.

The therapeutic effectiveness of neomycin was compared with the therapeutic effectiveness of streptomycin and mycerin under the same experimental conditions. The therapeutic preparations were given to the animals subcutaneously three times a day with an eight-hour interval between the injections for seven days. The daily dose of each preparation was equal to 18 milligrams. The injection of the preparation was begun 12 hours after the infection (prophylaxis), or 24, 48 or 56 hours after the infection (treatment).

The results of the experiments obtained from the treatment of 280 guinea pigs (22 in each experimental group) are shown in Table 2; 16 control guinea pigs died after three days.

As is seen from Table 2, the therapeutic effectiveness of neomycin is almost equivalent to that of streptomycin. True, with prophylaxis begun 12 hours after infection as well as when treatment is begun in the early stage of the disease, that is, 24 hours after infection, some advantage of streptomycin is seen: under these conditions, among the animals treated with

Table 2

Comparative Therapeutic Effectiveness of Neomycin, Streptomycin and Mycerin in Pneumonic Plague in Guinea Pigs

Препарат (1)	Число выживших свинок (в %) при начале лечения через (2)			
	12 часов (3)	24 часа (3)	48 часов	56 часов
Неомицин (4)	80	70	75	65
Мицерин (5)	60	80	68,5	70
Стрептомицин (6)	90	90	70	60

1. preparation; 2. number of surviving guinea pigs (in %) when treatment was begun after: 3. hours; 4. neomycin; 5. mycerin; 6. streptomycin.

streptomycin 10-20 percent more survived than after treatment with neomycin. However, in more neglected cases of the disease, that is, when treatment was begun 48 and 56 hours after infection, no such difference was noted. The effectiveness of mycerin in all experiments, when it was used for prophylactic purposes or for treatment of pneumonic plague in guinea pigs, was practically the same as that of neomycin, which was to be expected, because neomycin, colimycin and mycerin belong to the same group of antibiotics. Of the 88 guinea pigs treated with neomycin 63 survived; of the same number treated with mycerin, 61.

Testing of Antibiotics of the Tetracycline Series

The antibiotics aureomycin, terramycin and tetracycline have been tested for the experimental treatment of plague and, in addition, have been tested abroad for the treatment of people sick with plague.

It has been shown that the oral administration of these antibiotics for the treatment of experimental bubonic and pneumonic plague in doses permitted by the pharmacopoiea is ineffective (D. G. Savostin, Ye. I. Korobkova and others, 1958; Ye. V. Buntin and G. A. Zakharova, 1958; L. N. Makarovskaya, 1955; Ye. L. Semenova, A. L. Kartashova, 1957).

Considering the high degree of toxicity of preparations of the tetracycline series observed experimentally and clinically (M. Kh. Lepper and others, 1953; G. Ya. Kivman and A. M. Kharitonova, 1957; I. V. Isupov and V. K. Klochkova, see article in the present collection) and the fact that the highest daily dose for man should not exceed 2 grams a day according to the pharmacopoiea, it was decided to check these preparations for the treatment and emergency prophylaxis of bubonic and pneumonic plague in white mice with the use of tolerance therapeutic doses.

In the experiments 4,685 animals were used. As a standard of therapeutic effectiveness, as in all previous experiments, the therapeutic effectiveness of streptomycin was used. The following daily dosages were administered: 0.4-0.7-1-1.5 milligram. When converted to a person's weight this amounts to 1.2-2.1-3-4.5 grams a day. The first two doses do not exceed the daily doses provided by the pharmacopoiea; the other two are over the limit

of the therapeutic tolerance doses. These extra high doses were used purposely in the experiments with the aim of a more accurate evaluation of the treatment results with these antibiotics. The preparations were injected subcutaneously three times a day at eight-hour intervals for seven days.

The bubonic form of plague in white mice was produced by subcutaneous infection; the pneumonic form, by intranasal infection. For the infection massive doses of a virulent strain of plague microbe were used: for subcutaneous infection, from 200 to 1000 CLD; for intranasal infection, 25,000,000 and 50,000,000 microbes. This quantity for subcutaneous injection amounts to 100,000 and 200,000 CLD. It does not seem possible to determine one CLD for intranasal administration or to work with a smaller number of CLD's by this method of infection because of the marked reduction of the number of microbes in the infecting dose from the encounter with antagonistic microbes--common inhabitants of the respiratory tract (our observations)--and because of the protective function of ciliated epithelium of this tract (Smith P. N., McCamich J., 1957).

Before beginning the emergency prophylaxis and treatment experiment, the time of onset of the infectious process and dissemination of the plague pathogen in white mice was determined for both methods of infection. It was determined that in both cases 12 hours after infection the bacteriological signs of the disease are absent in white mice. Twenty-four hours after subcutaneous infection a distinct process is observed in the regional lymph nodes and, in various cases, dissemination of the infection is seen. After intranasal infection at this time the disease pathogen can be isolated from the regional lymph nodes, from the lungs, and in some of the animals at this time, just as after subcutaneous infection, dissemination of the infection is noted. Thirty-six and 48 hours after infection by either method a severe disseminated form of plague develops in the great majority of animals, and in many cases there is sepsis. In connection with this, the administration of the antibiotics 12 hours after infection was considered prophylactic. The use of the preparations 24, 36 and 48 hours after infection was considered treatment. Thereby, it should be emphasized once again that the majority of sick mice were in the phase of acute dissemination and sepsis by the beginning of treatment.

Death of the control (infected but untreated) white mice occurred in all experiments from 2.4 to 3.1 days after subcutaneous or intranasal infection. However, it should be noted that about 8 percent of the white mice in the control group also survived in special experiments, repeated many times, for the determination of dissemination of the infection, which should be taken into consideration in evaluating the results of the experiment. In connection with the existence of mice resistant to infection, the percentage of animals cured turns out to be too high by comparison with data obtained in the experiments on guinea pigs.

The data of the experiments are summarized in Table 3.

An analysis of these data makes it possible to state confidently that streptomycin in any of the dosages tested turned out to be, as always, the most effective in experiments of emergency prophylaxis as well as for the treatment of both clinical forms of plague. The comparatively low survival of white mice after treatment of bubonic plague with these antibiotics is

Table 3

Treatment and Prophylaxis of Bubonic and Pneumonic Plague in White Mice with Antibiotics of the Tetracycline Series and Streptomycin

Суточная доза антибиотиков, мг (1)	Количество выживших мышей (в %) (2) при лечении			
	биомицином (3)	террамицином (4)	тетрацикл. лином (5)	стрептомицином (6)
	Профилактика бубонной чумы (7)			
0,4	46,9	38,3	0,	69,4
0,7	78,3	57,2	20,0	80,9
1,0	78,5	72,9	10,0	87,1
1,5	95,0	85,0	50,0	90,0
	Лечение бубонной чумы (8)			
0,4	13,0	9,1	0	29,3
0,7	24,0	21,6	3,3	30,4
1,0	26,5	26,4	13,3	35,0
1,5	35,0	35,0	26,6	36,2
	Профилактика легочной чумы (9)			
0,4	0	0	Не лечили (11)	76,5
0,7	20,0	5,3	То же (12)	73,7
1,0	50,0	57,5	20,0	85,0
1,5	75,0	60,0	20,0	80,0
	Лечение легочной чумы (10)			
0,4	10,2	6,6	Не лечили	64,4
0,7	20,0	28,3	То же	66,1
1,0	40,9	46,7	26,6	72,6
1,5	61,7	63,3	40,0	73,3

1. daily dose of antibiotics, milligrams; 2. number of surviving mice (in %) in the case of treatment with; 3. aureomycin; 4. terramycin; 5. tetracycline; 6. streptomycin; 7. prophylaxis of bubonic plague; 8. treatment of bubonic plague; 9. prophylaxis of pneumonic plague; 10. treatment of pneumonic plague; 11. not treated; 12. the same.

explained by the fact that the majority of sick animals was, as has been stated above, in a state of very severe dissemination of the pathogen and sepsis at the beginning of treatment. Under these experimental conditions this percentage of recovery after streptomycin treatment is the usual one for guinea pigs. At the same time, in comparing these data with data obtained from the treatment of animals under the same experimental conditions with tetracycline, the complete ineffectiveness of this antibiotic is distinctly demonstrated.

As far as aureomycin and terramycin are concerned, for the evaluation of their therapeutic effectiveness a more detailed analysis of the results obtained from the use of different doses is needed, because when the data of

Table 3 are considered as a whole for the total number of animals cured, regardless of the dose of the preparation, the incorrect conclusion can be drawn that these antibiotics are effective for both cases of emergency prophylaxis and treatment of both clinical forms of plague. However, comparing the results of treatment according to dosages, the inadequacy of aureomycin and terramycin comes out distinctly, and the advantage of streptomycin is clearly seen when doses are used permitted by the pharmacopoiea. This is very clearly seen from Table 4, in which the results of treatment of 2,386 white mice with daily doses of 0.4 milligram-0.7 milligram (1.2-2.1 grams for man) are summarized and from Table 5, which depicts the data when daily doses of 1-1.5 milligram (3.0-4.5 grams for man) were used for the treatment of 2,272 white mice.

Table 4

Treatment and Prophylaxis of Bubonic and Pneumonic Plague in White Mice with Antibiotics of the Tetracycline Series and Streptomycin (Daily Doses of Antibiotics 0.4-0.7 mg)

Количество выживших мышей (в %) при лечении (1)			
окомицином (2)	террамицином (3)	тетрациклином (4)	стрептомицином (5)
(6) Профилактика бубонной чумы			
62,6	47,7	10	75,1
(7) Лечение бубонной чумы			
18,5	16,3	1,65	29,8
(8) Профилактика легочной чумы			
10	2,6	(10) Не лечили	75,1
(9) Лечение легочной чумы			
15,1	17,4	(10) Не лечили	65,2

1. number of surviving mice (in %) when treated with; 2. aureomycin; 3. terramycin; 4. tetracycline; 5. streptomycin; 6. prophylaxis of bubonic plague; 7. treatment of bubonic plague; 8. prophylaxis of pneumonic plague; 9. treatment of pneumonic plaguel 10. not treated.

Table 5

Treatment and Prophylaxis of Bubonic and Pneumonic Plague in White Mice with Antibiotics of the Tetracycline Series and Streptomycin (Daily Doses of Antibiotics 1-1.5 mg)

Количество выживших мышей (в %) при лечении (1)			
окомицином (2)	террамицином (3)	тетрациклином (4)	стрептомицином (5)
(6) Профилактика бубонной чумы			
86,7	78,9	30	88,5
(7) Лечение бубонной чумы			
30,7	30,7	19,9	35,5
(8) Профилактика легочной чумы			
62,5	58,7	20	82,5
(9) Лечение легочной чумы			
51,3	55,0	33,3	72,9

1-9. Same as for Table 4.

Analyzing the data of Tables 4 and 5, it should be noted that aureomycin, and, with greater reservation, terramycin in therapeutic doses can be prescribed only for the emergency prophylaxis and only in bubonic plague. The use of these preparations for the treatment of bubonic plague is less efficient, and in pneumonic plague both these antibiotics are absolutely ineffective under these conditions both with respect to the absolute percentage of positive results and by comparison with the results of streptomycin treatment. Good results in the prophylaxis and treatment of bubonic plague in white mice and satisfactory results for pneumonic plague can be obtained only when daily doses are prescribed which are very much greater than the doses permitted by the pharmacopoeia and, therefore, are toxic for man. For practical purposes, this makes very doubtful the expediency of utilizing terramycin and aureomycin for the treatment of plague.

Conclusions

1. The antibiotic monomycin is, in its therapeutic effectiveness, just as valuable a preparation for the treatment of plague as streptomycin, colimycin and mycerin.

2. The lower degree of toxicity of monomycin than that of streptomycin, colimycin and mycerin permits prescribing it in higher daily doses, which contributes to an increase in the number of persons recovering in case of delayed treatment of plague.

3. Therapeutic effectiveness of neomycin for the treatment of pneumonic plague in guinea pigs is, for practical purposes, equivalent to the therapeutic effectiveness of streptomycin and mycerin.

4. Of the antibiotics of the tetracycline series the aniibiotic tetracycline cannot be recommended in therapeutic doses for purposes of emergency prophylaxis or treatment of plague because of its ineffectiveness experimentally.

The antibiotic aureomycin and, less successfully, terramycin can be used only with the aim of emergency prophylaxis of bubonic plague. Both preparations are relatively ineffective for the treatment of bubonic plague, and for emergency prophylaxis and treatment of pneumonic plague they are entirely ineffective.

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Treatment of Bubonic and Pneumonic Plague in Guinea Pigs with Colimycin and Mycerin

D. G. Savostin (deceased), G. N. Lenskaya, Ye. I. Korobkova, V. M. Tumanskiy, A. M. Antonov, N. N. Ivanovskiy, N. K. Vereninova (deceased), V. N. Kuznetsova, L. A. Uroda, Ye. I. Smirnova, A. P. Yashchuk
(Saratov)

In the present work, done in 1954-1955, the group of authors set before themselves the task of studying the effectiveness of colimycin and mycerin in the experimental treatment of bubonic and pneumonic plague with the aim of supplementing the set of agents for curing this infectious disease.

The experiments on animals were preceded by a study of the direct action of the preparations on the plague microbe in test-tube experiments as well as a test of the toxicity and determination of the tolerance dose for guinea pigs in the case of colimycin and mycerin by comparison with streptomycin.

It had been determined by previous experiments that streptomycin in a concentration of 50 micrograms per cc after six hours completely suppresses the growth of all strains of the plague microbe used in the experiment. Compared with it, the bactericidal activity of colimycin and mycerin in the same concentration was higher: colimycin killed all the plague microbes in the concentration used for these experiments (100,000,000 microbes) in five hours; mycerin, in four hours (Table 1). Experiments for checking toxicity showed that colimycin and mycerin, like streptomycin, when administered for a month in a daily dose of 24 milligrams were readily tolerated by healthy guniea pigs. Recalculating for human weight, this dose is equal to 4.8 grams, that is, is close to the daily dose of streptomycin which has now been set in existing instructions on the treatment of patients with plague and emergency prophylaxis of those who have been in contact with them and which is prescribed for the treatment of primary pneumonic plague (5-6 grams a day).

After determining the degree of toxicity of these preparations a number of experiments were performed for the treatment of bubonic and pneumonic plague in guinea pigs with colimycin and mycerin.

In the first experiment the therapeutic effect of streptomycin was taken as the standard of therapeutic effectiveness, whereby guinea pigs infected subcutaneously with 10 CLD of a virulent strain of plague microbe and

Table 1

The Action of Streptomycin, Colimycin and Mycerin on the Plague Microbe in Test-Tube Experiments

Антибиотик (1)	Концентрация (2)	Рост культуры на агаровых пластинках при высеве из опытных пробирок через (3)							
		1 час (4)	2 часа (4)	3 часа	4 часа	5 часов (4)	6 часов	12 часов	24 часа
Стрептомицин (5)	100	++++	+++	+	+	−	−	−	−
Стрептомицин	50	++++	++++	+++	++	+	−	−	−
Стрептомицин	25	++++	++++	++++	+++	++	+	−	−
Стрептомицин	12,5	++++	++++	++++	++++	+++	++	+	−
Колимицин (6)	100	+	+	+	−	−	−	−	−
Колимицин	50	++	++	++	+	−	−	−	−
Колимицин	25	+++	+++	+++	++	+	−	−	−
Колимицин	12,5	++++	++++	+++	++	+	−	−	−
Мицерин (7)	100	+	+	+	−	−	−	−	−
Мицерин	50	+	+	+	−	−	−	−	−
Мицерин	25	+++	++	++	++	+	+	−	−
Мицерин	12,5	++++	++	++	++	++	+	−	−

Arbitrary designations: ++++ solid growth; +++, from 2000 to 500 colonies; ++, from 500 to 100 colonies; +, less than 100 colonies; -, no growth.

Key: 1. antibiotic; 2. concentration, micrograms per cc; 3. growth of the culture on agar plates, hours after plating out of experimental test-tubes; 4. hours; 5. streptomycin; 6. colimycin; 7. mycerin.

treated for 10 days with streptomycin should survive in 80 percent of the cases. Treatment was begun an hour after the infection. The antibiotics were injected subcutaneously into the guinea pigs at four-hour intervals six times a day. The daily dose of each antibiotic was equal to 12 milligrams which when converted to human weight is equal to 2.4 grams, that is, the dose provided for by instructions for streptomycin treatment of uncomplicated bubonic plague (Table 2).

In these experiments the high degree of effectiveness of colimycin and mycerin was also determined; these were not inferior to streptomycin. Thus, after infection, when treatment was begun after 48 hours 100 percent of the animals were cured with streptomycin and mycerin; 90 percent, by colimycin. When administered 72 hours after infection streptomycin cured 90 percent; colimycin, 100 percent; mycerin, 80 percent of the guinea pigs. All the control guinea pigs died with an average of lifespan of 6.4 days.

In evaluating the results of the given experiment the idea arose that when treatment was begun late possibly the high degree of effectiveness was achieved through the use of too high a daily dose of the therapeutic preparations, equal to 4.8 grams when converted to human weight, which is used for the treatment of pneumonic plague. In connection with this, in the next

Table 2

Comparative Therapeutic Effectiveness of Colimycin, Mycerin and Streptomycin in Bubonic Plague of Guinea Pigs

Препарат (1)	Суточная доза препарата, мг (2)	Число выживших свинок (в %) при начале лечения через (3)			
		1 час (4)	24 часа	48 часов	72 часа
Заражающая доза 10 DCl (5)					
Стрептомицин (6)	12	100	—	—	—
Колимицин (7)	12	100	—	—	—
Мицерин (8)	12	60	—	—	—
Стрептомицин (6)	24	—	100	—	—
Колимицин (7)	24	—	90	—	—
Мицерин (8)	24	—	90	—	—
Заражающая доза 100 DCl (9)					
Стрептомицин (6)	12	—	—	—	80
Колимицин (7)	12	—	—	—	80
Мицерин (8)	12	—	—	—	70
Стрептомицин	24	—	—	100	90
Колимицин	24	—	—	90	100
Мицерин	24	—	—	100	80

Note. The course of treatment was 10 days; the interval between injection of the preparations was four hours.

Key: 1. preparation; 2. daily dose of the preparation, milligrams; 3. number of surviving guinea pigs (in %) when treatment was begun after; 4. hours; 5. infecting dose, 10 CLD; 6. streptomycin; 7. colimycin; 8. mycerin; 9. infecting dose of 100 CLD.

experiment the daily doses of all three antibiotics were again cut in half, to 12 milligrams. Treatment of the guinea pigs was begun 72 hours after subcutaneous infection with 100 CLD of a virulent plague microbe culture and was given for 10 days at four-hour intervals. Despite such rigid experimental conditions for treatment with streptomycin and colimycin, 80 percent of the guinea pigs survived; when treated with mycerin, 70 percent survived.

Therefore, colimycin and mycerin, like streptomycin, were exceedingly effective preparations in the treatment of bubonic plague, and this included treatment begun late.

In subsequent experiments the therapeutic effectiveness of colimycin and mycerin were tested for the experimental treatment of pneumonic plague in guinea pigs. The guinea pigs were infected intratracheally with 100 and 1000 CLD of a virulent strain of the plague microbe. (Since it is impossible accurately to determine 1 CLD in the intratracheal method of infection with a very small number of microbes because of lack of standardization of the method (the presence of antagonists in the common microflora of the trachea and lungs), the number of microbes which killed the guinea pigs in 100 percent of the cases

by subcutaneous infection was taken as 1 CLD. For the strain of plague microbe used in experiments for treatment of pneumonic plague 1 CLD by subcutaneous infection was equal to 50 microbes). The treatment was given for 10 days at four-hour intervals. Daily doses of the preparations in the first experiment (after infection with 100 CLD) were equal to 24 milligrams; in subsequent experiments (after infection with 1000 CLD), 24 milligrams in one series; 12 milligrams in another. Treatment was begun 24 hours after infection, that is, before the onset of dissemination, 48 hours after the infection--during the period of development of the dissemination--and 72 hours after infection--during the phase of acute dissemination and sepsis (Table 3).

Table 3

Comparative Therapeutic Effectiveness of Colimycin, Mycerin and Streptomycin in Pneumonic Plague in Guinea Pigs

Препарат (1)	Суточная доза препарата, (2)	Число выживших свинок (в %) при начале лечения через (3)		
		24 часа (4)	48 часов	72 часа
(5) Заражающая доза 100 DCl				
(6) Стрептомицин	24	—	90	—
(7) Колимицин	24	—	80	—
(8) Мицерин	24	—	70	—
Заражающая доза 1000 DCl				
Стрептомицин (6)	24	—	100	30
Колимицин (7)	24	—	80	40
Стрептомицин (6)	12	90	95	30
Колимицин (7)	12	80	90	30
Мицерин (8)	12	80	90	20

Note. Course of treatment, 10 days; interval between injections of the preparations, 4 hours.

Key: 1. preparation; 2. daily dose of the preparation; 3. number of guinea pigs surviving (in %) when treatment was begun after; 4. hours; 5. infecting dose of 100 CLD; 6. streptomycin; 7. colimycin; 8. mycerin; 9. infecting dose of 1000 CLD.

As is seen from Table 3, in a case of intratracheal infection of guinea pigs with 100 CLD of a virulent strain of the plague microbe and when treatment was begun 48 hours after infection with daily doses of 24 milligrams, all three antibiotics gave the same good therapeutic effect: streptomycin cured 90 percent; colimycin, 80 percent; mycerin, 70 percent of the guinea pigs. The same good result was obtained when the infecting dose was increased to 1000 CLD and treatment was given with the same daily doses of the preparations 48 hours after infection: streptomycin assured recovery of 100 percent; colimycin, 80 percent of the animals. When treatment was begun

72 hours after infection, that is, during the period of acute dissemination and sepsis, streptomycin cured 30 percent; colimycin, 40 percent of the animals (mycerin treatment was not used in this experiment). The control guinea pigs died in 100 percent of the cases with an average lifespan of 3.6 days.

The third series of experiments was performed under even more unfavorable conditions. Aside from the increase in the infecting dose of the virulent strain of plague microbe from 100 to 1000 CLD, the daily dose of therapeutic preparations was reduced from 24 to 12 milligrams. Treatment of various groups of infected guinea pigs was begun 24, 48 and 72 hours after the infection.

As is seen from Table 3, under these experimental conditions when treatment was begun 24 hours after infection, streptomycin cured 90 percent; colimycin and mycerin, 80 percent of the animals each. When the preparations were injected 48 hours after infection, streptomycin assured the recovery of 95 percent; colimycin and mycerin, 90 percent of the animals. Finally, when treatment was begun after 72 hours, that is, essentially in the preagonal and agonal states, streptomycin and colimycin cured 30 percent; mycerin, 20 percent of the animals.

Therefore, the results obtained show that in the treatment of pneumonic plague, like its bubonic form, colimycin and mycerin are not inferior in their therapeutic activity to streptomycin.

After the study of the therapeutic effectiveness of colimycin and mycerin for the treatment of bubonic and pneumonic plague under the conditions described above, the significance of the length of the intervals between the separate injections of the preparations was checked in a new series of experiments. The guinea pigs were infected intratracheally with 1000 CLD of a virulent culture of the plague microbe and treated with the preparations tested for 10 days, injecting the antibiotics subcutaneously in a dose of 24 milligrams a day in equal parts in some groups at four-hour intervals; in others, at six-hour intervals; in still others, at eight-hour intervals. Treatment was begun 48 and 72 hours after the infection (Table 4).

From Table 4 it is seen that guinea pigs treated with streptomycin and colimycin begun 48 hours after infection survived in 80-90 percent of the cases when four-hour intervals were observed as well as when they were injected with these preparations at six- and eight-hour intervals. In the case of treatment begun 72 hours after infection, regardless of the length of the interval between injections of the preparations, 10 to 30 and 40 percent of the sick guinea pigs survived.

In the last series of experiments orientative investigations were made for determining the effectiveness of the combined action of streptomycin and colimycin for the treatment of pneumonic plague in guinea pigs. The animals were infected intratracheally with 1000 CLD of a virulent strain of the plague microbe and treatment was begun 48 hours after the infection. The course of treatment lasted 10 days; the daily dose of the preparations was equal to 24 milligrams. One group was treated with streptomycin alone; another group, with colimycin alone; a third group was treated with a mixture of streptomycin and colimycin, each used in half of the regular dose; the fourth group was treated alternately with streptomycin and colimycin (using full doses for

Table 4

Effect of the Intervals between the Injection of the Separate Doses of Antibiotics on the Effectiveness of Treatment of Pneumonic Plague in Guinea Pigs

Суточная доза, мг (1)	Интервал между введением, часы (2)	Число выживших свинок (в %) при начале лечения через (3)	
		48 часов (4)	72 часа
		Лечение колимицином (5)	
24	4	80	30
24	6	90	30
24	8	80	10
		Лечение стрептомицином (6)	
24	4	90	30
24	8	80	40

1. daily dose, mg; 2. interval between injections, hours; 3. number of guinea pigs surviving (in %) when treatment was begun after:; 4. hours; 5. treatment with colimycin; 6. treatment with streptomycin.

Table 5

Effectiveness of Combination of Streptomycin and Colimycin for Treatment of Pneumonic Plague in Guinea Pigs

(1) Препарат	Разовая доза, мг (2)	Число выживших свинок, % (3)
Стрептомицин (4)	8	90
Колимицин (5)	8	90
Стрептомицин и колимицин (смесь) (6)	4 4	80
Стрептомицин и колимицин (поочередно) (7)	8	70

1. preparation; 2. dose, mg; 3. number of guinea pigs surviving, %; 4. streptomycin; 5. colimycin; 6. streptomycin and colimycin (mixture); 7. streptomycin and colimycin (alternately).

each injection). The intervals between the injections of the preparations were equal to eight hours (Table 5).

From the data presented in Table 5 it is seen that with the combination

of such preparations as streptomycin and colimycin there is a reduction in the therapeutic effectiveness of each. Thus, in the case of treatment with streptomycin or colimycin alone 90 percent of the guinea pigs survived; among those treated with a mixture of these preparations, 80 percent survived; when streptomycin and colimycin were used alternately, 70 percent of the animals survived.

Conclusions

1. Colimycin and mycerin are complete substitutes for streptomycin for the treatment of bubonic and pneumonic plague in guinea pigs, assuring 80-100 percent recovery of the animals infected by subcutaneous or intratracheal methods.

2. For a favorable treatment result the early employment of the antibiotics is important, no more than 48 hours after infection. However, even when treatment is begun later (72 hours after infection) colimycin and mycerin (like streptomycin) protect up to 30-40 percent of the infected animals against death.

3. The therapeutic dose of colimycin and mycerin for guinea pigs is equal to 24 milligrams a day. Reduction of the daily dose to 12 milligrams does not reduce the therapeutic effect of these preparations. These doses of the antibiotics are nontoxic for healthy guinea pigs.

4. The therapeutic effectiveness of colimycin and mycerin is the same when the preparations are given to the guinea pigs every four hours or when used at six-hour or eight-hour intervals.

5. The high therapeutic effectiveness of colimycin and mycerin in the treatment of bubonic and pneumonic plague in guinea pigs makes it possible to recommend them for treatment and emergency prophylaxis of plague on a par with streptomycin.

Therapeutic Properties of Monomycin in Bubonic Plague in Guinea Pigs

G. A. Zakharova
(Saratov)

In a previous work the high therapeutic effectiveness of colimycin for the treatment of experimental bubonic and pneumonic plague in guinea pigs was indicated.

In June 1959 we made experimental observations on treatment of bubonic plague in guinea pigs with another antibiotic, monomycin. This antibiotic is a white powder, readily soluble in water.

In the preliminary experiment the toxicity of monomycin for guinea pigs was determined.

For this purpose 30 guinea pigs were used which were divided into three groups of 10 each. All animals were injected with monomycin three times a day at eight-hour intervals for 10 days. Guinea pigs of the first group were given 12 milligrams of the preparation a day; animals of the second group, 18

milligrams a day; animals of the third group, 24 milligrams a day (Table 1).

Table 1

Toxicity of Monomycin for Guinea Pigs

① Номер группы	② Суточная доза препарата, мг	③ Количество животных в группе	④ Число выживших свинок, %
1	12	10	100
2	18	10	100
3	24	10	100

1. number of group; 2. daily dose of the preparation, mg; 3. number of animals in the group; 4. number of surviving guinea pigs, %.

As is seen from Table 1, in the experiment on determination of toxicity all the animals survived regardless of the doses of the preparation used. No weight reduction of the guinea pigs was noted throughout the experiment; the animals were active and eagerly ate the food given.

The relatively low toxicity of monomycin for guinea pigs made it possible for us to proceed with performing the main experiment on treatment of bubonic plague with this preparation.

For this, 90 guinea pigs were used in the experiment which were divided into nine groups of 10 guinea pigs each. The animals of eight groups were treated; the ninth group served as a control.

The animals were infected subcutaneously with a 24-hour culture of the plague microbe (strain 708) in a quantity of 200 CLD (1 CLD is equal to 50 microbes). As a standard preparation for the treatment of plague streptomycin was used in our experiment. The therapeutic 24-hour doses of monomycin and streptomycin were equal to 18 milligrams; the antibiotics were given to the guinea pigs three times a day at eight-hour intervals for seven days.

The experimental groups of guinea pigs differed with respect to the time treatment of the plague-infected animals was begun; the treatment plan was the same for the groups when streptomycin or monomycin was used. The interval between infection and the beginning of treatment in the first and second groups was equal to 24 hours; in the third and fourth groups, 48 hours; in the fifth and sixth, 72 hours; in the seventh and eighth groups, 96 hours. The results of the experiment are shown in Table 2.

As is seen from Table 2, guinea pigs of the control group all died with an average lifespan of 4.5 days; in the first group of animals, treated with monomycin 24 hours after infection, one of the guinea pigs died at the beginning of the second day after infection. No plague microbe culture was isolated from it. The other animals of this group remained alive.

Among the guinea pigs of the second group, treated with streptomycin (the treatment was also begun 24 hours after infection), two animals died on the 10th and 15th days after infection; a plague microbe culture was isolated from each.

Table 2

Therapeutic Properties of Monomycin Compared with Streptomycin for the Treatment of Bubonic Plague

Номер группы (1)	Антибиотик (2)	Начато лечение после заражения через (3)	Средняя продолжительность жизни павших, дни (4)	Число выживших свинок, % (5)
1	Мономицин (6)	24 часа (9)	2,0	90
2	Стрептомицин (7)	24 "	12,5	80
3	Мономицин	48 часов	-	100
4	Стрептомицин	48 "	13,0	90
5	Мономицин	72 часа	5,0	50
6	Стрептомицин	72 "	10,7	30
7	Мономицин	96 часов	5,3	10
8	Стрептомицин	96 "	5,7	0
9	Контроль (нелеченые животные) (8)	—	4,5	0

1. number of the group; 2. antibiotic; 3. time after infection that treatment was begun; 4. average lifespan of animals which died, days; 5. number of guinea pigs surviving, %; 6. monomycin; 7. streptomycin; 8. control (untreated animals); 9. hours.

The early death of a guinea pig in the first group compared with the time of death of the controls (untreated) permits us to suppose that its death did not occur from plague but was more probably associated with unfavorable transportation conditions (high temperature replaced by a considerable cold spell, etc.) two days before the experiment.

Therefore, on the basis of the data obtained we can say that when treatment of experimental bubonic plague in guinea pigs is begun early with monomycin recovery is noted, apparently in all experimental animals, whereas streptomycin under the same conditions assures the survival of eight out of 10. This indicates the great effectiveness of monomycin (for the treatment of bubonic plague).

In the third group of guinea pigs, treated with monomycin 48 hours after infection, all the animals survived; in the fourth group of animals, treated with streptomycin under the same conditions, one animal died on the 13th day after infection. A plague microbe culture was isolated from this guinea pig.

Recovery of all the animals of the third group treated with monomycin 48 hours after infection, just as in the animals of the first group, treated 24 hours after the infection, once again corroborates the conclusion of the high degree of effectiveness of the antibiotic for bubonic plague when it is given in the initial stage of the disease.

Among the guinea pigs of the fifth group, treated with monomycin 72 hours after infection, five animals died with an average lifespan of five days. A plague microbe culture was isolated from all the animals. From the sixth

group of animals, treated at the same time with streptomycin, seven guinea pigs died with an average lifespan of 10.7 days. A plague microbe culture was isolated from all the animals which died. Death of the animals in these groups began even before the beginning of treatment (one in each group), and this indicates that treatment of the animals was essentially begun during the period of acute dissemination of the pathogen of the infectious disease and in the preagonal stage and, despite this, the use of monomycin made it possible to prolong the lives of part of the treated animals and to cure the disease in half of the experimental animals. In this case monomycin proved to be somewhat more effective than streptomycin, because the latter brought about the cure of only a third of the experimental animals under these conditions.

In the seventh and eighth groups of animals, treated 96 hours after infection, that is, in the agonal stage, because the majority of guinea pigs had died by this time (respectively, seven and eight), the results were the following. In the seventh group, of monomycin-treated guinea pigs two died with an average lifespan of 5.3 days; a plague microbe culture was isolated from both. In the eighth group both streptomycin-treated guinea pigs died with an average lifespan of 5.7 days, with isolation of a plague microbe culture. Therefore, according to the data obtained, despite the fact that treatment of the guinea pigs was begun in the preagonal period, one of the guinea pigs was cured with monomycin. This once again indicates the effectiveness of this preparation for the treatment of experimental bubonic plague even when it is used late. In this case also monomycin was somewhat more effective than streptomycin.

Conclusions

1. Monomycin is characterized by a low degree of toxicity when given to guinea pigs parenterally.
2. Monomycin is highly effective for the treatment of experimental bubonic plague in guinea pigs in cases where treatment is begun early (24 and 48 hours from the onset of the disease).
3. Monomycin leads to the cure of experimental bubonic plague during the period of dissemination and sepsis in half of the guinea pigs treated.
4. Monomycin contributes to the recovery of a certain percentage of the animals even when treated in the preagonal and agonal stages of bubonic plague.
5. Monomycin, according to the data of our experiment, is more effective for the treatment of experimental bubonic plague than streptomycin.

Therapeutic and Prophylactic Effects of Terramycin in Experimental Plague in White Mice

I. G. Lalazarova
(Saratov)

There is comparatively little information on the treatment of plague with

terramycin in the literature. Thus, McCrumb, Larson and Meyer (1953) established the fact that terramycin, along with other antibiotics, can give a therapeutic effect when it is used in experimental plague of mice. The successful use of terramycin for pneumonic plague is reported by Girard (1953); Mercier and McCrumb, (1953) and others.

With the aim of obtaining a more complete idea of the effect of terramycin on the course of plague infection in animals we performed experiments on white mice. In these experiments the intention was to study the following: 1) the course of the plague infection in white mice when treated with terramycin depending on the time at which treatment was begun after the infection and the size of the dose of the antibiotic; 2) the presence of immunity in animals surviving during the experiment; 3) change of the plague microbe cultures isolated from animals which died and which had been treated with terramycin.

For the purpose of solving the first problem 186 white mice of the same age, weighing 17-19 grams, were used. All animals were infected with a suspension of a virulent culture of the plague microbe 231 in a dose of 100 CLD (100,000 microbes) subcutaneously into the left thigh. After the infection the animals were divided, depending on the time at which treatment was begun after the infection and the dose of the antibiotic, into 11 groups of 16 mice each in the experimental groups and 26 in the control group.

For treatment of the animals crystalline terramycin hydrochloride was used with an activity of 819 units per milligram. The solution of the preparation was prepared in distilled water. Terramycin was injected intramuscularly into the right thigh three times a day for seven days. For the purpose of judging the stage of the infectious process in which the animals were before the beginning of treatment, simultaneously with the first injection of antibiotic into two successive groups of animals we sacrificed two mice of the control group. From the organs of these mice, the site of injection of the culture and the blood cultures and smear-impressions were made on solid nutrient media.

In addition, blood was seeded on Hottinger's bouillon (Table 1).

In mice killed one, eight, 16 and 24 hours after infection the culture was isolated only from the injection site; in mice killed after 48 hours the culture could be isolated from the injection site and a lymph node from one mouse; from all organs and blood, in another. Therefore, in mice killed prior to 24 hours, dissemination of the plague pathogen was not observed; it occurred only by 48 hours after the infection.

From Table 1 it is seen that the use of a culture in a dose of 100 CLD gave a 100 percent mortality of the control animals in three-four days. In the treated animals comparison of the results by groups showed that the best survival and average life span figures for the animals which died were obtained when treatment was begun early after the infection. Thus, in the second group of animals whose treatment was begun an hour after infection, with a daily dose of antibiotic equal to 1 milligram one out of 16 mice died (6.2 percent) with an average lifespan of 13 days, whereas in the ninth and 10th groups of animals, treatment of which was begun 48 hours after the infection, death occurred, on the average, in 75-87.5 percent with an average lifespan of those which died equal to 4.9-5.5 days. Treatment begun eight, 16 and 24

Table 1

Terramycin Treatment of White Mice Infected with Plague Microbe, Strain 231

Номер группы (1)	Срок начала лечения после заражения, часы (2)	Суточная доза антибиотика на 1 мышь, мг (3)	Число павших животных (4)	Число выживших животных (5)	Гибель мышей, % (6)	Средняя продолжительность жизни павших животных, дни (7)
1	1	0,5	8	8	50,0	12,1
2	1	1,0	1	15	6,3	18,0
3	8	0,5	4	12	25,0	11,2
4	8	1,0	3	13	18,8	14,0
5	16	0,5	4	12	25,0	10,5
6	16	1,0	6	10	37,5	13,0
7	24	0,5	4	12	25,0	9,0
8	24	1,0	2	14	12,5	8,5
9	48	0,5	14	2	87,5	4,9
10	48	1,0	12	4	75,0	5,5
11	Контроль (неле-ченные (8) животные)	—	16	—	100,0	2,7

1. number of group; 2. time of beginning treatment after infection, hours; 3. daily dose of antibiotic per mouse, mg; 4. number of animals which died; 5. number of animals which survived; 6. death of mice, %; 7. average lifespans of animals which survived, days; 8. control (untreated animals).

hours after the infection also gave better results, regardless of the dose of the antibiotic, than when it was begun 48 hours after the infection.

Therefore, there is a correlation between the time at which treatment is begun and the average lifespans of mice which died in the experiment: thus, where treatment was begun an hour after the infection using a dose of 1 milligram of terramycin a day the lifespan of the animals was equal to 18 days; with the same dose of antibiotic and with treatment begun after eight hours it was equal to 14 days; after 16 hours, to 13 days; after 24 hours, 8.5 days; after 48 hours, 5.5 days.

The use of the antibiotic in a dose of 1 milligram a day exerted a better effect than when it was used in a dose of 0.5 milligram. A certain relationship was also observed between the dose of the antibiotic and the average lifespan of the animals which died: in all cases, with the exception of one where treatment was begun after 24 hours, it was greater in animals which received 1 milligram of terramycin a day. In the control mice, dissected before the beginning of treatment of the experimental animals, no pathological changes were seen, with the exception of the animals killed 48 hours after the beginning of the infection. In the latter edema of the muscle was observed at the injection site of the culture and there was edema of the subcutaneous tissue of the lower portion of the abdomen; the regional inguinal lymph nodes were enlarged to the size of a millet grain. As has been pointed out, in one of these mice the infectious process was in the

stage of dissemination. In the control mice which died typical pathological changes were found on dissection. In all the mice which died which had been treated with terramycin there were areas of necrosis at the injection site of the culture. In the 10th group of the mice which died the culture could be isolated only from the injection site of the pathogen, whereas in the ninth group the culture was isolated from all organs and from the blood of those which died in the first six days of treatment; in those which died after six days, the same but only from the injection site. In other groups of mice which died in the first four-six days of treatment the plague microbe culture was isolated in four animals: in two, only from the injection site and in two, from all organs and blood. From mice which died on the fourth-12th day after the completion of treatment the culture was isolated from all organs and blood in all cases (26 out of 28), whereby in the 10th and ninth groups death of all the animals which died (with the exception of one in the ninth group) occurred during treatment, whereas of the 32 mice of the other groups a total of only four died during the treatment; the other 28 died, as has been pointed out above, on the fourth-12th day after the completion of treatment. Apparently, the seven-day period of treatment of plague-infected mice with terramycin was not entirely adequate.

From the data presented the conclusion may be drawn that terramycin acts in vivo on the plague bacillus bacteriostatically, because despite the treatment given, a plague microbe culture was isolated from all experimental mice which died as well as from controls. In part of the subcultures the biochemical and serological properties, virulence and resistance to terramycin were checked. Five of the subcultures were isolated from experimental animals (from the first, third, sixth, eighth and ninth groups); one, from a guinea pig of the control group. On examination all cultures were found to be identical; no difference was found between cultures of the plague microbe obtained from the treated mice which died and the culture isolated from the control mouse which died.

For the purpose of determining the effect of terramycin on the immunity of white mice which had been infected with plague in a dose of 100 CLD, the animals surviving the experiment were infected at different times (from 38 to 53 days) after completion of the treatment with the same virulent culture in a dose of 200 CLD subcutaneously. The mice weighed 18-21 grams (Table 2).

As is seen from Table 2, in the ninth and 10th groups all animals survived after reinfection. In the seventh group 80 percent of the animals survived; in the first, third, fifth and sixth groups about 40 percent of the mice, on the average, survived; in the eighth group, 16 percent; in the second, fourth and control groups all animals died.

The results of the experiment indicate a certain relationship between the strength of immunity and the time at which treatment is begun after infection: the later treatment was begun after infection the greater was the immunity. A certain relationship could also be noted between the strength of immunity and the dose of the antibiotic used for treatment. This is seen from a comparison of the results of infection of mice from the seventh and eighth as well as from the first, second, third and fourth groups of animals; with an antibiotic dose of 1 milligram a day the strength of the immunity was

Table 2

Strength of the Immunity of White Mice Infected with the 231 Strain of Plague Microbe and Surviving after Terramycin Treatment then Reinfected with the Same Strain

Номер группы (1)	Срок начала лечения животных при первом заражении, часы (2)	Количество мышей, выживших после лечения (3)	Период после окончания лечения до повторного заражения, дни (4)	Число павших животных (5)	Число выживших животных (6)	Выживаемость мышей, % (7)	Средняя продолжительность жизни павших животных, дни (8)
1	1	8	53	5	3	37,5	2,6
2	1	15	53	15	—	—	2,8
3	8	12	53	7	5	41,7	3,0
4	8	12	53	12	—	—	3,3
5	16	10	53	6	4	40,0	3,5
6	16	10	53	6	4	40,0	4,1
7	24	10	38	2	8	80,0	5,0
8	24	12	52	10	2	16,7	3,8
9	48	2	51	—	2	100,0	—
10	48	4	51	—	4	100,0	—
11	Контроль	14	—	14	—	—	2,6

1. number of group; 2. time of beginning treatment of the animals after the first infection, hours; 3. number of mice surviving after treatment; 4. period between the completion of treatment and the reinfection, days; 5. number of animals which died; 6. number of animals which survived; 7. survival of mice, %; 8. average lifespan of animals which died, days; 9. control.

less than in mice for whose treatment a dose of 0.5 milligram of the antibiotic was used a day. This phenomenon can apparently be explained by the fact that when treatment of the animals is begun early with high doses of the antibiotic the multiplication of the microbes in the body is delayed; therefore, immunity is greater in the animals whose treatment was begun later. The average lifespan of animals which died in this experiment was comparatively short and not much different from that in the control animals.

Conclusions

1. Terramycin possesses a pronounced therapeutic effect in the treatment of plague in white mice begun in the first 24 hours after infection. A terramycin dose of 1 milligram a day gives a better therapeutic effect than a dose of 0.5 milligram a day. The lifespan of the animals which died was greater with a dose of 1 milligram than with a dose of 0.5 milligram a day. The seven-day course of treatment of plague in white mice with the use of terramycin in these doses is clearly inadequate. Under these conditions terramycin acts only bacteriostatically on the plague bacillus in vivo.

2. Plague microbe cultures isolated from the white mice which died which had been treated with terramycin were the same as the original cultures in their properties.

3. White mice which survived plague infection because of treatment of them with terramycin acquire immunity to this infection. Thereby, a

relationship is noted between the time at which terramycin treatment is begun after infection with the plague microbe and the strength of immunity: the later the treatment was begun the greater was the immunity. Immunity was less in mice treated with terramycin in a dose of 1 milligram than in those which received it in a dose of 0.5 milligram a day.

4. Because of the bacteriostatic effect of terramycin on the plague bacillus in the treatment of plague-infected animals the best therapeutic effect can be obtained when the antibiotic is used early (in the first 24 hours), in an increased dosage and with long-term treatment.

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Preventive and Therapeutic Properties of Gamma-Globulin of Antiplague Sera Expressed as the PD_{50}

A. A. Trifonova, I. I. Derteva, N. K. Sidorova, Ye. P. Denisova, and L. A. Martens
(Saratov)

A comparative study of the effectiveness of the gamma-globulin fraction and the corresponding native antiplague sera, which we made in previous investigations, did not give distinct results (V. I. Kuznetsova, A. A. Trifonova and others, 1960). The variation in the data obtained is explained by considerable individual variations in sensitivity of experimental white mice to the plague microbe, even within the limits of a group of animals of the same weight and sex.

There is reason to believe that in the study of the effectiveness of one preparation or another on animals more reliable results are given by the LD_{50}, which characterizes the change in the reaction of all animals investigated, whereas the LD_0 or LD_{100} characterize the extreme deviations in the variation of the organisms, which may be associated with random differences in single individuals.

A comparative study of the prophylactic and therapeutic properties of native antiplague serum and its gamma-globulin fraction in the present investigation was made by the somewhat modified Sokhey method (Sokhey, 1932, 1935); Sokhey titrated antiplague sera on white mice in a dose which protected 50 percent of the experimental animals infected with a standard dose of a virulent strain of plague microbe.

In the present work the results of a study of 11 series of native antiplague serum and its gamma-globulin fraction are presented. Three series of antiplague serum were obtained as a result of immunization of horses intravenously; three, subcutaneously; three by the combined method.

The horses were immunized by the intravenous method gradually with increasing doses of a living avirulent culture of the plague microbe of the EV, 1 and 17 strains (from 5,000,000,000 to 150,000,000,000 microbes); in the subcutaneous method, with a filtrate of a virulent culture of the plague microbe of the 708 strain (from 5 to 150 cc); by the combined method, through the intravenous injection of half of the dose of living avirulent culture and subcutaneous injection of half of the dose of filtrate of the virulent culture. All the sera contained 0.5 percent chloroform as a preservative.

Two series of sera were mixtures of the sera which had been obtained in 1957 from different producers immunized by different methods six-nine months before purification. In these two series the preservative was quinosol, added in a concentration of 0.05 percent. Normal horse serum preserved with chloroform served as a control. The gamma-globulin fraction of the serum was isolated by the method of alcohol precipitation in the cold. The protein concentration in the purified preparations was always brought up to 10 percent.

The purity of the gamma-globulin fractions with respect to admixtures of other proteins was checked by the method of paper electrophoresis, which was conducted for 14 hours in a veronal buffer at a pH of 8.6 at a voltage of 4 volts per centimeter of length and with a current of 0.46 ma per centimeter of width of the paper strip. As it turned out, the gamma-globulin fraction of all the purified series of antiplague serum was electrophoretically homogeneous.

The anaphylactogenic properties of 11 series of native antiplague serum and its gamma-globulin fraction were checked by the method described in our previous work (V. I. Kuznetsova, A. A. Trifonova and others, 1960). The gamma-globulin fraction produced a less pronounced anaphylactic reaction in the guinea pigs, as a rule, than the corresponding native serum.

The preventive and therapeutic properties of the preparations were studied on white mice weighing 17-18 grams. Each dose of the native serum (0.05, 0.1, 0.3 and 0.5 cc) and gamma-globulin fraction (0.01, 0.03, 0.05, 0.1, 0.3 and 0.5 cc) was tested on 12 white mice (six males and six females). In the study of the preventive properties the native serum or its gamma-globulin fraction was injected an hour before the animals were infected subcutaneously with 5 CLD (500 microbes) of the plague microbe of strain 708. In the study of therapeutic properties the injection of the animals with these preparations was begun after 48 hours; the repeated injections of the serum in the same group of mice were given 72, 96 and 120 hours after the infection. At the time of beginning of treatment, that is, 48 hours after infection, dissemination of the infection was observed in 70 percent of the experimental mice. In the other animals the plague microbes were plated out only from the injection site of the culture and the regional lymph node.

Observations of white mice were made for 25 days. The experimental and control animals which died were examined bacteriologically by random selection. In all cases a plague microbe culture was isolated.

The LD_{50} or, more accurately, the PD_{50} (protective dose) of the preparations studied was calculated by the method of Reed and Muench separately for males and females and together for both groups.

The results of the study of the antiplague sera obtained after the

first course of immunization are shown in Tables 1 and 2.

Table 1

Preventive Properties of Native Antiplague Sera and Their Gamma-Globulin Fractions Expressed as the PD_{50}

Метод иммунизации (1)	Номер сыворотки (2)	Вид сыворотки (3)	Доза, защищающая 50% животных для (4) самок (5)	самцов (6)	тех и других (7)	Коэффициент концентрации (8)
Внутривенный (9)	453	Нативная (14)	0,210	0,400	0,250	6
		γ-Глобулин	0,038	0,045	0,042	
	454	Нативная	0,240	0,270	0,260	
		γ-Глобулин	0,050	0,065	0,057	4,5
	455	Нативная	0,220	0,220	0,220	
		γ-Глобулин	0,079	0,290	0,130	1,7
Подкожный (10)	448	Нативная	0,160	0,230	0,190	4,5
		γ-Глобулин	0,027	0,042	0,040	
	450	Нативная	0,230	0,500	0,500	1,1
		γ-Глобулин	0,180	>0,500	>0,500	
	451	Нативная	>0,500	>0,500	>0,500	1
		γ-Глобулин	>0,500	>0,500	>0,500	
Комбинированный (11)	447	Нативная	0,450	>0,500	>0,500	1,6
		γ-Глобулин	0,160	0,330	0,318	
	449	Нативная	0,460	>0,500	>0,500	7,6
		γ-Глобулин	0,050	0,076	0,067	
	452	Нативная	>0,500	>0,500	>0,500	10
		γ-Глобулин	0,100	0,037	0,047	
С хинозолем (12)	376+420	Нативная	0,500	>0,500	>0,500	5,5
		γ-Глобулин	0,060	0,230	0,091	
	395+400	Нативная	0,190	0,240	0,210	2,1
		γ-Глобулин	0,100	0,100	0,100	
Нормальная (13)		Нативная	>0,500	>0,500	>0,500	1
		γ-Глобулин	>0,500	>0,500	>0,500	

1. method of immunization; 2. number of serum; 3. type of serum; 4. dose protecting 50% of the animals in the case of: 5. females; 6. males; 7. both; 8. concentration factor; 9. intravenous; 10. subcutaneous; 11. combined; 12. with quinosol; 13. normal; 14. native gamma-globulin.

As is seen from Table 1, for the majority of series investigated the dose of gamma-globulin protecting 50 percent of the animals (PD_{50}) ranges from 0.04 to 0.310; the PD_{50} of native serum ranges from 0.19 to more than 0.5. Thereby, the gamma-globulin fraction of antiplague serum in nine out of 11 cases has preventive properties 1.7-10 times greater than the corresponding native serum. For two series, 450 and 451, it was impossible to find any advantages of the purified preparations over the native preparations, because in the doses investigated they did not protect the infected white mice

Table 2

Therapeutic Properties of Antiplague Sera and Their Gamma-Globulin Fractions Expressed as the PD_{50}

(1) Метод иммунизации	(2) Номер сыворотки	(3) Вид сыворотки	(4) Доза, защищающая 50% животных для (5) самок	(6) самцов	(7) тех и других	(8) Коэффициент концентрации
(9) Внутривенный	453	Нативная	0,300	0,160	0,220	2,5
		(14) γ-Глобулин	0,080	0,100	0,085	
	454	Нативная	0,370	0,310	0,330	3,7
		γ-Глобулин	0,088	0,095	0,090	
	455	Нативная	0,350	0,380	0,360	9
		γ-Глобулин	0,030	0,060	0,043	
(10) Подкожный	448	Нативная	<0,050	<0,050	<0,050	5
		γ-Глобулин	<0,010	<0,010	<0,010	
	450	Нативная	0,200	<0,050	0,110	1,5
		γ-Глобулин	0,070	0,160	0,080	
	451	Нативная	>0,500	>0,500	>0,500	1
		γ-Глобулин	0,440	0,400	0,410	
(11) Комбинированный	447	Нативная	0,270	0,360	0,320	4
		γ-Глобулин	0,100	0,060	0,080	
	449	Нативная	0,085	0,090	0,090	1,5
		γ-Глобулин	0,033	0,063	0,050	
	452	Нативная	<0,050	<0,050	<0,050	2
		γ-Глобулин	0,030	<0,010	0,026	
(12) С хинозолем	376+420	Нативная	0,085	0,330	0,190	3,5
		γ-Глобулин	0,033	0,128	0,054	
	395+400	Нативная	0,093	0,360	0,240	3
		γ-Глобулин	0,070	0,130	0,087	
Нормальная (13)		Нативная	>0,500	>0,500	>0,500	1
		γ-Глобулин	>0,500	>0,500	>0,500	

1-13. Same as for Table 1.

against death.

In the comparison of the PD_{50}'s of the antiplague sera investigated it is seen that in the present experiment sera obtained from horses immunized with vaccine strains of the plague microbe intravenously were the best.

The difference between the preventive power of preparations in males and females attracts attention. In the majority of cases (nine out of 11 series) the PD_{50} for females was less than for males. For series 451 these rules and regulations could not be demonstrated because of the poor quality of the serum, which did not protect the animals in the doses used. Serum 452, in which the PD_{50} was less for males than for females, constituted an exception.

The results of the study of the therapeutic properties of the same 11 series of sera are shown in Table 2.

From Table 2 it is seen that the single dose which cures 50 percent of the experimental animals of plague ranges from less than 0.01 to 0.09 for gamma-globulin; from less than 0.05 to 0.36 for native sera. The gamma-globulin fraction has therapeutic properties which are 1½-nine times greater than the corresponding native serum.

In the present experiment sera obtained as a result of immunization of horses subcutaneously had the best therapeutic properties. Of the three sera of this group only series 451 had weaker protective properties in experiments on the study of the preventive and therapeutic properties, approaching normal serum. The effectiveness of the antiplague sera obtained by subcutaneous and combined methods of immunization in the experiments on the treatment of plague was greater than that of the same sera used for prophylactic purposes. Conversely, sera obtained by the method of intravenous immunization were more effective in experiments on the prevention of plague than in experiments on treatment.

Sera prepared in 1957 six-nine months before purification and preserved with quinosol were not inferior in their protective characteristics to the sera prepared by methods of subcutaneous and combined immunization, preserved with chloroform and purified immediately after bleeding.

Conclusions

1. The effectiveness of the gamma-globulin fraction of antiplague serum, determined by the PD_{50} in experiments on the study of the preventive properties, ranges from 0.04 to 0.318, being greater than the preventive properties of the corresponding native sera by 1.6-10 times.

2. In experiments on the study of therapeutic properties the PD_{50}'s of the gamma-globulin fraction of antiplague serum ranged from less than 0.01 to 0.09, being more effective than the corresponding native sera by 1½-nine times.

3. Antiplague sera obtained by the method of intravenous immunization possessed more pronounced preventive properties; sera obtained by methods of subcutaneous and combined immunization are more effective in experiments on the treatment of animals.

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Antigenic Structure of Brucellas in Connection with the Problem of Specific Prophylaxis and Therapy

M. S. Drozhevkina
(Rostov-na-Donu)

The extensive and successful utilization of vaccines for therapeutic and prophylactic purposes in brucellosis is responsible for the need for further perfection of the preparations used. It is well known that the quality of the bacterials depends to a considerable degree on their utilization for preparation of strains which are complete in an antigenic respect, the correct selection of which is possible only on the basis of knowledge of the antigenic structure of the pathogen of this disease.

For a long time the idea was common that the brucellas had a relatively simple antigenic structure. Thus, the presence of a total of two somatic antigens--A and M--present in various proportions in various types of this microorganism was generally accepted for the S forms of brucellas.

In recent years, a considerably more complicated antigenic structure has been demonstrated in brucellas. Thus, Wolf and Dinger (1951), in a strain of brucella isolated from a sick person, found a thermostable antigen accounting for the inagglutinability of this culture in the specific serum. In 1954, we found an additional surface somatic antigen in dissociated strains of brucellas.

This antigen is similar in many properties to the Vi-antigen of microbes of the colon group and we therefore called it the Vi-antigen of brucellas. Its presence in cultures of brucellas is comfirmed by agglutinin tests with pure O- and Vi-sera with the utilization of living and heat-killed antigens, by the results of the test with bacteriophage, with the trypaflavine test, by obtaining Vi-sera from rabbits through immunization of them with cultures containing this antigen as well as by the presence of the Vi-antibodies in the sera of people and agricultural animals sick with brucellosis. According to our data, the majority of freshly isolated brucella cultures carry the Vi-antigen. It can also be preserved for a long time in museum cultures but in smaller quantities. A high content of the Vi-antigen in museum strains of brucellas is achieved through constant cultivation of them on coagulated egg yolk. This antigen, like the Vi-antigen of microbes of the colon group, is very labile and is readily destroyed from the effect of various physical and chemical antigens (M. S. Drozhevkina, 1956).

By means of chemical treatment of a mass of microbes of the B. melitensis 512 strain in the V form by the somewhat modified Webster method, proposed for the isolation of Vi-antigen from microbes of the colon group, the antigen which we have described was isolated in the pure form by A. M. Konnova, T. I. Kharitonova and N. P. Prostetova (1959).

A study of the properties and chemical structure of this preparation made in our laboratory by T. I. Kharitonova showed its specificity. Along with great similarity there are also certain differences between the Vi-antigen of brucellas and the Vi-antigen of B. typhi abdominalis.

Depending on the content of Vi-antigen, brucella cultures are in V, VW

and W forms. In the study of the antigenic structure of brucellas in different forms the inhomogeneity of their somatic thermostable antigens was demonstrated. Along with antigens common to all forms of brucellas there are also thermostable antigens specific for each form of brucella in a certain quantity. In addition, in the set of antigens of the V form of brucellas there is an additional thermolabile antigen (Vi-antigen) which is completely absent from the W forms (M. S. Drozhevkina, 1954, 1956). Somewhat later, Renoux and Mahaffen (1955) described the presence of two additional somatic antigens, r and Z. The r antigen was characteristic of the r forms of brucellas. The Z antigen is a specific antigen of the New Zealand variety of brucellas and is found in a rough phase also in B. melitensis and B. abortus.

The papers of Bruce and Jones (1959), Olitzki and D. Sulitzani (1958), I. I. Dubrovskaya (1957, 1960), V. K. Antonova (1958) and others attest to the existence of several antigens in brucellas. The possibility has not been ruled out that all these investigators described the same accessory thermolabile surface somatic antigen characteristic of some of the strains of brucellas under different names.

These new data open up prospects for the study of the role of the various antigenic complexes in the immunogenesis of brucellosis and determine the possibility of creating more effective therapeutic and prophylactic preparations.

In connection with what has been presented, our task was the testing of brucella vaccine prepared from brucella strains different in their antigenic structure. The vaccines were prepared from the B. melitensis 512 strain in the V form, rich in the Vi-antigen, from the W form of the same strain without the Vi-antigen, and from a mixture of them. The strains used in the experiment were different not only in their content of Vi-antigen but also had some components of the O antigen specific for each form.

In six unitypical experiments on 180 guinea pigs the preventive properties of vaccines prepared from these strains by different methods were tested. The vaccine was injected subcutaneously into the guinea pigs in three injections. The dose of vaccine for the first injection was equal to 500,000,000; the second, 1,000,000,000; for the third, 2,000,000,000 microbes; the intervals between the injections were equal to four-five days. Twenty-one days after the last injection all experimental and control guinea pigs were infected by subcutaneous injection with 10 infecting doses of a strain of B. melitensis 535 (one infecting dose was equal to two microbes). The animals were dissected and examined bacteriologically 30 days after the infection.

The summarized results of the experiment are shown in Table 1.

As is seen from Table 1, all the vaccines made from brucellas possessing the Vi-antigen by methods which spare this antigen possessed distinctly expressed preventive properties. Thereby, the vaccines made from the V or W forms separately did not completely guarantee against infection of animals immunized with them. Vaccines made of a mixture of the V and W forms, particularly those which were made by methods which spare the Vi-antigen (acetone-treated and irradiated), proved to be much better. These vaccines protected all animals immunized with them against infection.

The following experiment was directed at clarifying the role of various brucella antigens isolated through chemical treatment of a mass of microbes

Table 1

Degree to Which Brucellas Could be Plated out of Internal Organs of Guinea Pigs Immunized with Vaccines Prepared from Strains of Different Antigenic Structures 30 Days after their Infection with 10 Infecting Doses of B. melitensis 535

Штаммы, из которых приготовлена вакцина (1)	Гретая (2)	Формалинизированная (3)	АД вакцина (4)	Спиртовая (5)	Ацетоновая (6)	Облученная (7)
Br. melitensis 512 V	0	0,42	0,21	0,21	0,10	0
Br. melitensis 512 W	0,35	0,14	0,03	0,35	0,15	0
Br. melitensis 512 V+W	0,21	0,03	0,07	0,10	0	0
(8) Контроль (невакцинированные животные)	0,42	0,53	0,42	0,50	0,46	0,46

1. strains from which the vaccine was prepared; 2. heat-killed; 3. formalinized; 4. AD vaccine; 5. alcohol; 6. acetone; 7. irradiated; 8. control (unvaccinated animals).

of the B. melitensis 512 strain in the V form. For the purpose of extraction of the Vi-antigen a method was used which had been proposed in the work by A. M. Konnova, T. I. Kharitonova and N. P. Prostetova (1959). In general outlines it amounts to the following.

The extract obtained after treatment of a mass of microbes with trichloroacetic acid was dialyzed from the dialysate. After centrifugation and separation of the insoluble precipitates, the Vi-antigen was precipitated by means of uranyl acetate. The precipitate was dissolved in 2 percent citric acid and dialyzed. Reprecipitation and solution were repeated four-five times, until the end product was free of impurities giving a positive biuret test. After separating the precipitate of the Vi-antigen from the centrifugate the O antigen was precipitated with four volumes of alcohol.

Guinea pigs were immunized three times by subcutaneous injection of the antigens diluted in a small quantity of physiological saline solution. The dose of antigen for the first injection was 0.004 milligram; for the second, 0.01 milligram; for the third, 0.05 milligram. The intervals between the injections were equal to five days. Twenty-one days after the last injection the animals were infected with five infecting doses of B. melitensis 599 (one infecting dose was two microbes). Thirty days after the infection of guinea pigs they were dissected, and a bacteriological examination was performed (Table 2).

As is seen from Table 2, subcutaneous immunization of guinea pigs three times with these antigens and subsequent infection of them with a virulent culture of the B. melitensis type showed that separate immunization with the Vi- and O antigens does not lead to complete protection of the animals against infection. It only restrains the dissemination of the process and the seeding of the body with the pathogen. Much better results are given by immunization of guinea pigs with the Vi- and O antigens simultaneously; however, this does not protect all the experimental animals against infection.

It is very possible that this is connected with the loss of a certain portion of the O antigens during the course of chemical treatment of the mass of microbes. Therefore, in the next experiment we tested the immunogenicity

Table 2

Immunogenicity of Various Brucella Antigens Obtained by Chemical Treatment

(1) Антиген, использованный для иммунизации	(2) Номер морской свинки	(3) Результат бактериологического исследования: (4) лимфатические узлы: (5) правый паховый	(6) левый паховый	(7) правый подмышечный	(8) левый подкрыльцовый	(9) правый паховый	(10) левый паховый	(11) селезенка	(12) печень	(13) легкое	(14) кровь	(15) костный мозг	(16) моча
(17) Vi-антиген	4389	+	—	—	—	+	+	+	—	—	—	—	—
	4390	+	—	—	—	+	—	—	—	—	—	—	—
(18) О-антиген	4399	+	—	—	—	+	+	+	—	—	—	—	—
	4400	+	—	—	—	+	+	+	+	—	—	—	—
(19) Vi+О-антигены	4395	—	—	+	—	—	—	—	—	—	—	—	—
	4396	—	—	—	—	—	—	—	—	—	—	—	—
(20) Контроль (неиммунизированные)	5456	+	+	+	+	+	+	+	+	—	—	+	—
	5457	+	+	—	+	+	+	+	+	+	—	+	+
	5453	+	+	+	+	+	+	+	+	—	—	+	—

1. antigen used for immunization; 2. No of guinea pig; 3. results of bacteriological examination; 4. lymph nodes; 5. right inguinal; 6. left inguinal; 7. right axillary; 8. left axillary; 9. right paraaortic; 10. left paraaortic; 11. spleen; 12. liver; 13. lung; 14. blood; 15. bone marrow; 16. urine; 17. Vi-antigen; 18. O antigen; 19. Vi- plus O antigens; 20. control (nonimmunized).

of the vaccine made up of the Vi-antigen and a typical strain of brucellas killed with acetone and heat. For this purpose the B. melitensis 364 strain in the W form was used, which, as we believe, should contain a complete set of somatic antigens in the killed form. Data in the literature attesting to the high degree of immunogenicity of typhoid vaccine consisting of a combination of Vi-antigen with acetone-dried typhoid culture (A. de Barbieri and M. Scaevola and others, 1956) also constituted the basis for performing this experiment.

The guinea pigs were immunized in the same way as in the previous experiment, with the difference only that a killed brucella culture was added to the diluted Vi-antigen in the following quantities: 500,000,000 for the first injection; 1,000,000,000, for the second; and 2,000,000,000 microbes for the third.

Twenty-one days after the last injection, the immunized guinea pigs and control animals were infected with five infecting doses of B. melitensis 599. Dissection and study of these animals 30 days after infection showed the high degree of immunogenicity of the vaccine, which was made up of the Vi-antigen and the heat-killed culture. The vaccine made up of a combination of the Vi-antigen with the same culture but killed through the use of acetone proved to be somewhat poorer (Table 3).

Table 3

Preventive Properties of Brucella Vi Vaccine

Антиген, использованный для иммунизации (1)	Номер морской свинки (2)	(3) Результаты бактериологического исследования												
		(4) место заражения	(5) лимфатические узлы: (6) правый паховый	(7) левый паховый	(8) правый подмышечный	(9) левый подмышечный	(10) правый парааортальный	(11) левый парааортальный	(12) селезенка	(13) печень	(14) легкое	(15) кровь	(16) костный мозг	(17) моча
(18) *Первый опыт*														
(19) Vi-антиген+ Br. melitensis 364, убитая нагреванием	4391	—	—	—	—	—	—	—	—	—	—	—	—	—
	4392	—	—	—	—	—	—	—	—	—	—	—	—	—
	4394	—	—	—	—	—	—	—	±	—	—	—	—	—
(20) Vi-антиген+ Br. melitensis 364, убитая в результате применения ацетона	4397	—	+	+	—	+	+	+	±	—	—	—	—	—
	4398	—	—	—	—	—	—	—	—	—	—	—	—	±
(21) Контроль (неиммунизированные)	5456	+	+	+	+	+	+	±	+	±	—	—	+	—
	5457	+	+	+	+	+	+	+	—	±	+	—	+	+
	5458	+	+	+	+	+	+	+	+	+	—	—	+	—
(24) *Второй опыт*														
(14) Vi-антиген+ Br. melitensis 364, убитая нагреванием	4836	—	—	—	—	—	—	—	—	—	—	—	—	—
	4837	—	—	—	—	—	—	—	—	—	—	—	—	—
	4838	—	—	—	—	—	—	—	—	—	—	—	—	—
	4839	—	—	—	—	—	—	—	—	—	—	—	—	—
	4840	—	—	—	—	—	—	—	—	—	—	—	—	—
(22) Контроль Br. melitensis 364, убитая нагреванием	4841	—	—	—	+	—	—	—	—	—	—	—	—	—
	4842	+	—	—	±	—	—	—	—	—	—	±	—	±
	4843	+	+	+	—	—	—	—	+	—	—	—	—	—
	4844	—	+	—	—	—	±	—	—	—	—	±	—	—
	4845	—	—	—	—	—	—	-	—	—	—	—	—	—
(20) Vi-антиген+ Br. melitensis 364, убитая ацетоном	4821	—	—	—	—	—	—	—	—	—	—	—	—	—
	4822	—	—	—	—	—	—	—	—	—	—	—	—	—
	4823	—	—	—	—	—	—	—	—	—	—	—	—	—
	4824	—	—	—	—	—	—	—	—	—	—	—	—	—
	4825	—	—	+	+	—	—	—	+	—	—	—	—	—
(23) Контроль Br. melitensis 364, убитая ацетоном	4826	±	—	—	—	—	—	—	—	—	—	±	±	—
	4827	—	+	—	±	—	—	—	—	±	±	—	±	±
	4828	+	+	—	—	—	—	+	±	±	—	±	±	±
	4829	—	±	+	—	—	±	±	±	—	±	±	±	±
	4390	—	+	—	—	±	—	±	±	—	±	±	—	±
(21) Контроль (неиммунизированные)	5810	+	—	—	—	—	±	±		±	±	-	-	—
	5811	+	—	—	—	—	±	±	±	-	-	—	-	—
	5812	-	+	—	—	—	—	+	±	±	±	—	-	—
	5813	+	±	+	—	—	—	±	±	—	—	—	±	±
	5814	—	±	±	—	±	+	±	±	—	—	—	—	±

[Table 3, continued next page]

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/Table 3, continued from previous page/ 1. antigen used for immunization; 2. number of guinea pig; 3. results of bacteriological examination; 4. site of infection; 5. lymph nodes; 6. right inguinal; 7. left inguinal; 8. right axillary; 9. left axillary; 10. right paraaortic; 11. left paraaortic; 12. spleen; 13. liver; 14. lung; 15. blood; 16. bone marrow; 17. urine; 18. first experiment; 19. Vi-antigen plus B. melitensis 364 killed by heat; 20. Vi-antigen plus B. melitensis 364 killed through the use of acetone; 21. control (nonimmunized); 22. control B. melitensis 364, killed by heat; 23. control, B. melitensis 364 killed by acetone; 24. second experiment.

After a year the experiment was repeated on a large number of animals. Although there was a reduction in the virulence of the strain of B. melitensis 599 used for infection in this period of time, still clear-cut data were obtained which were in complete agreement with the results of the first experiment (see Table 3). This time also injection of the vaccine made of the Vi-antigen and the heat-killed B. melitensis 364 culture protected all the guinea pigs against brucellosis.

The data obtained represent the basis for further experiments on the testing of the therapeutic effectiveness of brucellosis Vi Vaccine as well as the possibility of utilizing it for purposes of emergency prophylaxis of brucellosis.

The second series of experiments was on the study of antibrucellosis sera. Apparently, sera obtained from the immunization of animals with whole antigens should possess the most effective therapeutic and prophylactic properties. With the aim of determining the effect of the antigen composition on the quality of the serum, we (M. S. Drozhevkina and T. I. Kharitonova, 1956) used brucella strains which were different antigenically for the immunization of animals. Vi plus O serum obtained as a result of immunization of the animals with strains of B. melitensis in the V and W forms was the best. This serum contained a more complete set of antibodies (O and Vi). When tested in vitro, it had a bactericidal effect and activated the phagocytic activity of leukocytes. In experiments on animals the serum showed very good preventive properties. Injection of it three times contributed to localization of brucellas at the primary complex with subsequent sterilization of the brucellosis-infected animals. This determines the promise of studies on the preparation and testing of such serum with the aim of emergency prophylaxis and possibly, therapy of brucellosis.

The factual material presented here only in a general form, accumulated at the laboratories of our institute in the past five years, are evidence of the great practical significance of the latest data on the antigenic structure of brucellas.

Study of the preventive properties of the vaccines made from brucellas strains of different antigenic structures as well as the Vi and O antigens isolated showed that the effectiveness of brucellosis vaccine is conditioned by the presence of O and Vi antigens in it simultaneously and of O and Vi antibodies in the serum.

The quality of the therapeutic brucellosis vaccine is directly related to the proper selection of strains of brucellas and methods of preparation of it. The material which we obtained is evidence of the need for including

brucellas in the commercial strains along with the W and V forms. The combination of them alone will assure the presence of a complete set of antigens characteristic of the brucellosis microbe. Therefore, study of the preventive properties of the vaccines made of brucellas strains of different antigenic structures as well as study of the isolated antigens indicates the great part of surface-somatic antigen in the creation of immunity.

The quality of the therapeutic brucellosis vaccine and serum depends on the proper selection of strains of brucellas and proper methods of preparing them. The testing of the brucellosis vaccine and sera prepared by the author is recommended for the therapy and emergency prophylaxis of brucellosis.

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The Effect of Different Antibiotic Combinations on the Cultivation of Brucellas in Vitro

N. B. Igonina
(Saratov)

There are quite a number of papers on treatment of brucellosis with different antibiotics, but the authors' data are controversial.

Less controversial and more encouraging results have been obtained by a number of authors from the application of combined antibiotic therapy.

Thus, S. Ye. Shapiro (1954, 1955), Ye. A. Shayreva (1956), T. Kh. Nadzhimiddinov (1956, 1957) and V. M. Madzhidov (1957) used streptomycin in combination with synthomycin successfully clinically for the treatment of patients with brucellosis. T. Kh. Nadzhimiddinov obtained a good therapeutic effect when biomycin [aureomycin] was added to the combination of streptomycin and synthomycin [racemic chloramphenicol]. However, the best results, in this author's opinion, were obtained from the use of streptomycin with terramycin. A good therapeutic effect from the use of streptomycin in combination with terramycin was also obtained by Werner and Knight (1950), Herrell and Barber (1952). Ye. A. Shyyreva (1956), I. N. Ivashurova (1957) and A. M. Yartseva (1958) recommend using streptomycin in combination with aureomycin for the treatment of brucellosis.

M. Ye. Adamova (1957) reports the fair therapeutic effect of synthomycin in combination with biomycin. A. M. Tselishchev (1956) obtained a "complete immediate effect" from the clinical use of a combination of levomycetin [laevo-rotary chloramphenicol] with streptomycin or biomycin and streptomycin with simultaneous administration of specific serum.

In experiments on white mice Ye. I. Kaytmazova and N. I. Givental' (1957) showed the therapeutic effectiveness of a combination of antibiotics, biomycin with ecmoline [antibiotic from fish liver] and terramycin with ecmoline.

It should be noted that many authors consider it advisable for the treatment of brucellosis to use antibiotics in combination with vaccine or vitamins or all three ingredients in combination (I. A. Chervinskiy, 1947; A. F. Bilibin, 1957; A. M. Tselischev, 1956; A. M. Yartseva, 1956; M. Ye. Adamova, 1957; I. L. Bogdanov, 1955, 1957; Z. V. Yermol'yeva and Ye. I. Kaytmazova, 1957; N. N. Ipatova, 1957; G. M. Marueshvili, 1957; S. Sh. Pinkus, 1957 and others). All authors recommend that the antibiotic therapy be conducted in the acute septic or septic-metastatic period of the disease as well as in the exacerbation phase of chronic brucellosis.

In the present work we set for ourselves the task of studying the combined effect of the antibiotics colimycin, mycerin, streptomycin and terramycin in various combinations and doses on the brucellosis microbe under test-tube conditions. The accomplishment of this task was dictated by the fact that in the present work on the study of the action of these antibiotics per se on brucellas (N. B. Igonina, 1960) a clearly expressed antibacterial activity of the first three antibiotics was obtained.

We used the antibiotics in the following combinations: colimycin + mycerin; colimycin + streptomycin; mycerin + streptomycin; colimycin + terramycin; mycerin + terramycin; streptomycin + terramycin.

The preparations were given in equal doses of 12.5, 6.25 and 3.12 gamma per cc. In selecting the doses we were guided by the fact that in the previous experiments colimycin, mycerin and streptomycin used per se in doses of 12.6 gamma per cc produced sterilization of the cultures, as a rule, after 24 hours of contact with the culture; in smaller doses they exerted only a bacteriostatic effect on the brucellas. We used terramycin in higher concentrations (from 12.5 to 100 gamma per cc), because in the experiment smaller doses of it exerted no bactericidal effect on the brucellas.

The experiments were performed with the same series of liver bouillon

and agar (pH of 6.9). A virulent strain of brucellosis microbe (487b) was seeded in the bouillon in a quantity of 10,000,000 microbes per cc of the medium, and antibiotics were immediately added. Bouillon which did not contain antibiotics in which the same number of microbes had been seeded as in the experimental test-tubes served as a control. The cultures were incubated at 37°. Material was plated out of all the test-tubes (in quantities of 0.1 cc) on plates containing liver agar every day for five days. The absence of growth on agar plates was evidence of the loss of viability of the brucella under the influence of antibiotics.

We compared the results of the present experiments with data obtained in experiments on the study of the action of the antibiotics per se on a culture of brucellas under conditions which we have described in our previous report (N. B. Igonina, 1960).

From Table 1 it is seen that colimycin, mycerin and streptomycin used in these combinations and doses completely suppressed the growth of brucellas after 24 hours of contact with the culture. However, the bactericidal activity of colimycin and mycerin in combination with terramycin was somewhat reduced by comparison with the utilization of them by themselves. Thus, when colimycin and mycerin were used by themselves in doses of 12.5 gamma per cc the brucella cultures were sterilized as early as after 24 hours of contact, while in combination with terramycin in doses from 50 to 12.5 gamma per cc this occurred only on the second day. Streptomycin in a dose of 12.5 gamma per cc in combination with terramycin, used in a dose of 25 gamma per cc, also reduced the bactericidal activity from that in the basic experiments.

We performed the subsequent experiments with the aim of determing the time of occurrence of a complete bactericidal effect by the same combinations of antibiotics on the brucellosis microbe.

With this aim in view, we performed a series of experiments by the same method, plating out the cultures (in a quantity of 0.1 cc) from the experimental test-tubes onto agar plates every two hours for a day as well as after 48 and 72 hours (Table 2).

From Table 2 it follows that all antibiotic combinations in the first few hours of contact with the culture of brucellas exerted only a bacteriostatic effect; the bactericidal effect was observed after longer contact.

The bactericidal effect of colimycin in combination with mycerin in doses of 12.5 gamma per cc each was expressed after 10-12 hours' exposure; in doses of 6.25 gamma per cc each, after 20 hours of exposure, and in doses of 3.12 gamma per cc each, after a 24-hour exposure. Let us recall that these antibiotics per se in doses of 12.5 gamma per cc inhibited the growth of brucellas only after a 20-22-hour exposure.

Bactericidal activity of colimycin in combination with streptomycin, using doses of 12.5 gamma per cc, was expressed after eight hours of exposure; in 6.25 gamma per cc each, after 20 hours; and in doses of 3.12 gamma per cc each, after 24 hours of exposure. These antibiotics per se in a dose of 12.5 gamma per cc each sterilized the cultures only after 18-22 hours of contact with the culture. Therefore, through the combined application of antibiotics their bactericidal characteristics were manifested much more quickly.

The bactericidal effectiveness of mycerin in combination with streptomycin was demonstrated almost at the same time as in the combination of

Table 1

Antibacterial Effect of Antibiotic Combinations with Respect to Developing Brucellas in Relation to the Duration of Contact

(1) Длительность контакта, сутки	(2) Антибактериальная активность комбинаций антибиотиков в дозах, γ/мл													
	(3) колимицин + мицерин аа от 12,5 до 3,12	(4) колимицин + стрептомицин аа от 12,5 до 3,12	(5) мицерин + стрептомицин аа от 12,5 до 3,12	(6) колимицин 12,5 + террамицин в дозе			(7) мицерин 12,5 + террамицин в дозе			(8) стрептомицин 12,5 + террамицин в дозе			(9) контроль
				50	25	12,5	50	25	12,5	50	25	12,5	
1	-	-	-	+	+	++	+	+++	+++	-	±	+	++++
2	-	-	-	-	+	+	-	+	+	-	-	+	++++
3	-	-	-	-	-	-	-	-	-	-	-	-	++++

Arbitrary designations: ++++ continuous growth; +++, growth up to 1000 colonies; ++, up to 500 colonies; +, up to 100 colonies; -, no growth.

Key: 1. duration of contact, days; 2. antibacterial activity of antibiotic combinations in doses, gamma per cc; 3. colimycin +mycerin in doses of 3.12 gamma per cc to 12.5 gamma per cc each; 4. colimycin +streptomycin in doses from 3.12 to 12.5 gamma per cc each; 5. mycerin +streptomycin, 3.12 to 12.5 gamma per cc each; 6. colimycin, 12.5 +terramycin in a dose of:; 7. mycerin, 12.5 +terramycin in a dose of:; 8. streptomycin 12.5 +terramycin in a dose of; 9. control.

Table 2

Time of Expression of Antibacterial Activity of Antibiotic Combinations with Respect to Developing Brucellas

(1) Длительность контакта, часы	(2) Антибактериальная активность комбинаций антибиотиков в дозах, γ/мл																					(9) Контроль
	(3) колимицин + мицерин аа			(4) колимицин + стрептомицин аа			(5) мицерин + стрептомицин аа			(6) колимицин 12,5 + террамицин в дозе				(7) мицерин 12,5 + террамицин в дозе				(8) стрептомицин 12,5 + террамицин в дозе				
	12,5	6,25	3,12	12,5	6,25	3,12	12,5	6,25	3,12	100	50	25	12,5	100	50	25	12,5	100	50	25	12,5	
2	++	++	+++	++	++	++++	+++	++++	++++	+	+++	+++	++++	+	++	+++	+++	+	-	+++	+++	+
4	+	++	+++	+	++	++++	+	++++	++++	+	+	++	+++	+	++	++	++	±	+	+	++	+
6	+	+	+++	+	++	++++	+	++	++++	+	+	++	+++	+	+	+	+++	-	+	+	+	-
8	+	+	++	-	+	+++	+	++	++++	+	+	++	+++	+	+	+	++	-	+	+	+	-
10	+	+	+	-	+	+	+	+	+++	+	+	+	+++	+	-	+	++	-	+	+	+	-
12	-	+	+	-	+	+	-	+	+++	+	+	+	+++	+	+	+	++	-	+	+	+	+
14	-	+	+	-	+	+	-	+	++	+	+	+	+++	+	+	+	+	-	+	+	+	+
16	-	+	+	-	+	+	-	+	++	+	+	+	+++	+	+	+	+	-	+	+	+	+
18	-	+	+	-	+	+	-	+	+	+	+	+	+++	+	+	+	+	-	+	+	+	+
20	-	-	+	-	-	+	-	-	+	+	+	+	+++	+	+	+	+	-	-	+	+	+
22	-	-	+	-	-	+	-	-	+	+	+	+	+++	+	+	+	+	-	-	-	+	+
24	-	-	-	-	-	-	-	-	-	-	+	+	+++	-	+	-	+	-	-	-	+	+
48	-	-	-	-	-	-	-	-	-	-	-	-	+++	-	-	-	+	-	-	-	-	+
72	-	-	-	-	-	-	-	-	-	-	-	-	+++	-	-	-	+	-	-	-	-	+

Designations are the same as Table 1.

Key: 1, 2, 6, 7, 8, 9. Same as Table 1. 3. colimycin +mycerin each in a dose of; 4. colimycin +streptomycin, each in a dose of; 5. mycerin +streptomycin, each in a dose of.

mycerin with colimycin.

With the use of colimycin and mycerin in combination with terramycin the bactericidal activity of the antibiotics included in the combination was reduced by comparison with when they were used per se. While per se colimycin in a dose of 12.5 gamma per cc had a bactericidal effect on the cultures of brucellas after 22 hours of contact, in combination with terramycin (in a dose of 50-12.5 gamma per cc) it did not show a bactericidal effect even in 48-72 hours. Mycerin per se in a dose of 12.5 gamma per cc sterilized the cultures after 20 hours of contact, but in combination with terramycin in a dose of 50-12.5 gamma per cc it showed a bactericidal effect only after two or three days of contact.

In our experiments streptomycin was an exception; its bactericidal activity in combination with terramycin in a dose of 100 gamma per cc was considerably increased compared with its application per se. In these doses streptomycin in combination with terramycin sterilizes the culture of a brucellosis microbe as early as after six-eight hours of contact. With reduction of the dose of terramycin in this combination the bactericidal effect of streptomycin is reduced by comparison with its administration per se. Therefore, as our experiments showed, terramycin is apparently an antagonist of colimycin and mycerin with respect to the antibacterial effect on brucellas. In combination with streptomycin terramycin (in a dose of 100 gamma per cc) exerted a synergistic effect. Despite the fact that after the use of terramycin in combination with colimycin and mycerin their sterilizing effect is reduced, the bactericidal effect of terramycin in combination with these antibiotics is increased. Thus, in a dose of 100 gamma per cc per se it manifested only a bacteriostatic effect; in combination with colimycin or mycerin this preparation showed bactericidal activity after 24 hours of exposure. In combination with streptomycin this dose of terramycin sterilized the cultures as early as after six-eight hours of contact with the culture.

In the subsequent experiments we set before ourselves the aim of studying the effect of different combinations of colimycin, mycerin, streptomycin and terramycin on a developing (two-day) bouillon culture of brucellas.

For this purpose subcultures from the cultures in the experimental test-tubes were made on agar plates every day for five days. Antibiotics in this experiment were used in the same combinations as in the first. Based on our previous data, which showed that the antibacterial effect of the antibiotics with respect to a mature culture of brucellas is manifested much less vigorously than in the case of a young one, we used colimycin, mycerin and streptomycin in a somewhat greater concentration than in the previous experiments, 25 instead of 12.5 gamma per cc (Table 3).

From Table 3 it is seen that colimycin in combination with mycerin, used in a concentration of 25 gamma per cc each, had a bactericidal effect on a culture of brucellas after two days of exposure, whereas per se they sterilized the cultures only after four days of exposure.

Colimycin in combination with streptomycin and mycerin in combination with streptomycin gave almost the same results in their action on the brucellas. These antibiotics, used in doses of 25 gamma per cc each, exerted a bactericidal effect on the second-third day of contact with the culture of brucellas; in doses of 12.5 gamma per cc, on the fourth day, and in concentrations of

Table 3

Antibacterial Effect of Antibiotic Combinations on Developing (Two-Day) Culture of Brucellas Depending on the Duration of Contact

(1) Длительность контакта, сутки	(2) Антибактериальная активность комбинаций антибиотиков в дозах, γ/мл																								(9) Контроль
	(3) колимицин + мицерин аа				(4) колимицин + стрептомицин аа				(5) мицерин + стрептомицин аа				(6) колимицин 12,5 + терramицин в дозах				мицерин 12,5 + терramицин в дозах				стрептомицин 12,5 + терramицин в дозах				
	25	12,5	6,25	3,12	25	12,5	6,25	3,12	25	12,5	6,25	3,12	100	50	25	12,5	100	50	25	12,5	100	50	25	12,5	
1	++	+++	+++	+-	-	+++	+++	+++	++	-++	+++	+++	++	+++	+++	++	++	+++	+++	+-	++	+++	+++	+-	++
2	-	+	++	++-	-	+	+++	+++	-	-	+++	+-+	+	++	++	+-	+	+-	++	+++	+	-	+	++	++
3	-	+	++	+-	-	+	++	+++	-	-	+++	++	+	+	+	+	-	-	+-	++	-	-	+	+	++
4	-	-	+	-	-	-	+	++	-	-	++	+	+	-	+	+	-	-	-	+	-	-	+	+	++
5	-	-	-	-	-	-	-	+	-	-	-	-	+	-	-	+	-	-	-	+	-	-	-	-	++

Arbitrary Designations are the same as in Table 1.

Key: 1-9. Same as for Table 2.

6.25 and 3.12 gamma per cc, only on the fifth day.

Colimycin or mycerin in combination with terramycin, by contrast with experiments in which they were used per se, not only failed to increase the antibacterial properties but even decreased them: per se they had a bactericidal effect after four days of contact; in combination with terramycin, after five days.

The bactericidal effect of streptomycin, used in combination with terramycin, increased appreciably. Thus, used in a dose of 12.5 gamma per cc it did not per se show any bactericidal effects for four days; in combination with terramycin, on the other hand, where the dose of the latter was 100-50 gamma per cc it had a bactericidal effect on brucellas after three days of exposure.

Conclusions

1. In the study of the effect of a combination of antibiotics (colimycin +mycerin, colimycin +streptomycin and mycerin +streptomycin) on a developing bouillon culture of brucellas an increase was found in the bactericidal activity of these combinations compared with their utilization in the pure form.

2. The bactericidal activity of colimycin or mycerin, used in combination with terramycin and by contrast with their utilization per se, is reduced with respect to young brucella cultures.

3. A terramycin concentration of 100 gamma per cc is synergistic for streptomycin. The sterilizing effect of streptomycin in this case is increased two-three times.

4. Colimycin, mycerin and streptomycin, used in different combinations and doses also showed a more active antibacterial effect on already developed brucella individuals (two-day bouillon culture).

5. The bactericidal effect of colimycin and mycerin when each was used in combination with terramycin was reduced with respect to two-day brucella cultures.

6. In an experiment with a two-day culture the bactericidal effect of streptomycin, used in combination with terramycin (dose of 50-100 gamma per cc) was increased by almost two times.

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Pathological Changes in the Organs of Dwarf Susliks Infected with Plague in a State of Hibernation

N. D. Dobrokhotova and V. N. Lobanov
(Ural'sk)

The course of plague infection in the bodies of dwarf susliks in a state of hibernation may be of a lingering character. Pathological changes occurring in this infectious process have been inadequately studied.

A. A. Churilina (1915), infecting dwarf susliks experimentally during the period of their hibernation, concluded that under these conditions the rodents mentioned become sick with the "chronic form of plague," the main characteristics of which are the presence of tubercles in the parenchymatous organs and cachexia. In addition, A. A. Churilina observed a "secondary inflammatory process" in the lungs of a suslik which had died four months and 21 days after infection. Under the impression left by this work in the literature mention began to be made of the existence of a "chronic nodular" form of plague in susliks.

Ya. Ye. Braul (1931) made a study of the nodules in the organs of dwarf susliks, considering them most important and even the only sign of "chronic plague" in these rodents. Ya. Ye. Braul had the organs of five susliks, caught during a summer plague epizootic. In the spleens and livers of the animals which he investigated small grayish-white nodules were seen. On histological examination it was found that they consisted of spheroidal accumulations of plague microbes surrounded by a belt of polynuclear cells. The author did not find any granulomas in the parenchymatous organs of susliks, which are characteristic of chronic infections. In our opinion, the nodules in the liver and spleen of susliks are not a sign of chronic plague, because many investigators have observed the same structures in their organs in acute and subacute forms of plague during epizootics among susliks (S. M. Nikanorov, 1925; V. M. Tumanskiy, 1947 and others).

The picture of the pathological changes in dwarf susliks which have died of plague during the spring-summer epizootics, according to the data of many authors, is distinguished by great variety. I. S. Tinker (1940) believes that in the first half of intense epizootics among these animals the septic form of plague predominates with a high mortality rate and with poorly

expressed pathological changes in the organs. In the second half of the epizootic enlargements of lymph nodes, primary buboes, signs of degeneration of the liver, whose color consists of various shades of clay-yellow, are encountered. At the same time, secondary lesions are encountered in the lungs (areas of necrosis and pneumonia); enlargement of the spleen and adhesions of it to surrounding tissue, "necrotic foci" in it. Toward the end of the epizootic there is an increase in the number of cases with a benign course of plague. The course of the pathological process in plague of dwarf susliks in the autumn-winter has been little studied. Therefore, histological studies of the organs of these animals infected with plague while in a state of hibernation should be of significance for throwing light on this subject.

Personal Observations

In the autumn-winter of 1949/50 we infected 109 dwarf susliks; 84 of them were in a state of hibernation, and 25 had not gone into hibernation yet. The animals were infected subcutaneously with a suspension of a two-day culture of the plague microbe, strain 586, in doses of 1000, 10,000 and 100,000 and 1,000,000 microbes in a volume of 0.5 cc of physiological saline solution. The minimum lethal dose of this strain for the guinea pig was equal to 1000 microbes. Of 109 animals 97 died at various periods and 12 were killed after their spring awakening from hibernation.

The organs of 59 susliks were studied histologically. In this group there were 29 susliks from which plague microbe cultures had been isolated, 11 animals which had been killed and 19 which had died, from which the culture was not obtained.

The suslik organs were fixed in 10 percent formalin. The sections were stained with hematoxylin-eosin, picrofuchsin, for fibrin (by the Cockel method) and for detection of microbes (by the Unna-Pappenheim method).

Of 29 plague-infected susliks 15 died from four to 15 days after the infection; almost all of them had received large doses of the plague microbe (100,000 and 1,000,000 microbes). None of the 15 animals became sick while in hibernation; some were infected in a waking state and did not go into hibernation; others awakened from hibernation after their infection. Cultures of the organs and blood of these susliks showed good growth of the plague microbe with the exception of two cases where no bacteriemia was observed.

Susliks which were in deep hibernation (including those infected in a waking state which shortly afterwards went into hibernation) died of plague after a longer period of time--from 27 to 250 days. In 11 of them dissemination of plague infection was observed in the winter as well as in the spring after awakening (eight susliks).

In Table 1 the most important pathological changes observed in the organs of the experimental susliks are presented with an indication of their lifespans. With a lingering pathological process and subsequent exacerbation of it the same macroscopically visible changes were encountered in the organs as in susliks with the acute disease which died in the early periods: injection of the subcutaneous blood vessels, degeneration of the liver (the color of it was gray-yellow, brown-gray or brown-yellow and the tissue was flabby);

hyperemia and grayish-white nodules in the spleen: from isolated nodules to numerous ones, from the size of a dot to a grain of millet. Nodules were encountered in the susliks no sooner than the 11th day after the infection. Cachexia and dense infiltrates at the injection site were observed in the case of a lingering pathological process. In all cases pathological changes were noted in the lungs: hyperemia, hemorrhages, areas of necrosis and foci of pneumonia. In four cases adhesions of the lungs to the diaphragm or parietal pleura were observed in the plague-infected susliks, which in two cases were combined with areas of necrosis and lung abscesses.

As far as the pathological picture in the 33 animals which died is concerned, from which no culture was obtained (of these the organs of 19 animals were examined histologically), the following macroscopic changes were observed: in 21 there was wasting to the point of cachexia; in 13 degenerative changes of the liver were noted; in five, injection of the subcutaneous blood vessels; in six, hyperemia of the spleen; nodules in the liver and spleen were noted in two susliks. In the lungs there were areas of densification in three susliks and hyperemia in 13; in eight susliks adhesions of the lungs to the adjacent tissues were found (to the pleura and diaphragm). In eight of the 11 susliks killed no changes were observed in the organs, and in only three of them was enlargement of the spleen and hyperemia of the lungs noted.

These changes in the organs were in complete agreement with those described above and were observed in susliks from which the plague culture was obtained. However, in these cases the diagnosis was not corroborated either by culture or by biological test.

Histological Studies

For convenience of description of the results obtained we divided the susliks investigated into three groups: 1) susliks from which the plague microbe culture was obtained; 2) susliks which died but in which the diagnosis of plague could not be confirmed bacteriologically; 3) killed susliks with a negative culture.

In the first group there were also 29 susliks, from which a culture was isolated. Of these 14 died after a long hibernation. The results of study of the organs of these animals are the following.

In the lungs of 12 animals pneumonia was found: in five susliks it was confluent or lobar; in seven, focal. Thereby, in the alveoli an accumulation of exudate was seen, chiefly serous-hemorrhagic or hemorrhagic in type. Along with plasma and red blood cells, sloughed-off alveolar epithelial cells and neutrophils were seen in the majority of cases in larger or smaller numbers. Large accumulations of microbes were found in the lumina of the blood vessels, capillaries and alveoli and around the blood vessels and capillaries. Marked circulatory disorders were also noted in the form of stasis, exudation of plasma, and irregular blood distribution. In the walls of the blood vessels signs of necrosis and necrobiosis, inflammatory infiltrates and plasma impregnation were observed. Around the blood vessels there were infiltrates consisting chiefly of macrophages and lymphocytes.

In the liver in three of the 14 susliks examined areas of necrosis

were observed (in two, multiple micronecrotic areas; in one, single macroscopic areas); in one there were scattered microabscesses, and in 10, cloudy swelling of the liver cells.

In the spleens of two animals there was hyperplasia; in two, areas of necrosis; in the other 10, no essential changes were found.

In the kidneys of one suslik there were abscesses; in 13, cloudy swelling of the tubular epithelium.

Of the 15 susliks which became sick in the waking state and died in the period between the fourth and 15th day the following data were obtained. In the lungs of seven susliks confluent and lobar pneumonia were found; in four, bronchopneumonia. On histological examination pneumonia was demonstrated characteristic of plague, as in the animals described above in which red blood cells were predominant in the exudate; in two susliks there were extensive areas of necrosis in the foci of pneumonia. In two animals only marked circulatory disorders were observed (congestion, stasis) in the lungs with perivascular inflammatory infiltrates; in one there was hemorrhage into the lungs; in two there was interstitial pneumonia.

In the liver micronecrotic areas were observed in three susliks; there were hemorrhages in one; productive nodules in one. In 11 susliks there was cloudy swelling of the liver cells.

In the spleens of seven susliks there were areas of necrosis; in three, hyperplasia; in five, no considerable changes were noted. In the kidneys of one suslik there were hemorrhages; in 11, cloudy swelling of the tubular epithelium.

Therefore, in 29 susliks, which were in long-term hibernation as well as those which became sick in the waking state, changes typical of plague were found in the organs. This was confirmed in every case by positive results of culture (Table 2).

In the second group there were 19 susliks which died, from which the plague microbe was not obtained.

In these susliks superficial changes were noted, chiefly in the form of cloudy swelling of parenchymatous organs. In three foci of pneumonia were observed but without areas of necrosis, without accumulations of microbes or deep-seated injuries to blood vessels. On the basis of this as well as the negative results of cultures, it may be supposed that in these animals the pneumonia, apparently, was not of a plague nature.

The same may be said about killed susliks, which we put into the third group. In all 11 susliks of this group, on histological examination, no changes characteristic of plague or any deep-seated lesions in the organs were found.

Conclusions

1. In experimental susliks which died of plague in the winter as well as after awakening in the spring morphological changes were found characteristic of acute plague.

2. We did not observe processes of a chronic nature in the organs in the form of scars, encapsulated abscesses, cicatrizing granulomas, or others in any of the experimental animals.

3. In susliks which died of plague the following most important

Table 1

The Basic Pathological Changes of Suslik Organs Depending on the Duration of the Infectious Process

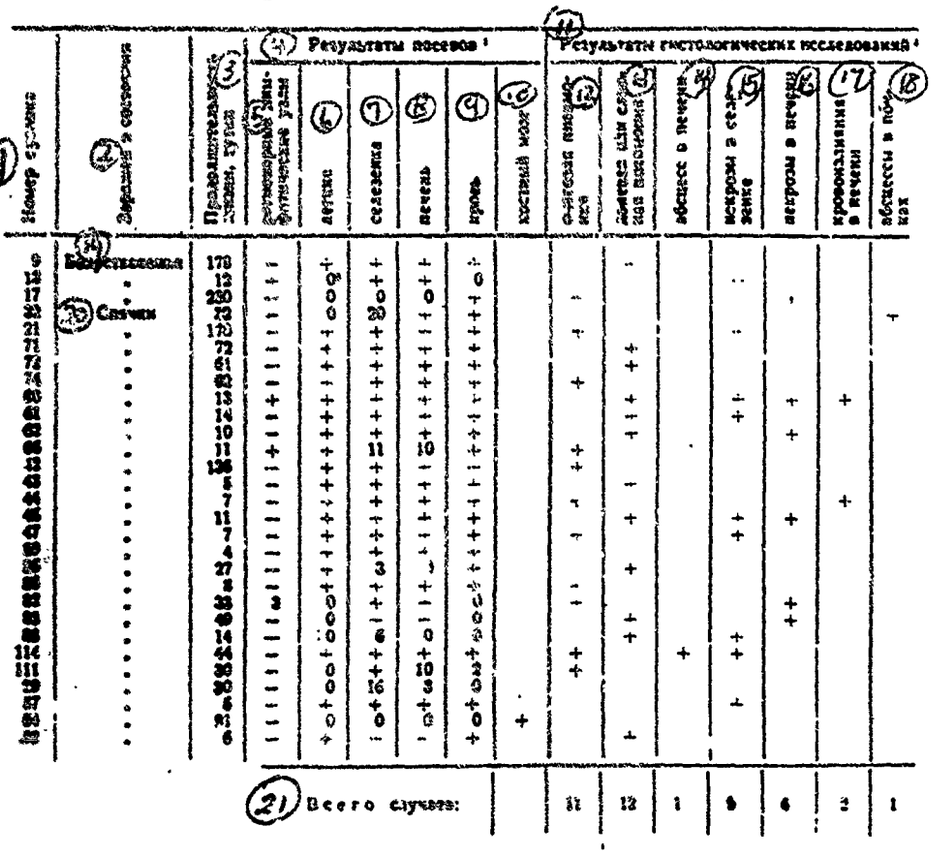
Особенность течения инфекции (1)	Число сусликов (2)	Продолжительность жизни, сутки (3)	Истощение (4)	Наличие подкожных кровоизлияний (5)	Число животных с макроскопически видимыми изменениями (6)														Число животных с гистологическими изменениями (22)						
					кишечник (7)	место инъекции (8)		увеличение регионарных лимфатических узлов (11)	селезенка (12)			печень (16)			легкие (19)										
						некроз язвы (9)	плотный инфильтрат (10)		увеличена (13)	гиперемия (14)	узлы (15)	дистрофия (17)	узлы (18)	кровоизлияния	кровоизлияния (18)	гиперемия (14)	некрозы (22)	ателектазы (21)	пневмония очаговая (23)	пневмония сливная, долевая (24)	некрозы в селезенке (25)	некрозы в печени (26)	кровоизлияния в печени (27)	абсцесс в печени (28)	абсцесс в почках (29)
(30) Острая форма чумы в ранние сроки	8	4—10	—	5	—	—	—	5	2	2	—	3	—	—	1	6	—	—	3	3	2	1	1	—	—
	5	11—15	—	3	1	2	—	3	2	4	1	5	—	—	—	1	1	1	1	3	3	2	1	—	—
(31) Бактериемия не обнаружена	2	12—11	—	—	—	1	—	—	—	—	2	1	1	—	—	—	—	—	—	1	2	—	—		—
	3	30—72	1	1	—	1	—	—	—	1	—	1	—	1	1	—	—	—	1	1	—	2	—		—
(32) Генерализация инфекции в поздние сроки	7	27—72	3	1	2	2	2	—	—	2	1	4	—	3	—	1	1	3	3	3	1	—	1	1	1
	4	135—280	2	2	—	1	—	—	—	—	1	—	1	1	—	1	—	—	3	1	1	1	—	—	—
(33) Всего . . .	29		6	12	3	7	2	8	4	9	5	14	2	5	2	9	2	4	11	12	9	6	3	1	1

1. characteristics of the course of the infection; 2. number of susliks; 3. lifespan, days; 4. wasting; 5. injection of subcutaneous blood vessels; 6. number of animals with macroscopic changes; 7. hyperemia of the intestines; 8. site of injection; 9. necrosis of the ulcer; 10. dense infiltrate; 11. enlargement of the regional lymph nodes; 12. spleen; 13. enlargement; 14. hyperemia; 15. nodules; 16. liver; 17. degeneration; 18. hemorrhages; 19. lungs; 20. areas of necrosis; 21. pneumonia; 22. number of animals with histological changes; 23. bronchopneumonia; 24. confluent, lobar pneumonia; 25. areas of necrosis in the spleen; 26. areas of necrosis in the liver; [Table 1, continued next page]

Table 1, continued from previous page 27. hemorrhages in the liver; 28. abscess in the liver; 29. abscess in the kidneys; 30. acute form of plague in the early period; 31. no bacteriemia found; 32. dissemination of the infection in the late periods; 33. total.

Table 2

Results of Microbiological and Histological Study of Susliks from Which the Plague Microbe Culture was Isolated



30, no growth (the figures designate the number of colonies which grew out; the organs were not examined).

Key: 1. number of suslik; 2. infected in a state of; 3. lifespan, days; 4. results of the cultures (bacteriological examinations: +abundant growth of the culture); 5. regional lymph nodes; 6. lungs; 7. spleen; 8. liver; 9. blood; 10. bone marrow; 11. results of histological examinations (histological examinations: +, presence of changes); 12. bronchopneumonia; 13. lobar or confluent pneumonia; 14. liver abscess; 15. areas of necrosis in the spleen; 16. areas of necrosis in the liver; 17. hemorrhages in the liver; 18. abscesses in the kidneys; 19. waking; 20. hibernation. 21. total cases.

changes were found: pneumonia, hemorrhages into the lungs, areas of necrosis in the liver and spleen, liver abscesses, hemorrhages of the liver, kidneys and suprarenal glands.

At the same time, degenerative changes were observed in the internal organs, chiefly in the form of cloudy swelling of the parenchymatous organs.

4. With respect to frequency and significance in the development of the infection the main position was occupied by pneumonia. In our experiments, in 12 out of 29 susliks there was lobar or confluent pneumonia; in 11, bronchopneumonia with vascular changes characteristic of plague, areas of necrosis, and accumulations of microbes.

5. In animals infected with plague and which died without bacteriological confirmation of the diagnosis, in the majority of cases only cloudy swelling of the parenchymatous organs was found; in three susliks pneumonia characteristic of plague was not found.

6. On microbiological and morphological studies the same results were obtained in our experiments.

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MICROBIOLOGY

Directed Variation of the Plague Microbe under the Influence of Streptomycin with the Aim of Obtaining Vaccine Strains

Z. Ye. Malinina
(Saratov)

Since the time of the discovery of the plague pathogen many facts have been recorded indicating variation of the plague microbe (D. K. Zabolotnyy, 1907; A. A. Bezsonova and G. N. Lenskaya, 1929; M. P. Pokrovskaya, 1935; Ye. I. Korobkova, 1937; N. N. Zhukov-Verezhnikov, 1940; V. M. Tumanskiy, 1955).

At the present time, studies on the directed variation of the plague microbe are acquiring great importance for the search for avirulent strains possessing immunogenic properties.

The majority of vaccine strains of the plague microbe has been found by chance among museum cultures. Strains obtained under the influence of various factors (temperature, aging, frequent subcultures) have not always justified themselves, because in the experiments conditions necessary for the production of vaccine strains were not created in all cases.

To date, no reliable methods have been worked out for obtaining vaccine strains with very good immunogenic properties from highly virulent cultures.

The subject of the present communication is the study of the possibility of obtaining living vaccines from virulent strains of the plague microbe by means of directed variation of it under the influence of streptomycin.

The choice of streptomycin as a factor in directed variation of the plague microbe was not made by chance. In studies begun in 1950 it was determined that this antibiotic, in its physicochemical properties and mechanism of action, permits the experimenter to use it in any dosages (from the so called bactericidal to growth-stimulating doses) depending on the purposes and to obtain the necessary results in accordance with this. Our experiments show that under the influence of streptomycin, depending on the method in which the antibiotic is used, the following may be obtained: 1) variants of the plague microbe resistant to streptomycin which have lost their virulent properties; 2) streptomycin-resistant variants of the plague microbe with the same virulent properties, and 3) streptomycin-dependent variants which require the presence of streptomycin in the medium for their growth.

Changes in the structure of the colonies and cell morphology of the plague microbe were first observed when the experiments were performed for determining the sensitivity of the plague microbe to streptomycin and for determination of the nature of its effect. Thereby, the changes were recorded under test-tube conditions as well as in the bodies of animals treated with streptomycin. When the plague microbe cultures which had been subjected to the effect of streptomycin were plated out from the test-tubes onto agar plates a heterogeneous colony structure was observed (Fig 1). The colonies were different in size, shape, markings and color. In smears prepared from cultures of the altered together with the typical bipolar staining cells a multitude of altered cells was noted in the form of thick tortuous

filaments (Fig 2).

During the course of the work we also observed dissociative variation. By acting on the plague microbe culture with streptomycin, we were able to produce dissociation of the culture into S and R variants (Fig 3).

On the basis of the observations which we made the conclusion may be drawn that all strains of the plague microbe, after even brief contact with streptomycin (for 20 hours), change the morphology of their colonies and their cell structure. Despite the rapidity of occurrence and variety of forms of the colonies, the changes recorded were unstable. Subcultures of the altered colonies on media which did not contain the antibiotic quite rapidly were converted into typical plague microbe colonies. The brevity of existence of the altered colonies is understandable. The changes in the colonies were observed only as long as a factor producing these changes--streptomycin--was present in the culture. For the purpose of producing more stable changes in the plague microbe the longer effect of the preparation is needed. The latter found its expression in experiments on obtaining streptomycin-resistant variants of the plague microbe. For the purpose of obtaining streptomycin-resistant variants of the plague microbe from streptomycin-sensitive variants we used a somewhat modified method of passages of sensitive cultures through media containing increasing streptomycin concentrations. As the result of a comparatively small number (8-10) passages plague microbe variants were obtained whose resistance to streptomycin was increased by 10,000 times. Their initial sensitivity was equal to 1.5-3 units; after passages of the cultures they grew well in Hottinger's bouillon containing 15 milligrams of streptomycin. In the experiments 40 strains of plague microbe were used which were different from one another in their cultural characteristics, origin and virulence. Among the strains there were avirulent strains, those with moderate virulence and highly virulent strains which killed the guinea pigs when they were injected with 10-100 microbes.

At the time of their formation streptomycin-resistant variants were very much different from the initial strains in the cell morphology and colony structure. When they were subsequently kept on media containing streptomycin or media without it the morphological characteristics of the variants were the same as the maternal culture. The variants resistant to streptomycin show a slower growth rate than the sensitive cultures only in the first generations. Both in the sensitive cultures and in the streptomycin-resistant strains the minimum inoculation dose needed to produce a culture was almost the same.

Analyzing the material showing differences in biochemical activity between sensitive and resistant cultures, the absence of any principles in this respect should be emphasized in the resistant plague microbe cultures. In some strains, as the result of adaptation to streptomycin, we could note an increase in biochemical activity (strain 709); in others, a decrease of it was observed (strain 565); and in still others, the biochemical activity did not change at all.

The streptomycin-resistant cultures and the sensitive variants showed the same relationship to bacteriophage. Cultures sensitive and resistant to streptomycin were lysed in the same titers by both plague and pseudotuberculosis bacteriophages. Nor did we observe any differences in the

serological properties of the streptomycin-resistant and sensitive cultures. During the course of study of the comparative activity of oxidation-reduction systems in streptomycin-adapted and sensitive cultures of the plague microbe we noted that cultures resistant to streptomycin more rapidly reduce methylene blue than the sensitive cultures. The results of the experiments give us the basis for supposing that the change of the streptomycin-sensitive cultures of the plague microbe into streptomycin-resistant cultures is apparently associated with the activation of the system of dehydrogenases. The results of experiments on the determination of the oxidation-reduction capacity of cultures sensitive and resistant to streptomycin in the presence of streptomycin introduced additionally into the experiment as well as specially performed experiments on the dehydration of glucose by resistant and sensitive cultures under relatively anaerobic conditions speak for this idea.

We studied the stability of acquired resistance to streptomycin by the method of passages of resistant cultures through media which did not contain streptomycin, by the method of passaging resistant cultures through the bodies of sensitive animals as well as by the preservation of resistant cultures for six-seven years on media which did not contain the antibiotic. The data obtained show that even repeated (from 25 to 50) subcultures of the streptomycin-resistant cultures on media without the antibiotic did not lead to a reduction in the resistance of the culture to streptomycin. It was impossible to note any reduction in the resistance of resistant cultures even after passage 10 times through the bodies of guinea pigs or white mice; keeping the cultures for a long time (six-seven years) on media without streptomycin also failed to lead to a reduction in the resistance of the strains to the antibiotic.

The virulence of streptomycin-sensitive strains and their resistant variants was studied on white mice. For the purpose of determining the minimum lethal dose of various plague microbe cultures the method of biological titration on animals was used (Table 1).

Table 1

The Virulence of Streptomycin-Resistant and Sensitive Cultures of the Plague Microbe in an Experiment on White Mice

Штамм (1)	(2) Отношение к стрептомицину	(3) Число животных	(4) Минимальная убивающая доза, количество микробных тел
709	Чувствительные (5)	5	1000
709	Устойчивые (6)	5	100 000 000
708	Чувствительные (5)	5	100
708	Устойчивые (6)	5	100 000 000
751	Чувствительные	5	100
751	Устойчивые	5	100 000 000
565	Чувствительные	5	1000
565	Устойчивые	5	1000 000 000

1. strain; 2. relationship to streptomycin; 3. number of animals; 4. minimum lethal dose, number of microbes; 5. sensitive; 6. resistant.

An analysis of the experimental data shows that the virulent properties of all the strains studied decrease sharply compared with the virulence of streptomycin-sensitive maternal cultures.

Even more significant results were obtained from the testing of the innocuousness of the resistant plague microbe cultures in experiments on guinea pigs. All the streptomycin-resistant strains (709, 696, 819, 815, 806, 751, 807, 810, 813, 708) were found to be innocuous. Guinea pigs infected subcutaneously with 15,000,000,000-20,000,000,000 microbes all survived.

The immunogenic properties of cultures of the streptomycin-resistant 709 strain were tested on guinea pigs. In the experiment 27 animals were used which were divided into three groups. The first group of animals (nine) was immunized with 1,000,000,000 microbes; the second group (eight), with 500,000,000; the third group (10), 250,000,000 microbes. At the end of 21 days after immunization all the animals were subjected to a control infection with a highly virulent culture of the 708 strain (400 MLD). Five control guinea pigs were infected with the same quantity of the culture.

Guinea pigs immunized with the streptomycin-resistant culture of the 709 strain all remained alive, whereas all the control animals died five-six days after the infection (Table 2).

Table 2

The Immunogenic Properties of the Streptomycin-Resistant Variant of the Plague Microbe of the 709 Strain

Иммунизирующая доза (1)	Заражающая доза (DlM) (2)	Число животных (3)	Из них после заражения (4)	
			погли (5)	остались (6)
1 млрд. (7)	400	9	0	9
500 млн. (8)	400	8	0	8
250 млн. (8)	400	10	0	10
Контроль (9)	400	5	5 (5—6-й день) (10)	0

1. immunizing dose; 2. infecting dose (MLD); 3. number of animals; 4. of these, the number which following the infection. . .; 5. died; 6. survived; 7. billion; 8. million; 9. control; 10. fifth-sixth day.

Therefore, 100 percent survival of the immunized animals after administration of known high doses of a virulent plague culture attests to the very good immunogenic properties of the streptomycin-resistant variant, 709.

Conclusions

1. As the result of the gradual adaptation of it to streptomycin the plague microbe markedly changes its metabolism. First there is a change in its relationship to the antibiotic, a reorganization of the enzyme apparatus of the microbe. In the streptomycin-resistant variant the system of dehydrogenases characterizing the anoxidative component of cell respiration is

activated, there is a change in its biochemical activity and virulence decreases sharply. The newly acquired properties are stably maintained by the plague microbe and are transmitted by heredity.

2. Streptomycin-resistant avirulent cultures maintain their immunogenic properties completely.

3. Streptomycin can be used as a factor in directed variation with the aim of obtaining vaccine strains (avirulent and immunogenic) of the plague microbe. Thereby, the avirulent strains are comparatively easily obtained from highly virulent strains. This method has the advantage that the avirulent strain is obtained in quite a short time.

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The Action of Mycerin on the Toxin and Toxin-Production of the Plague Microbe

V. A. Zaytsev
(Saratov)

According to data in the literature (A. F. Bilibin, 1958; G. P. Rudnev, 1959; K. V. Bunin, 1958; K. M. Loban, 1958; N. V. Vorotyntseva, 1953; K. I. Kondrashkina, 1953 and others), with antibiotic treatment of some infectious diseases a reduction of intoxication is noted. The mechanism of this phenomenon remains unclarified to date. A number of investigations (V. N. Derkach, 1956, 1957; V. A. Lyashchenko, 1960; N. F. Kalinichenko, 1955; M. K. Shcheglova, 1953; L. P. Guseva, 1954; M. R. Nechayevskaya, 1955; Yu. V. Solov'yeva, 1947; S. M. Navashin, 1959; S. M. Navashin and A. I. Braude, 1959 and others) render the opinion that detoxication is conditioned in such cases by the antitoxic capacity of the antibiotics. According to the data of other investigators (E. A. Gal'perin, 1950; P. R. Kolomeytsev and A. Ye. Nikitina, 1951; A. N. Shneyerson, 1958; Ye. V. Chernokhvostova and others, 1957), it is the result of a disorder in the production of bacterial toxins produced by

antibiotics.

As is well known, in plague the intoxication of the organism developing as the result of the propagation and breakdown of bacteria, is of a pronounced nature and together with the bacteriemia determines the entire symptom complex and outcome of the disease (G. P. Rudnev, 1938; Meyer and Jawetz, 1954). The elimination of toxicosis in the treatment of plague mitigates the course and the severity of the disease. A good antitoxic effect is exerted by plague serum, but it is not very effective therapeutically. In the literature available to us we have encountered only several papers on the study of the mechanism of action of antibiotics on the plague microbe toxin. Quan, Chen and Meyer (1950) established the fact that chlorotetracycline, aureomycin and oxytetracycline, given prophactically four-12 hours before injection of the plague microbe toxin, protect mice against death. Streptomycin, which in their experiments gave the greatest therapeutic effect, did not protect the mice against lethal intoxication. According to the data of Ye. N. Aleshina and L. N. Makarovskaya (1959), streptomycin possesses a certain inactivating effect against the plague toxin, whereby it is more pronounced than in the case of biomycin.

In the papers of workers at the "Mikrob" Institute the antibiotic mycerin [neomycin-like antibiotic] was appraised highly for the treatment of experimental plague in laboratory animals. However, some factors in the mechanism of the therapeutic effect of the preparation have been inadequately studied; specifically no study at all has been made of the effect of mycerin on the plague microbe toxin or toxin formation. The aim of the present investigations was the study of some problems dealing with the effect of mycerin on the toxin and toxin formation of the plague microbe.

First of all, in experiments performed in vitro a study was made of the inactivating effect of mycerin on the plague toxin.

The toxin was obtained by a method described by Ye. I. Korobkova (1957). The avirulent EV strain was seeded on Hottinger's slant agar in matrasses (pH of 7.1) with 0.4 percent glycine. The cultures were incubated at 28-30° for two days. The slimy culture which grew out was carefully triturated in condensation water. The microbe suspension obtained (180,000,000,000-200,000,000,000 microbes per cc) was aspirated into test-tubes and put into a refrigerator for three-four days, after which it was centrifuged. In the yellowish, slightly opalescent supernatant fluid used as the toxin the minimum lethal dose (MLD) was determined on white mice weighing 17-20 grams. One MLD of the toxin in 0.1 cc of physiological saline solution was mixed with different doses of mycerin--1, 2 and 3 milligrams--dissolved in 0.1 cc of physiological saline solution. In all cases, with mixing of the mycerin and toxin solutions a turbidity appeared immediately; subsequently a precipitate came down from it in the form of fine clumps. After a 30-minute exposure at room temperature (18-20°), a three- and 24-hour exposure at 28°, these mixtures in a volume of 0.2 cc were injected into white mice subcutaneously. In a similar way control solutions of the toxin and mycerin were prepared (the results of the experiments are shown in Table 1).

As is seen from Table 1, mycerin possesses an appreciable detoxifying effect which increases with increase of the dose of antibiotic, whereby the neutralizing effect does not depend on the temperature or the period of

Table 1

Effect of Mycerin on 1 MLD of the Plague Microbe Toxin

(1) Доза мицерина, мг	(2) Длительность экспозиции (в часах) при температуре		(3) Число мышей	(4) Число павших мышей	(5) Средняя продолжительность жизни павших мышей, часы
	18–20°	28°			
1	0,5		10	8	38.6
2	0,5		10	5	38.2
3	0,5		10	4	20.5
(6) Контроль (1 Dlm токсина)	0,5	3	10	9 5	20,6 23
1		3	5	2	28,5
(7) Контроль (1 Dlm токсина)		3	5	5	18
1		24	5	3	67
3		24		2	28
(8) Контроль (1 Dlm токсина)		24	5 5	4	18

Note. The control animals (those which received only mycerin) all survived.

1. dose of mycerin, mg; 2. duration of exposure (in hours) at a temperature of; 3. number of mice; 4. number of mice which died; 5. mean lifespan of mice which died, hours; 6. control (1 MLD of the toxin).

contact between mycerin and the toxin. Thus, with a dose of 3 milligrams of mycerin and an exposure time of 24 hours at 28°, two of the five mice used in the experiment died, whereas in the control group four out of five mice died; the same relationships were noted after a dose of 3 milligrams of mycerin under conditions in which the contact was at room temperature: of 10 mice used in the experiment only four died, whereas in the control group nine out of 10 mice died. In the analysis of the data the impression is gained that the mean lifespan of the mice which died, which were given a mixture of the toxin and mycerin, was somewhat greater than in the control animals which received the toxin alone.

At the second stage of the investigations we performed experiments in vivo on the study of the detoxifying effect of mycerin with experimental intoxication of the white mice with plague toxin. The experiments were performed in the following way. One group of white mice received mycerin (3 milligrams once) and the toxin (1 MLD) simultaneously (in different parts of the body). Another group of white mice were given mycerin (1 gram) intramuscularly three hours after the injection of the toxin (1 MLD) and subsequently twice at six-hour intervals. The control mice received only the toxin.

It was determined that mycerin, both after simultaneous injection with the toxin and three hours after injection of the toxin, exerted no detoxifying effect. This is confirmed by the lifespans of the experimental animals,

which were not much different from those of the controls (all the mice died in 21 hours).

The subsequent experiments were given over to clarification of the prophylactic effect of mycerin in experimental intoxication in white mice. With this aim in view, each experimental mouse was injected with 2 milligrams of mycerin in a volume of 0.1 cc of physiological saline solution. The control mice received the 0.1 cc of physiological saline solution only. After two hours, both the experimental and control mice were given different dilutions of toxin (Table 2).

Table 2

Prophylactic Effect of Mycerin in Intoxication by the Plague Microbe

(1) Доза токсина, (мл)	(2) Подопытные мыши			(6) Контрольные мыши		
	(3) число	(4) число павших	(5) средняя продолжительность жизни павших, часы	(3) число	(4) число павших	(5) средняя продолжительность жизни павших, часы
0,006	10	1	42	10	4	24
0,012	10	5	23	10	7	21
0,025	10	9	26,6	10	10	18
0,05	10	10	18	10	10	16

1. dose of toxin (cc); 2. experimental mice; 3. number; 4. number which died; 5. average lifespan of those which died, hours; 6. control mice.

As follows from Table 2, mycerin shows a poorly expressed prophylactic effect in intoxication of white mice by the plague toxin. Thus, with a toxin dose of 0.012 cc five out of 10 experimental mice died and seven out of 10 control mice died; with a toxin dose of 0.006 one mouse out of 10 experimental mice died; in the control group, as the result of the utilization of this toxin dose four out of 10 mice died. In addition, in the experimental mice a very slight increase in the lifespan was noted.

The effect of mycerin on toxin-production of the plague microbe was studied in the following way.

The toxin was obtained by the method described above of Ye. I. Korobkova from cultures of the plague microbe (EV strain) grown out on a medium containing a subbacteriostatic concentration of mycerin (0.5 gamma ler cc). A toxin obtained from cultures grown out without the antibiotic served as a control.

The number of bacteria participating in toxin production was determined nephelometrically, by comparing the density of the bacterial suspension of the plague microbe culture grown out in the presence of mycerin with the density of the bacterial suspension obtained from the medium without the antibiotic. The comparison was made in accordance with the optical standard and measured platings of the various dilutions (10^{-4}, 10^{-9}) on agar plates with subsequent count of the colonies which grew out.

Both nephelometrically and by means of counting the colonies it was determined that the bacterial suspension in the control group (180,000,000,000-200,000,000,000) contained twice as many microbes as in the experimental group (90,000,000,000-110,000,000,000). Therefore, mycerin in this concentration inhibited the growth and multiplication of the plague microbe.

With the aim of making the conditions the same the control bacterial suspension was diluted two times with physiological saline solution before centrifugation, that is, its density was brought up to the density of the suspension in the experiment. After centrifugation, in experiments on white mice the minimum lethal doses of the supernatant fluids obtained were determined (Table 3).

Table 3

Titration of Plague Microbe Toxin of EV Strain Grown Out on Medium Containing Mycerin (0.5 gamma per cc) and Without It

Опыт (1)			Контроль (2)		
Доза токсина (3)	число животных (4)	число павших животных (5)	доза токсина (3)	число животных (4)	число павших животных (5)
0,1	2	2	0,1	2	2
0,05	2	1	0,05	2	2
0,025	2	1	0,025	2	2
0,012	2	0	0012	2	2
0,006	2	0	0,006	2	2
0,003	2	0	0,003	2	1
0,0015	2	0	0,0015	2	1

1. experiment; 2. control; 3. dose of toxin; 4. number of animals; 5. number of animals which died.

As is seen from Table 3, the minimum lethal dose (0.006 cc) of toxin obtained from the plague microbe grown out on medium without mycerin was 16.6 times less than the minimum lethal dose (0.1 cc) of toxin obtained from the plague microbe grown out on medium with a subbacteriostatic concentration of mycerin.

Therefore, it was determined by our experiments that mycerin suppresses the toxin-producing function of the plague microbe together with an inhibition of its growth and propagation.

In conclusion, we should point out the following. Ye. V. Chernokhvostova and others (1957) believe that antibiotics do not neutralize the bacterial toxin. The difference between the mortality of the animals in the control group (those which received toxin) and in the experimental group (those which received the toxin and the antibiotic) may depend, in their opinion, on the activation of an infection with extraneous bacteria in the laboratory animals under the influence of the toxin which usually occurs in a late manner; this should aggravate the intoxication and increase the mortality of animals in the control group. If the antibiotic used suppresses this infection, a false impression may be gained about the effect of the antibiotic on intoxication.

We cannot agree with this viewpoint, because in our experiments the extraneous flora were not, as a rule, plated out from the organs of animals which died of the toxin or from the toxin and mycerin.

Conclusions

1. Mycerin in vitro possesses a poorly expressed capacity of neutralizing the plague microbe toxin.
2. In the treatment of experimental intoxication in white mice caused by injection of the toxin mycerin proved to be ineffective.
3. Mycerin, given to white mice prophylactically (two hours before injection of the toxin), shows a slight protective effect against experimental intoxication.
4. Subbacteriostatic concentrations of mycerin inhibit the toxin production by plague bacteria.

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The Effect of Some Human Body Tissues on the Properties of Cholera Vibriones and Cholera Bacteriophages

O. M. Petrunina, N. V. Polyakova and L. V. Makaridze
(Saratov)

The problem of increasing and stabilizing the specific properties of pathogenic microbes remains unsolved to date and is undoubtedly of current importance. As is well known, when kept on synthetic nutrient media the specific characteristics of many pathogenic microbes show a tendency toward reduction (sometimes to the point of complete loss), which depends on the nature of the microbes and the conditions under which they are kept. This process is reversible at a certain stage. By using different methods of cultivation, for example, passages through animals, it is sometimes possible not only to restore but even increase such characteristics of pathogenic microbes as virulence, immunogenicity and others.

Of the many methods proposed for this purpose cultivation and maintenance of the cultures on nutrient media in which animal body tissues have been included deserve special attention. Thus, Ye. I. Korobkova (1958), by means of drying the EV vaccine strain in the organs of infected animals without intermediate subcultures on synthetic nutrient media in combination with other techniques, was able to maintain or even increase the immunogenicity of this strain. Similar results were obtained by V. M. Tumanskiy, N. V. Uryupina and V. A. Knyazeva (1958) by means of long passages of the culture through chick embryos.

According to Shaw's data (1957), cholera vibriones dried in a medium containing 5 percent human blood possessed better immunogenic properties than those dried in a medium without blood.

For the vibrio of cholera, which is a typical anthroponosis, the choice of the animal--the tissue donor--is particularly important, because passages in a medium unfavorable for the cholera vibrio can be the reason for a change in its properties. Taking into consideration what has been stated, for the cultivation of cholera vibriones we used human blood and nutrient media, to which some human body tissues were added (liver, spleen, small intestine and bile).

The media for the cultivation of strains of cholera vibriones were prepared in the following way: pieces of spleen or liver were poured over with alcohol and flamed. The outer burned layer was removed and the remaining mass was ground up and triturated in a sterile mortar containing sterile sand, with the addition of a small quantity of physiological saline solution. Sections of the small intestine were washed several times with physiological saline solution, ground up and triturated also. The triturated mass was added in quantities of 1-1.5 cc to test-tubes containing 5 cc of semiliquid agar, which was sterilized by means of heating for 30 minutes for three days straight at 60°. The blood was poured out in a sterile manner into the test-tubes.

The experiments were performed on six Inaba strains (54, 76, 78, 110, 2.55) and six Ogawa strains (14, 18, 22, 25, 28 and 48) which possessed

properties typical of the S form of cholera vibriones. In the first experiment the cultures were subcultured every six-eight days; in the subsequent work, once a month; between the subcultures the experimental and original cultures were kept at 8-10°; the former were kept on the test media; the latter, on Martin's semiliquid agar.

During the course of work, a dynamic check was made of the cultural, morphological, biochemical, agglutinogenic and hemolytic properties, viability, virulence and lysability of all the subcultures.

As the experiments showed, the morphology of the subculture colonies of the cholera vibriones obtained as the result of passages in blood and on media containing liver, spleen, small intestine, corresponded to the typical S form. The subcultures obtained produced delicate shiny transparent, slightly bluish colonies with even margins on the agar plates, which under low power had a smooth or finely granular structure. The colonies were easily separated from the surface of the agar. Therefore, in passages on the test media, the colony morphology of the cholera vibriones was definitely improved, particularly in the case of passages in media containing blood and media containing spleen and intestine. In bouillon the subcultures obtained grew with a formation of a delicate membrane and a uniform turbidity. An exception was constituted by subcultures obtained as the result of the passaging on a medium containing 30 percent bile. In all subcultures the colonies were turbid, rough, with an uneven margin, with a coarsely granular texture, that is, similar to the R form in their morphology. Cholera vibriones from such colonies showed agglutinative growth in bouillon. We did not make a more detailed study of these colonies.

The biochemical characteristics of the cholera vibrio subcultures obtained as the result of passaging in experimental media were the same as those of the original cultures: they broke down glucose, mannitol, maltose and sucrose with the formation of acid, did not ferment arabinose, gave a positive nitrosoindole test and positive test for indole and hydrogen sulfide.

The viability of the cholera vibriones in the original cultures and in the subcultures obtained as the result of passaging on experimental media was checked for a year by means of periodic plating-out on agar plates from the test-tubes containing cultures of the generations being studied kept at 8-10° (Table 1).

As is seen from Table 1, the greatest viability was shown by subcultures cultivated and kept in blood--of 12 subcultures nine showed growth after nine months, and two of them were viable for more than a year.

The least viability was noted in subcultures passaged and kept on the control medium, semiliquid Martin's agar: all 12 subcultures died after being kept for six months.

As is well known, great importance is ascribed to the hemolytic properties of cholera vibriones; this characteristic is elevated to the rank of a diagnostic sign. According to the data of the majority of authors, cholera vibriones do not hemolyze sheep erythrocytes. Some investigators believe that cholera vibriones do not possess hemolysins but during the course of their activity they give off an enzyme, protease, into the surrounding medium which splits blood and changes the red color of sheep's blood to a yellow-green (Ye. I. Korobkova, 1959). Others, on the other hand, point to the fact that

Table 1

Viability of Cholera Vibriones Cultivated on Experimental Media

(1) Среда, на которой культивировали и хранили холерных вибрионов	(2) Число субкультур в опыте	(3) Число субкультур, давших рост при высеве через				
		(4) 3 месяца	4 1/2 месяца	6 месяцев	9 месяцев	12 месяцев и более (5)
(6) Полужидкий агар с селезенкой	12	12	10	8	0	0
(7) Полужидкий агар с печенью	12	12	6	5	0	0
(8) Полужидкий агар с тонким кишечником	12	12	9	1	0	0
(9) Кровь	12	12	12	12	7	2
(10) Контроль (полужидкий агар Мартена)	12	6	4	0	0	0

1. medium on which the cholera vibriones were cultivated and kept; 2. number of subcultures in the experiment; 3. number of subcultures which showed growth when plated out after; 4. three months; 5. 12 months or more; 6. semiliquid agar containing spleen; 7. semiliquid agar containing liver; 8. semiliquid agar with small intestine; 9. blood; 10. control (semiliquid Martin's agar).

cholera vibriones are capable of hemolyzing the red blood cells of some animals. Thus, Bhattacharya and Roychandbury (1959) established the fact that 19 freshly isolated Ogawa and eight Inaba strains hemolyzed human red blood cells but did not hemolyze sheep erythrocytes, while 12 out of 27 strains hemolyzed rabbit red blood cells. Pinghui (1959) notes that in his experiments cholera vibriones showed hemolytic properties with respect to human erythrocytes.

In our studies we checked the hemolytic properties of all subcultures dynamically on solid and liquid nutrient media.

In the case of culture on agar plates (Martin's agar, pH of 7.6) containing 3 percent human and rabbit blood, clear pink areas were observed around the colonies of cholera vibriones, both in the case of the original cultures and in the subcultures passaged in blood and on media containing spleen, liver and small intestine, which at the end of two-three days became decolorized. Thereby, the decolorized zones were wider containing rabbit blood. On agar containing sheep's blood hemolyzed green zones appeared around the colonies of all cultures. Therefore, the facts which we obtained confirm data in the literature to the effect that cholera vibriones possess hemolytic properties with respect to human and rabbit erythrocytes and contain the enzyme protease, which decolorizes sheep erythrocytes.

The cholera vibriones of some subcultures did not show hemolytic properties against human erythrocytes after passaging in bouillon containing 30 percent bile. This fact as well as the undesirable variation of the colony

morphology of these subcultures made us exclude medium with 30 percent bile from the experiments.

The presence of hemolysins against sheep and human erythrocytes in all cholera vibrio cultures was determined in liquid medium (peptone water) in three-hour, 24-hour and three-day cultures.

In these experiments it was noted that the cholera vibriones of the experimental subcultures which had been grown for three hours caused complete hemolysis of human erythrocytes, whereas the control cultures (original generations) hemolyzed human erythrocytes to the same degree only in the 24-hour and three-day cultures.

The virulence of the experimental subcultures was checked simultaneously with subcultures of the original strains, cultivated and kept in semiliquid Martin's agar. For this purpose white mice weighing 16-17 grams were used, which were infected intraperitoneally with doses of 200,000,000, 400,000,000 and 600,000,000 microbes. The result was read after 48 hours. In six of the nine experimental subcultures checked the virulence was increased compared with the initial culture. Thus, a subculture of the 110 strain cultivated on media containing spleen, liver and in blood, in a dose of 400,000,000 microbes caused the death of five infected white mice, whereas three mice died from the initial culture injected in the same dose. Subcultures of the 14 and 2 strains, obtained as the result of cultivation on media containing liver, spleen and in blood as well as subcultures of the 78 strain cultivated on medium containing spleen and in blood, and strains 18 and 48, cultivated on media with spleen, caused the death of four-five animals in a dose of 600,000,000 microbes, whereas the initial cultures were either entirely innocuous in this dose or caused the death of only one-two animals (Table 2).

Table 2

Virulence of Cholera Vibriones of Initial and Experimental Subcultures

(1) Среда культивирования	(2) Число павших животных в зависимости от заражающей дозы (в млн. микробных тел) штаммов																	
	110			14			2			78			18			48		
	600	400	200	600	400	200	600	400	200	600	400	200	600	400	200	600	400	200
(3) Полужидкий агар с селезенкой	—	5	5	5	0	0	5	0	0	4	3	1	5	4	0	5	3	0
(4) Полужидкий агар с печенью	—	5	3	5	2	0	5	2	0	—	—	—	—	—	—	—	—	—
(5) Кровь	—	5	4	4	2	2	4	1	0	5	0	3	—	—	—	—	—	—
(6) Контроль (полужидкий агар Мартена)	—	3	2	0	0	0	1	0	0	2	1	2	1	1	0	1	1	

Note. Five white mice were infected in each experiment.

1. culture medium; 2. number of animals which died depending on the infecting dose (in millions of microbes) of strains; 3. semiliquid agar with spleen; 4. semiliquid agar with liver; 5. blood; 6. control (Martin's semiliquid agar).

As is well known, the characteristics of bacteriophage and the biological characteristics of the microbes on which the bacteriophage is being passaged are interdependent. The most active bacteriophage is obtained on strains with the most pronounced specific characteristics. Therefore, in bacteriophage production it is customary to use freshly isolated strains. This principle has been made a regulation in instructions on the production of bacteriophage.

All methods adopted in laboratory practice for improving the properties of cholera vibriones, namely, animal passages, passages through chick embryos (O. M. Petrunina, 1958), passages through the isolated intestinal loop of guinea pigs (A. G. Nikonov and others, 1958) make it possible to obtain bacteriophage with higher activity on such strains.

After determining the fact that the specific characteristics of cholera vibriones change in a direction desirable for our purposes (better morphology, greater viability, more pronounced hemolytic properties, increase in virulence) as the result of passaging through the experimental media which we were testing, we were right in supposing that the activity of cholera bacteriophages seeded on such cultures would be greater.

Based on this, we passaged two standard and polyvalent cholera bacteriophages on cholera vibriones of the original and experimental subcultures in Martin's bouillon. The activity of the cholera bacteriophages was checked by the Appelman method and by counting the plaques on two-layered agar by the Grazzia method (Table 3).

Table 3

Titers of Cholera Bacteriophages Passaged on Different Subcultures

(1) Бактериофаг	(2) Количество корпускул фага в 1 мл среды при культивировании его на культурах, полученных путем пассажей на				
	(3) полужидком агаре Мартена (контроль)	(4) среде с селезенкой	(5) среде с печенью	(6) среде с тонким кишечником	(7) в крови
A	$2 \cdot 10^7$	$2 \cdot 10^8$	$2 \cdot 10^8$	$2 \cdot 10^8$	$2 \cdot 10^8$
B	$2 \cdot 10^8$	$2 \cdot 10^9$	$4 \cdot 10^9$	$8 \cdot 10^9$	$2 \cdot 10^9$
(8) Поливалентный	$2 \cdot 10^8$	$4 \cdot 10^8$	$4 \cdot 10^8$	$6 \cdot 10^8$	$2 \cdot 10^8$

1. bacteriophage; 2. number of phage corpuscles per 1 cc of the medium when they are grown on cultures obtained by means of passages on; 3. Martin's semiliquid agar (control); 4. medium containing spleen; 5. medium containing liver; 6. medium containing small intestine; 7. in blood; 8. polyvalent.

As is seen from Table 3, the activity of phages obtained as the result of passages on the experimental subcultures exceeded that of phages obtained from the initial cultures by 10 and 100 times.

Then, we passaged the phages on experimental subcultures in experimental media. From sources in the literature it is well known that the synthesis of

new bacteriophage corpuscles occurs from the DNA not only of the bacterial cells but also of the constituents of the nutrient medium. The more completely the nutrient medium satisfies the requirements of the phage + bacterium combination, the more actively will the production of phage corpuscles occur and the more active they will be. The experimental nutrient media which we tested contain components of a natural medium for cholera vibriones and, therefore, for the cholera bacteriophage also. It is particularly important to adapt the cholera bacteriophage to bacterial cells grown out on media containing components of human tissues, because, according to Willy (1959), phage in the presence of blood, pus or feces is ineffective.

Triturated masses of organs (prepared in a sterile manner) were added to test-tubes containing Martin's bouillon. Simultaneously, 0.2 cc of phage and 0.4 cc of a suspension of a three-four-hour bouillon culture of cholera vibriones were seeded on 10 cc of medium and blood. The cultures were incubated at 37° for 18 hours. Then the phages grown out on subcultures of the cholera vibriones in medium containing human organs were filtered through L3 and L5 candles, and were preserved in the blood with quinosol in a dilution of 1:10,000. After cultivation on the cholera vibriones in the test media the bacteriophages were passaged one-three times on the same cultures in Martin's bouillon.

After passage on the vibriones in the experimental media the phages possessed lytic activity equal to or even lower than the phages obtained by means of passages in Martin's bouillon on the initial cultures. The phage titer dropped particularly sharply as a result of passage (on a culture) in blood. However, after two-three serial passages in Martin's bouillon the lytic activity increased by 100-200 times over the activity of phages passaged on cultures by the usual method (Table 4).

Table 4

Titers of Bacteriophages Passaged on a Culture in Experimental Media

(1) Среда культивирования	(2) Количество корпускул фагов в 1 мл среды							
	(3) после пассажа на вибрионах в опытной среде				(4) после трех пассажей на вибрионах в бульоне			
	A	B	D	поли-валентный (5)	A	B	D	поли-валентный (5)
(6) Бульон Мартена с селезенкой	$6 \cdot 10^7$	$2 \cdot 10^8$	$2 \cdot 10^8$	$2 \cdot 10^8$	$2 \cdot 10^9$	$2 \cdot 10^8$	$2 \cdot 10^9$	$2 \cdot 10^9$
(7) Бульон Мартена с печенью	$2 \cdot 10^8$	$2 \cdot 10^9$	$2 \cdot 10^7$	$2 \cdot 10^6$	$6 \cdot 10^8$	$4 \cdot 10^9$	$2 \cdot 10^9$	$2 \cdot 10^9$
(8) Бульон Мартена с тонким кишечником	$2 \cdot 10^8$	$2 \cdot 10^8$	$2 \cdot 10^8$	$2 \cdot 10^7$	$4 \cdot 10^8$	$8 \cdot 10^9$	$3 \cdot 10^9$	$8 \cdot 10^8$
(9) Кровь	$2 \cdot 10^5$	$2 \cdot 10^4$	$2 \cdot 10^4$	$2 \cdot 10^4$	$2 \cdot 10^9$	$2 \cdot 10^9$	$2 \cdot 10^9$	$2 \cdot 10^9$

1. culture medium; 2. number of phage corpuscles per cc of the medium; 3. after passage on vibriones in the experimental medium; 4. after three passages on vibriones in bouillon; 5. polyvalent; 6. Martin's bouillon with spleen; 7. Martin's bouillon with liver; 8. Martin's bouillon with small intestine; 9. blood.

Apparently, in medium containing tissues the adsorption of phage corpuscles on tissue components occurs. This is particularly pronounced in blood. Possibly, adsorption is observed on passaging the cholera bacteriophage with cholera vibriones through the isolated loop of a guinea pig intestine, because, according to the data of A. G. Nikonov (1958), the phage titer after passages by this method decreases sharply when titrated in bouillon by the Appelman method.

For the purpose of confirming this idea we performed a comparative titration on two-layered agar, using cholera bacteriophages of several types obtained by means of cultivation in blood with subsequent passage of them on a culture in Martin's bouillon (Table 5).

Table 5

Titers of Bacteriophages Passaged (on a Culture) in Blood and with Subsequent Passage in Bouillon

(1) Среда культивирования	(2) Количество корпускул фагов различных типов в 1 мл среды				
	С (3)	РВ (4)	Д (5)	У (6)	Е (7)
(8) Кровь	$2 \cdot 10^3$	$2 \cdot 10^6$	$2 \cdot 10^4$	$6 \cdot 10^3$	$2 \cdot 10^3$
(9) Бульон Мартена	$8 \cdot 10^8$	$4 \cdot 10^9$	$2 \cdot 10^5$	$4 \cdot 10^9$	$4 \cdot 10^9$

1. culture medium; 2. number of phage corpuscles of different types in 1 cc of medium; 3. S; 4. RV; 5. D; 6. U; 7. Ye; 8. blood; 9. Martin's bouillon.

From Table 5 it is seen that the titer of phages of types S, U and Ye after a single passage on a culture in bouillon increased sharply from $N \cdot 10^3$ to $N \cdot 10^8$ and $N \cdot 10^9$ corpuscles per cc of medium. Similar data were obtained with phages of types RV and D.

Conclusions

1. As a result of passaging keeping them on media containing liver, spleen, small intestine and in human blood, cholera vibriones develop the most pronounced specific characteristics (more distinct colony morphology, increase in viability and in hemolytic characteristics).

2. Cultivation of cholera vibriones on media containing 30 percent bile leads to a variation of these properties in the culture.

3. Culture of the cholera bacteriophage in blood or in media containing spleen, liver or human small intestine with cholera vibriones grown out in the same media increases its activity.

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The Effect of the Freeze-Drying Process on the Cholera Vibrio

M. S. Naumshina and L. P. Guseva
(Saratov)

At the present time, considerable attention is being given to keeping museum strains of bacteria in the dried state. Freeze-drying of microorganisms in the Soviet Union and abroad is being studied by many investigators. In their papers they throw light on problems of the effect of low temperatures, the drying conditions and residual moisture on the survival bacterial cultures and present data on the preservation of the properties of the strains being studied when they are kept for a long time in the dry state in a vacuum. However, all these investigations deal mainly with vaccine strains (BCG, tularemia, brucellosis), microbes of the colon-typhoid group and cocci.

The problem of the effect of the drying conditions on the cholera vibrio and stabilization of its main properties during the course of standing has been inadequately completely discussed in the literature. In the papers published by foreign authors there are indications only of a low survival of the cholera vibrio (from 0.005 to 10 percent) after drying in the following stabilizers: physiological saline solution, distilled water, beef extract, egg yolk, serum, albumin, globulin and built-up Mist. dessicans medium (R. Fry, 1956; R. Greeves, 1956). Only Zheleva (1958) gives data on the

stabilization of the basic characteristics of the cholera vibriones during 23 months of being kept in the dry form.

In the present study the aim was to obtain dry cultures of the cholera vibrio with a high survival and minimum residual moisture with the aim of further observation of preservation of the basic characteristics of the strains studied.

For the purpose of carrying out this aim a study was made of the effect of various stabilizers, low temperatures and the freeze-drying process on the viability of the cholera vibrio, and a determination was also made of the residual moisture of the cultures at different stages of drying.

Method

Six strains of the cholera vibrio were used in the experiment: 2, 14, 48, 54, 119, 125; the last two strains were obtained from Pakistan in 1958; the others had been kept in the museum laboratory for a long time. All the properties of the strains studied were typical. As stabilizers the following were used: sucrose-gelatin-agar medium, gelatin with lactose, milk, milk with lactose, inactivated normal horse serum and egg yolk diluted with physiological saline solution in a ratio of 1:10.

First, the original suspension in physiological saline solution was prepared from a 20-hour agar culture and this was diluted with the corresponding stabilizer to 10,000,000,000 microbes per cc. The suspension was poured out in a sterile manner in quantities of 0.2 cc into ampules with a capacity of 1 cc. The ampules containing the microbe suspension were frozen with dry ice (-76°) for 30 minutes, and then were connected to the IEM [Institute of Experimental Medicine] header-drier apparatus designed by Dolinov and covered with dry ice. After one hour of drying in a vacuum (50-100 microns) the ampules were taken out of the dry ice. In this way, they were in the dry ice for a total of 1½ hours. The entire drying process lasted 20 hours.

Depending on the experimental conditions, during the course of the drying or after it, the survival of the microbes and the residual moisture of the dry cultures were recorded. Determination of the number of viable microbes prior to drying was carried out by means of diluting the contents of one ampule with Martin's bouillon (pH 7.6) to 10^{-7} with subsequent plating out from the sixth and seventh solutions in quantities of 0.1 cc on plates containing Martin's agar (pH 7.6). After 24 hours of incubation at 37° the number of colonies which grew out on the plates and the corresponding number of living cells in a volume of 0.2 cc of the bacterial suspension in the ampule were counted. In a similar way, a determination was made of the number of viable microbes in dry cultures. Thereby, the contents of the ampule (0.2 gram of the dry residue) was diluted with 2 cc of Martin's bouillon, obtaining a dilution of 1:10. The method of dilution described, seeding on plates and counting the colonies which grew out was used in further observations also. In each study the number of viable cells was determined simultaneously in two ampules, after which the averages were computed.

The Results of the Investigations

In experiments on the clarification of the various factors influencing the survival of the cholera vibrio it was determined that the stabilizers which we used exerted no harmful effect on the cholera vibrio at a temperature of 18-20°. It was impossible to find a decrease in the number of living cells as the result of freezing the bacterial suspension at -76° for one-six hours. Therefore, the duration of freezing also failed to exert an appreciable effect on the viability of the microbes. Under low vacuum conditions (50-100 microns) after an hour at -76° a reduction was observed in the number of living microbes in the culture being dried ranging from 3.1 to 27.8 percent (Table 1).

Table 1

Survival of Cholera Vibrio and Residual Moisture after 20 Hours of Drying in Various Stabilizers at a Freezing Temperature of -76°

Момент исследования (1)	Выживаемость в % при стабилизаторе (2)					
	желатина с лактозой (3)	сахарозо-желатино-агар (4)	молоко с лактозой (5)	молоко (6)	сыворотка (7)	желток (8)
До опыта (9)	100	100	100	100	100	100
После часа высушивания (10)	80,2	73,3	92,4	96,9	72,5	72,2
После 20 часов высушивания (11)	23,6	13,1	26,9	0,44	0,75	0,0005
Остаточная влажность (12)	2,2	2,6	1,4	2,4	4,52	1,4

1. time in the investigation; 2. survival in % in the following stabilizers:; 3. gelatin with lactose; 4. sucrose-gelatin-agar; 5. milk with lactose; 6. milk; 7. serum; 8. yolk; 9. before the experiment; 10. after an hour of drying; 11. after 20 hours of drying; 12. residual moisture.

From Table 1 it is seen that after 20 hours of drying the greatest survival of the vibriones was found in milk with lactose (26.9 percent). A very low survival was obtained in milk (0.44 percent), serum (0.75 percent) and egg yolk (0.0005 percent). When egg yolk was used as a stabilizer foaming of the ampule contents was observed after removal of the carbon dioxide; therefore, the ampules had to be frozen again. Possibly, this explains the low survival of microbes dried in the yolk. An idea of the mortality rate dynamics of the cholera vibrio during the course of freeze-drying is given by the data shown in Table 2.

In Table 2 the results of the experiments on the determination of the survival of four strains of cholera vibrio (2, 48, 119, 125) dried in gelatin with lactose and in milk with lactose are shown. Reduction in the

Table 2

Survival of Cholera Vibrio in the Drying Process

	(1) Число живых микробных тел (в %) и остаточная влажность (в %)												
	(2) до опыта	(3) в процессе высушивания в течение часов											
		1	2	3	4	5	6	8	10	12	14	16	18	20
(4) Желатина с лактозой	100	80,2	21,3	51,3	75,0	58,5	64,3	51,2	43,0	24,0	26,1	25,4	29,2	23,6
(5) Остаточная влажность	—	—	—	—	—	—	3,57	3,35	2,6	2,76	1,77	1,87	1,79	2,2
(6) Молоко с лактозой	100	92,4	33,3	82,1	63,6	59,1	56,8	64,1	53,7	59,1	40,0	40,6	60,3	26,9
(5) Остаточная влажность	—	—	—	—	—	—	4,3	3,9	2,7	2,8	1,7	1,6	1,9	1,4

1. number of living microbes (in %) and residual moisture (in %); 2. before the experiment; 3. during the course of drying for. . hours; 4. gelatin with lactose; 5. residual moisture; 6. milk with lactose

number of viable cells two-three hours after connecting up the header is not a true reduction, because in the subsequent hours of drying the number of living cells increases sharply. Such a phenomenon apparently depends on the condition of the microbe suspension being dried, which after two hours becomes thickened and gelatinous, dissolves poorly in bouillon, as a result of which there is a reduction in the number of cells plated out. After four-five hours or more the ampule contents dry, and are readily soluble, and the percentage of living microbes determinable in the culture increases. A certain discrepancy in the percentage of living cells in the ampule after four hours of drying may be explained by the imperfection of the counting method used. Nevertheless, the number of viable cells decreases considerably after 12-14 hours and particularly 20 hours of drying. It should be noted that during the course of drying the rate of extinction of the cells is different in the various strains of cholera vibrio (Table 3).

Table 3

Survival of Cholera Vibrio as a Function of the Strain and the Stabilizer

(1) Штамм	(2) Число живых микробных тел (в %) при стабилизаторе		
	(3) сахарозо-желатино-агар	(4) желатина с лактозой	(5) молоко с лактозой
2	8,9	30,0	23,2
14	0,05		
48	8,7	59,1	38,0
54	28,5		
119		36,5	24,0
125		16,7	23,2

1. strain; 2. number of living microbes (in %) in the following stabilizer:; 3. sucrose-gelatin-agar; 4. gelatin with lactose; 5. milk with lactose.

In the studies of I. P. Druzhinina there are data attesting to better survival of the plague microbe when the preliminary freezing temperature of -30° is used. We checked this principle with regard to the cholera vibrio. For this purpose, strains 2, 48, 119 and 125 were dried simultaneously in a single header in gelatin with lactose; on one side of the header the ampules were frozen and dried for the first 1½ hours at -76°; on the other side, at -30°.

When the bacteria are kept in a dry form in a vacuum for a long time the residual moisture of the dry cultures is of great importance. The majority of authors believes that when dry cultures are kept the residual moisture should not exceed 2-4 percent (S. G. Kolesov, 1952 and others). When various stabilizers are used for the drying the relationship between the residual moisture and the drying medium had to be determined, and the dynamics of change in the latter had to be studied during the course of drying of the culture. We determined the residual moisture by the method of completing the drying at 100° for an hour. The results of determination of the residual moisture of the dried cholera vibrio cultures when various drying media were used are shown in Table 1. A low percentage of residual moisture was obtained when the cultures were dried in milk with lactose and egg yolk (1.4 percent); a high percentage, in dry cultures with serum (4.52 percent).

During the drying process the residual moisture was determined every two hours (see Table 2). It was found that the main mass of moisture is removed after six hours of drying; by 10 hours it amounts to 2.6-2.7 percent. At this time, quite a high percentage of living cells is preserved in the culture (69.7-43 percent). In the subsequent hours of drying the index of residual moisture decreases very slowly, but at the same time an accelerated extinction of the cholera vibrio cells is observed. At the end of the drying the residual moisture drops to 1.4-2.2 percent; thereby, the survival amounts to 26.9-23.6 percent. On the basis of the figures obtained for the residual moisture and the survival rate of the cholera vibrio we consider it efficient to reduce the drying time to 10 hours with maintenance of our experimental conditions.

Conclusions

1. Of the stabilizers studied the best with respect to maintaining the viability of the cholera vibrio during the course of drying and with respect to the residual moisture content are the following: milk with 10 percent lactose (26.9 percent survival, 1.4 percent residual moisture) and gelatin with 15 percent lactose (23.6 percent survival, 2.2 percent residual moisture).

2. In drying various strains of the cholera vibrio in one stablizer with the maintenance of all other conditions the same variations in survival are observed, which depend, apparently, on the individual characteristics of the strain.

3. The routine of preliminary freezing and subsequent drying at -30° preserves more viable vibriones than the routine at -76°.

4. When cholera vibrio cultures are dried for 10 hours in milk with lactose and gelatin with lactose the residual moisture is equal to 2.6-2.7

percent and the survival is 43-63.7 percent. This permits us to recommend the reduction in the duration of freeze-drying of the cholera vibrio to 10 hours with the maintenance of our experimental conditions.

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IMMUNOLOGY

The Infection Phase of Immunity in Animals Inoculated with EV Vaccine

L. V. Samoylova
(Saratov)

The problem of the nature of immunity conferred by living plague vaccine has been inadequately studied.

There is reason to believe that in the first few days after inoculations, when the microbes are circulating in the organs and tissues of the inoculated animals, the living vaccine creates a state of infection immunity, which then changes into actual immunity (Ye. I. Korobkova, 1956). It has been proved that in the bodies of animals inoculated with living plague vaccine an active propagation of microbes occurs and the early production of immunity is noted (M. N. Aliyev, 1958).

It may be supposed that the duration and strength of the immunity created are determined by the level of the vaccine process, that is, by factors in actual immunity: by the presence of living microbes and by tissue reactions which they produce.

In the present studies we determined the period for which microbes of the EV vaccine strain are present in the bodies of inoculated animals, the duration of the infection phase of immunity and the relationship between the dose of vaccine and the period needed for the development of immunity.

The experiments were performed on 365 white mice weighing 18-20 grams and 49 guinea pigs weighing 300-400 grams. As a vaccine a two-day agar EV culture was used, which was injected subcutaneously into the right hind extremity. For white mice inoculation doses of 30,000, 50,000, 100,000 and 150,000 microbes were used in accordance with the TsGNKI [Central State Scientific Testing Institute] optical standard; for guinea pigs, a single dose of 500,000 microbes. The time that the vaccine microbes were in the bodies of inoculated animals was determined on the basis of isolation of the culture from organs and tissues of animals on differential diagnostic media. The material studied (tissue from the injection site of the vaccine, regional lymph node, heart, spleen, liver and lungs) was seeded by impressions on agar containing hemolyzed blood (5 percent) and gentian violet (1:100,000). At the same time, pieces of material were inoculated into test tubes containing semiliquid agar (0.15 percent) with hemolyzed blood (0.3 percent) and gentian violet (1:200,000) by the Ye. I. Korobkova method. Blood was taken from the heart with a pipet after cauterizing the surface of the organ with an incandescent spatula. The cultures were incubated at 28°. The results were read for five days.

Experiments on White Mice

The survival time of the EV vaccine strain in the bodies of white mice was determined in the following way. The animals were inoculated with the doses mentioned above in a volume of 0.2 cc, killed and dissected one, three, six and 24 hours after inoculation, and then every day until the 15th.

20th day, using five mice at each time.

The first group of mice (90) was inoculated with a dose of 100,000 microbes. As the experiments showed, a culture of the vaccine strain was isolated from these animals from the injection site for as long as 11 days; from the regional lymph node, to the second day; from the liver, for four-nine days; from the spleen, from 24 hours to nine days; from the blood, on the fourth and fifth days; and from the lung, on the fifth day.

Vaccine microbes injected into laboratory animals are isolated with difficulty by the usual bacteriological methods. It is possible that bacteria of the vaccine strain which are in the organs and tissues of inoculated animals cannot be found by the methods which we use. It is well known that the resistance of the animal organism to different microbes, particularly to the vaccine strain of the plague microbe, is reduced by cortisone. This afforded the basis for assuming that after intramuscular injection of 3 milligrams of cortisone into a white mouse 18 hours before dissection the percentage of plague microbe cultures isolated would increase.

In an experiment on white mice treated with cortisone and inoculated with 100,000 microbes, it was possible to show that the vaccine strain is preserved in the body for as long as 17 days after the inoculation. For the purpose of obtaining comparable results in subsequent experiments, we injected cortisone into all animals. Under the same condition, in a similar experiment with an inoculation dose of 150,000 microbes, the EV culture was obtained from the injection site for as long as 17 days; from the regional lymph node, as long as four days; from the liver, on the 11th and 12th days; from the spleen, on the fourth-sixth and 10th-15th days; from the blood, on the fifth day. The culture was not isolated from the lungs. After vaccination of mice with 50,000 microbes, the EV culture was isolated for as long as 12 days, whereby it was isolated from the injection site for as long as eight days; from the regional lymph node, as long as seven days; from the liver, from three to 12 days; from the spleen, on the third-fifth-seventh and 9th-10th days; from the blood, from six hours to two-seven days; from the lungs, on the third day. From mice inoculated with 30,000 microbes the inoculation culture could be obtained from the injection site for as long as six days; from the regional lymph node, on the first day; from the liver, from three hours to four-six days; from the spleen, on the third-seventh and 10th days; from the blood, from three-six hours to three-six days; from the lungs, on the sixth day.

After vaccination, regardless of the dose of bacteria used, the greatest seeding was observed four-six-seven days after the inoculation. Later, the bacteria of the vaccine could be detected only in isolated cases.

In white mice treated with cortisone, after subcutaneous inoculation of the EV vaccine in doses of 150,000 and 100,000 microbes, the culture of the vaccine strain was maintained in the body for as long as 17 days; after the inoculation of 50,000 microbes, as long as 12 days, and after the inoculation of 30,000 microbes, as long as 10 days. Therefore, there is a direct relationship between the inoculated dose of vaccine and the survival of microbes in the body.

Therefore, the vaccine process (infection phase of immunity) occurs over a period of 10-17 days in white mice depending on the dose of the vaccine.

Experiments on Guinea Pigs

For the purpose of determining the time of maximum seeding of the bodies of guinea pigs with the EV vaccine, the following experiment was performed. Guinea pigs (34) were injected subcutaneously with the vaccine in a dose of 500,000 microbes in 0.5 cc. Two, six and 24 hours and every day for as long as 17 days after the inoculation, two guinea pigs were killed and their organs and tissues were seeded in the same way as in the experiment with white mice (Table 1).

Table 1

Seeding of Organs and Tissues of Guinea Pigs with Plague Microbes after Subcutaneous Injection of 500,000 Microbes of Living EV Plague Vaccine

Вскрыты после прививки (1)	(2) Число животных, от которых выделена культура											
	(3) при введении вакцины в объеме 0,5 мл						(4) при введении вакцины в объеме 0,2 мл + 10 мг кортизона					
	(5) место введения	(6) лимф. узел	(7) печень	(8) селезенка	(9) кровь	(10) легкие	место введения	лимф. узел	печень	селезенка	кровь	легкие
2 часа (11)	2	—	—	—	—	—						
6 часов (11)	2	—	—	—	—	—						
1 сутки (12)	2	2	1	2	1	—						
2 суток (12)	2	—	—	1	—	—						
3 »	2	—	—	—	—	—						
4 »	2	—	—	—	—	—						
5 »	—	—	—	—	—	—						
7 »	—	—	—	—	—	—						
8 »	—	—	—	—	—	—						
9 »	2	—	—	—	—	—						
10 »	—	—	—	—	—	—	3	3	—	—	—	—
11 »	—	—	—	—	—	—	—	2	—	—	—	—
12 »	—	—	—	—	—	—						
13 »		—	—	—	—	—						
14 »		—	—	—	—	—						
15 »		—	—	—	—	—	—	1	—	—	—	—
17 »		—	—	—	—	—						
18 »							1	2	—	—	—	—
21 »							—	—	—	—	—	—

1. Time dissection performed after inoculation; 2. Number of animals from which culture was isolated; 3. After injection of the vaccine in a volume of 0.5 cc; 4. After injection of the vaccine in a volume of 0.2 cc and 10 milligrams of cortisone; 5. Site of injection; 6. Lymph node; 7. Liver; 8. Spleen; 9. Blood; 10. Lung; 11. Hours; 12. Days.

As is seen from Table 1, from tissue cultures taken from the injection site of the vaccine the culture was isolated for as long as nine days. After 24 hours, the culture was found in the regional lymph node, spleen, liver and blood; after 48 hours, in the spleen.

With this aim in view, we performed a somewhat modified experiment on another 15 guinea pigs. The EV vaccine in a dose of 500,000 microbes was injected subcutaneously in a volume of 0.2 cc. Eighteen hours before dissection the guinea pigs were injected intramuscularly with 10 milligrams of cortisone. Ten, 12, 15, 18 and 21 days after the inoculation three guinea pigs were dissected at each time, and a culture of the vaccine strain could be isolated from the injection site on the 10th and 18th days and from the regional lymph node, on the 10th, 12th, 15th and 18th days. The culture could not be isolated from the internal organs.

The pathological changes in the guinea pigs were expressed in slight hemorrhages at the injection site of the vaccine, sometimes areas of necrosis, enlargement of the regional lymph node and spleen one-six days after vaccination. On the basis of the study made it was possible to determine that microbes of the EV strain are retained in the bodies of guinea pigs as long as 18 days after the inoculation.

Comparing the results of isolation of the culture from the bodies of white mice and guinea pigs on the media utilized in the experiments, it should be noted that frequently, in the absence of growth on agar media in the plates, we observed the growth of microbes in test tubes containing semiliquid agar by the Ye. I. Korobkova method. In many cases, on this medium, growth appears 24-48 hours sooner than on solid agar. In addition, on this medium bacteria can be detected at a time when the cultures of organs remained sterile on the agar. On semiliquid agar containing blood and gentian violet the microbe grows out 24 hours after inoculation in the form of punctate colonies suspended in the agar and a thin whitish film on the surface of the medium. After 48 hours, the colonies increase in size; the film becomes coarser. The percentage of cultures isolated from semiliquid agar in all cases was higher than on the solid medium (Figs 1 and 2).

Time of Creation of Immunity

In the second series of experiments the aim of the investigations included the study of the time at which immunity was created, depending on the dose of living plague vaccine.

The experiments were performed on 684 white mice, divided into two groups. The first group of mice was inoculated subcutaneously with a two-day agar culture of the EV vaccine, using doses of 30,000, 50,000, 100,000 and 150,000 microbes. The second group was inoculated with the same doses of vaccine but killed by heating for an hour at 60° (the sterility of the killed vaccine was confirmed by a bacteriological test). For the purpose of comparative study of the time at which immunity occurred after the injection of living and killed vaccines, white mice were infected with 200 CLD of a virulent culture of the plague microbe (708 strain). Different groups of mice were infected daily, beginning with the fourth to 12th day and then on the 15th, 17th, 19th and 21st days after inoculation. Of the animals which died after infection a plague microbe culture was obtained on agar containing

gentian violet and blood. The experiments showed that mice inoculated with living vaccine acquire immunity to plague as early as on the fourth day after inoculation; those vaccinated with the killed vaccine all die.

On Fig 3 the survival curves of the vaccinated white mice are shown after infection. The wave-form nature of the curves cannot be overlooked; this can apparently be explained by the action of the plague endotoxin on the bodies of white mice. Possibly, a superimposition of the effect of the vaccine on that of the virulent culture occurs in their body. The time at which immunity occurs, as is seen from the curves, depends on the dose of vaccine. After inoculation with a larger dose (within the limits of the average doses which we used) immunity occurs more quickly.

The time at which immunity was created in the guinea pigs inoculated subcutaneously with a dose of 500,000 microbes of living and killed cultures of the EV vaccine strain was determined by means of subcutaneous infection of them with 400 CLD (40,000 microbes) of a plague microbe culture of the 708 strain five, seven, 10, 19 and 21 days after inoculation. At these times five guinea pigs from each group were infected. The control animals were not vaccinated. In all, 75 animals were used in the experiment. Five days after the inoculation three guinea pigs out of five immunized with living vaccine survived. Immunity was formed completely by the seventh day. All of the control guinea pigs and guinea pigs inoculated with killed vaccine died. Therefore, the production of immunity was brought about only after immunization with living vaccines; after the inoculation of killed vaccine no immunity was observed.

On the basis of the investigations which we have presented, we can conclude that the phase of immunogenesis where living vaccine microbes survive, multiply and seed the organs and tissues of the animal should be considered the infection phase of immunity, which changes into the postinfection phase.

Conclusions

1. Beginning with the first few days after inoculation of the EV vaccine, through the propagation of vaccine microbes in the bodies of animals, a state of resistance is created in the latter which can be regarded as the infection phase of plague immunity.

2. The rate of development of immunity is directly related to the degree of spreading and propagation of the living immunogenic vaccine in the organs of inoculated animals.

3. After immunization of white mice with EV vaccine in doses of 30,000, 50,000, 100,000 and 150,000 microbes a culture of the corresponding microbes can be isolated for as long as 17 days. With increase of the dose of the vaccine, the period in which microbes can be isolated is lengthened. After vaccination of guinea pigs with a dose of 500,000 microbes a culture could be obtained from them for 18 days.

4. Comparative isolation of plague microbe cultures from the bodies of inoculated animals on solid medium and semiliquid agar containing gentian violet and blood showed the advantage of the latter medium.

5. Under the influence of cortisone injected into the vaccinated animals 18 hours before they were sacrificed, the bacteria are isolated in a

higher percentage of cases than in the investigation of animals not given this hormone.

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Study of the Antigenic Composition of the Plague Microbe (Pasteurella Pestis EV of the NIIEG) by the Method of Diffusion in Gel

V. L. Pustovalov, N. G. Ovanesova, A. M. Konnova and L. I. Kolesnikova
(Rostov-na-Donu)

The study of the antigenic composition of the plague microbe is of great interest from the viewpoint of subsequent isolation of these substances, study of their chemical composition and immunogenic properties.

The development of the method of diffusion in gel by Oudin (1948) and Ouchterlony (1949) was an important step in the analysis of the antigenic structure of microorganisms (Yu. Z. Gendon, 1958).

The diffusion in gel method has been used in recent years for the study of antigens of the plague microbe.

Ransom and others (1955) studied the antigenic composition of five different strains of the plague microbe and obtained 10 precipitation zones.

Chen and Meyer (1955) studied the antigenic composition of a number of plague microbe strains and fractions of them as well as those of one strain of the pseudotuberculosis microbe. It was found that the avirulent but antigenic strain A1122 contained the 1 Baker fraction and others (Baker and others, 1947, 1952), while the avirulent but nonantigenic strains 14 and TRU contain practically no 1 Baker fraction.

Bhagavan, Chen and Meyer (1956) found seven antigens in virulent and

avirulent strains of the plague microbe, five of which were common to antigens of the pseudotuberculosis microbe.

Crumpton and Davies (1956) found 10 antigens in the avirulent Tjiwidej strain. A precipitation band was identified which corresponded to the capsular antigen as well as a band corresponding to the plague toxin and antigen, the specificity of which was determined by the polysaccharide component.

Burrows and Bacon (1956) observed 12-18 precipitation bands in performing the test with living plague microorganisms. In all virulent strains as well as in EV 76 they found V and W antigens which inhibit phagocytosis.

S. I. Zaplatina (1958) studied the antigenic composition of a number of fractions of virulent (177) and avirulent (EV 76) strains of the plague microbe as well as the pseudotuberculosis microbe (strain 498). In another study S. I. Zaplatina (1957) reported the presence of seven antigens in living plague cultures of different origins, of which four were common to antigens of pseudotuberculosis cultures. V. V. Akimovich and T. Ya. Dobrotsvetsova (1960) established the fact that virulent and toxic strains of the plague microbe have a minimum of five antigenic components.

Lawton and others (1960) found 16 antigens in the plague microbe and 13 antigens in the pseudotuberculosis microbe; 11 antigens were common to both species. Among the common antigens an L antigen was discovered, which the authors consider immunogenic.

In the present work we made an attempt to determine the total number of antigens in the EV avirulent strain of P. pestis (NIIEG [Scientific Research Institute of Epidemiology and Hygiene]) by the method of diffusion in gel and to study their distribution by fractionation of substances extracted from the bacterial mass.

In this work the EV avirulent strain of P. pestis (NIIEG) was used, which was grown out for three days at 37° on casein-hydrolysate agar (pH, 7.2) containing 5 percent yeast autolysate solution (Ye. M. Gubarev, S. I. Zaplatina, A. M. Konnova, 1956). The bacteria were washed off the surface of the agar with physiological saline solution containing 0.001 M phosphate buffer (pH, 7.1) and killed by the addition of two volumes of acetone cooled to -35° to the suspension. After two days of keeping in a refrigerator (2°) the bacterial mass was separated by centrifugation, then washed on a filter with cold acetone and dried in a vacuum (5-10 millimeters of mercury).

The acetone-water centrifugate gave a precipitate, after the addition of another three volumes of acetone to it, which was collected, dialyzed and dried by the freeze-drying method. This substance, readily soluble in physiological saline solution and distilled water, was called the R precipitate.

The plan for extraction and fractionation of antigens of the plague microbe with ammonium sulfate, proposed by Baker and others (1952), for isolation of fractions 1A, 1B and the toxic fraction was used here for the study of the distribution of the antigens in these fractions. With this aim in view, 129.2 grams of an air-dried bacterial mass was extracted serially three times with 2.5 percent sodium chloride solution (pH of 7.1). By means of allowing it to stand (with periodic shaking) for 24 hours, three extracts were obtained, which were separated from the bacterial mass by centrifugation (2°) at 3000 revolutions a minute for two hours. The first extract (E_1) had a volume of 1370 cc; the second (E_2), 1405 cc; the third (E_3), 1040 cc. Determination of the dry residue in samples of each extract showed that the

content of dry matter, after subtracting the sodium chloride content in the extract E_1, amounted to 17.9 milligrams per cc; in the extract E_2, 6.4 milligrams per cc; in the extract E_3, 1 milligram per cc. Then all the extracts were combined. As the result, 3815 cc of combined extract was obtained, which according to calculation contained 32.3 grams of solid matter (25.0 percent of the original bacterial mass). Substances which were in the combined extract were subjected to fractionation with ammonium sulfate (Fig 1). All the fractions were freed of the ammonium sulfate admixture by dialysis and were dried by the freeze-drying method.

As the result of addition of ammonium sulfate to 25 percent saturation, fraction α_1 was isolated which contained a large quantity of agar from the nutrient medium.

After 30 percent ammonium sulfate saturation the crude 1A fraction was isolated, which was then reprecipitated several times in order to eliminate agar impurities (fractions a_2, a_3, a_4) and the admixture of other antigens (fractions C_1, C_2 and C_3). The purified 1A fraction (1A4 precipitate) contains protein and polysaccharide, giving a positive biuret test and a positive Molisch test.

After 40 percent ammonium sulfate saturation the crude 1B fraction was isolated, which was then reprecipitated, eliminating fraction 1A (fractions K, L, M) and the toxic fraction (fractions γ_1, γ_2 and γ_3). The purified 1B fraction (precipitate 1B4) represents a protein substance without an admixture of polysaccharide (positive biuret test and negative Molisch test).

After 67 percent ammonium sulfate saturation the toxic fraction was isolated. The substance remaining in the centrifugate was dialyzed, frozen-dried, and called the residual fraction.

The yield and chemical characteristics of the fractions are shown in the Table.

The extracts of the bacterial mass (E_1, E_2 and E_3), the precipitate R as well as all fractions isolated were studied by the method of diffusion in gel after Ouchterlony (1949) with the aim of demonstrating the total number and distribution of antigens of the plague microbe.

As a gel, 1 percent agar in aqueous solution containing 0.85 percent sodium chloride, 0.25 percent phenol and 0.001 M phosphate buffer (pH, 7.2) were used. Into each Petri dish 40 cc of hot agar solution was poured. After the setting of the gel in the agar plate, wells of a diameter of 15 or 18 millimeters each were made in the agar plate with a sharp cylindrical punch. For the purpose of sealing two drops of fused agar solution was adde to the bottom of each well. The wells were arranged according to patterns corresponding to the experimental condition. The distance between the wells was 15 or 20 millimeters. Experiments on diffusion in gel were performed by two methods. In the first method, A, samples of the substances were dissolved in 0.5 cc of physiological saline solution and introduced into the corresponding wells once. At the same time, 0.5 cc of plague agglutinating serum was added to the corresponding well. Where wells 18 millimeters in diameter were used, samples of the substances were dissolved in 1 cc, and each solution was introduced into the corresponding well once. In this case, 1 cc of serum was introduced into a certain well. In this work serum of the Saratov "Mikrob" Institute was used (series 66 and 68, titer 1:2000) obtained from the immunization of horses with mixtures of cultures of P. pestis 1, 17 and

Properties of Fractions Isolated from Extract of P. Pestis EV (NIIEG)

Наименование фракции (1)	(2) Вес, г	(3) Содержание азота, %	(4) Содержание фосфора, %
a_1	0,1876		0,03
a_2	0,5020	8,10	0,03
a_3	1,3323	9,73	0,01
a_4	0,0232	—	—
(5) $Ц_1$	0,4220	10,42	0,11
$Ц_2$	0,5624	11,09	0,03
$Ц_3$	0,0301	—	—
1A4	0,9006	10,58	0,05
1B4	0,2280	14,03	0,03
К	0,7094	9,77	0,03
Л	0,0340	—	—
М	0,0283	—	—
γ_1	0,7890	8,70	0,01
γ_2	0,0465	8,50	0,03
(6) Токсическая	5,2095	—	0,16
(7) Остаточная	2,9980	—	0,19
Р (8)	2,9343	—	0,15

1. Name of fraction; 2. Weight, grams; 3. Nitrogen content, %; 4. Phosphorus content, %; 5. C_1; 6. Toxic; 7. Residual; 8. R.

EV. The plates were closed over with covers, to the inner surfaces of which filter-paper disks were attached which were of a diameter equal to the size of the plate. The plates were put into the horizontal position in an incubator at 37°. Every day the filter paper in the covers of the plates was moistened with distilled water. In the second method, B, samples of the substances were dissolved in 1 cc of physiological saline solution and introduced into the respective wells (15 millimeters in diameter) for four days. Thereby, on the first day 0.5 cc of the solution was introduced into the well, and the remaining quantity was added until the well was filled on the next three days.

In exactly the same way, for four days, serum was added to the corresponding well. During the next 14 days physiological saline solution was added to all wells (with a pH of 7.1). The plates were closed with covers, on the insides of which filter-paper disks 4 centimeters in diameter were attached with adhesive tape, and they were put into an incubator at 37°. The filter-paper disks were moistened with distilled water every day. We used the method described, B, for more effective separation of the precipitation bands. These bands are formed more gradually, and with the addition of physiological saline solution they separate from one another somewhat, apparently because of different degrees of ultrafiltration of the particles of precipitate through the gel. The final reading of the test was made on the 20th day of the experiment.

In a number of cases, for the purpose of more accurate interpretation of the antigenic composition, plague agglutinating serum which had been absorbed by the 1A fraction was used. For the preparation of it 25 milligrams of the 1A fraction was added to 10 cc of serum. The mixture was carefully triturated in a test tube with a glass rod and allowed to stand in an incu-

bator at 37° for three hours, mixing it periodically. After this, the precipitate was centrifuged at 16,000 revolutions per minute at a temperature of 2°. The absorbed serum was kept in the refrigerator until it was used. Such serum did not give any precipitation zones when it was checked by the method of diffusion in gel against fractions 1A and 1B.

Special experiments in which physiological saline solution, an extract of sterile nutrient medium for the cultivation of plague microbe as well as normal horse serum were used did not show the production of any precipitation zones for a month's observation.

It should be noted that a comparison of the antigenic composition of living bacteria (P. pestis EV of the NIIEG), used in a quantity of 75,000,000,000 in a well (in a volume of 0.5 cc of physiological saline solution) containing a suspension of the same quantity of these microorganisms killed with acetone, showed a very similar picture of precipitation bands (Fig 2).

On Fig 2 the precipitation zones corresponding to the 1 Baker fraction are near the well containing microorganisms, because the quantity of bacteria used is too small for the creation of these zones at the usual place at which they are located.

Study of the antigenic composition of the extracts E_1, E_2 and E_3 showed that a large number of antigens is present in them (Fig 3). With the aim of systematization we designated these precipitation bands with letters of the Russian alphabet, beginning with the well containing the solution of antigens and going toward the well containing serum. Change in the concentration of the substances in extracts from E_1 to E_3 made it possible to detect the number of antigens which have confluent precipitation bands (П). Thus, the largest precipitation band (П) in the extracts E_1 and E_2, the corresponding 1 Baker fraction and others (1952) are distinctly separable into four bands in the extract E_3. Study of the behavior of this band on Fig 4 as well as on a large number of other plates makes it possible to suspect the presence of substances in it which, when the experiments are performed by the B method (see Fig 3), give as many as six precipitation bands. In this way, the inhomogeneity of the 1 Baker fraction and others is demonstrated.

On the outer side of the band described (see Fig 3), going toward the well containing E_1, there is a broad precipitation band which divides into three zones (МНО) at E_2. Further to the outside there are two closely situated bands, ZИ; further to the periphery there is a broad band which at E_2 is divided into zones ЖЕ and АГ; then come the bands Б, Б and, at the actual well containing the solution of antigens, the band А. On the inner side of the thick-line П there is another broad precipitation band, which consists of two components, УФ. Between this band and the fraction 1 band there is a small zone, Т. To the inside of the band УФ, toward the well containing serum, three other zones are seen, Х, Ц and Ч.

It should be noted that the thick precipitation band П contains two other precipitation bands, РС, which coincide with it in their positions. Performing the diffusion in gel test with plague agglutinating serum absorbed by the 1А fraction makes it possible distinctly to demonstrate the existence of the РС zones (Fig 5). On Fig 6 the bands К and Л, lying between zones ZИ and МНО, are clearly seen.

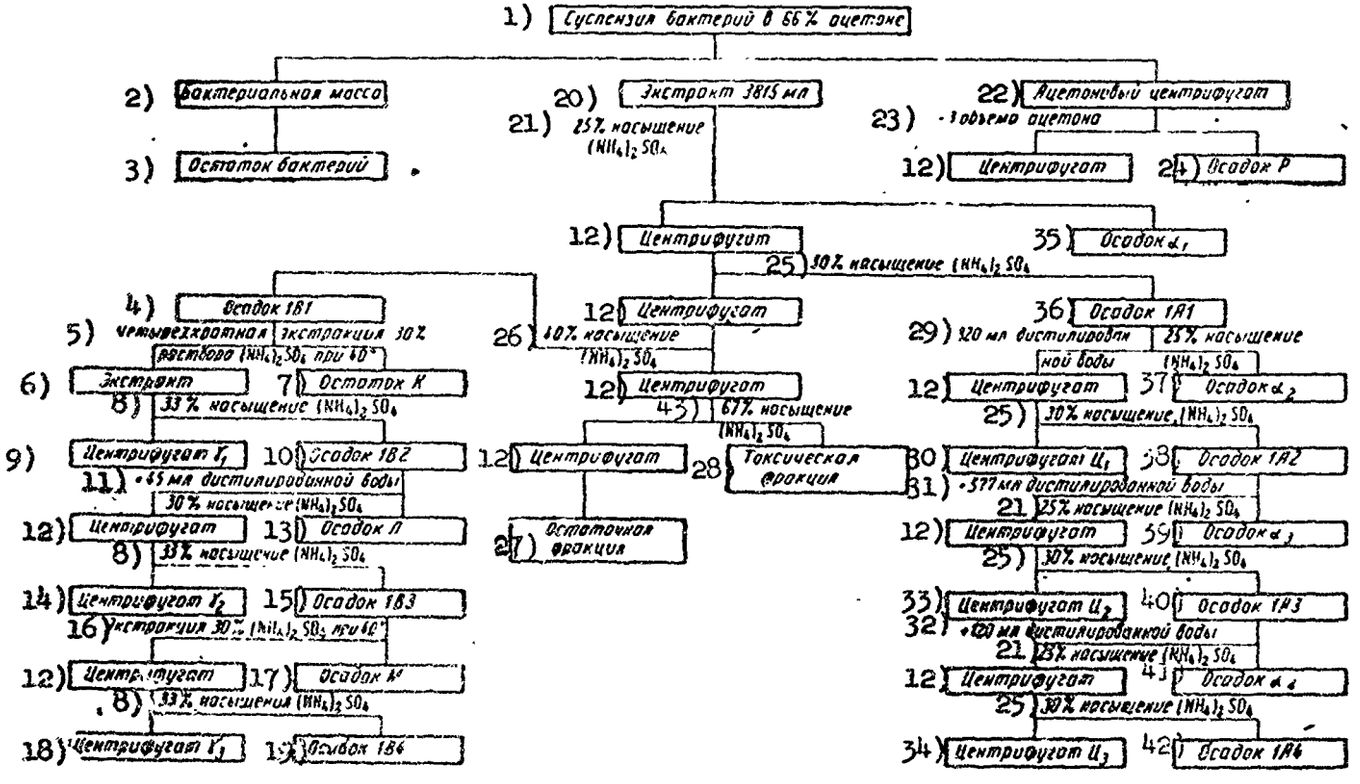

Fig. 1.

[Key on following page]

Fig 1. Fractionation of Antigenic Substances of an Extract from P. Pestis EV (NIIEG). 1. Bacterial suspension in 66% acetone; 2. Bacterial mass; 3. Bacterial sediment; 4. Precipitate 1R1; 5. Extraction four times with 30% ammonium sulfate solution at 40°; 6. Extract; 7. Sediment K; 8. 33% ammonium sulfate saturation; 9. Centrifugate r_1; 10. Precipitate 1B2; 11. + 45cc of distilled water, 30% saturation with ammonium sulfate; 12. Centrifugate; 13. Precipitate L; 14. Centrifugate r_2; 15. Precipitate 1B3; 16. Extraction with 30% ammonium sulfate at 40°; 17. Precipitate M; 18. Centrifugate r_3; 19. Precipitate 1B4; 20. Extract, 3815 cc; 21. 25% saturation with ammonium sulfate; 22. Acetone centrifugate; 23. Three volumes of acetone; 24. Precipitate R; 25. 30% ammonium sulfate saturation; 26. 40% ammonium sulfate saturation; 27. Residual fraction; 28. Toxic fraction; 29. 320 cc of distilled water and 25% ammonium sulfate saturation; 30. Centrifugate C_1; 31. 577 cc of distilled water, 25% ammonium sulfate saturation; 32. 120 cc of distilled water, 25% ammonium sulfate saturation. 33. Centrifugate C_2; 34. Centrifugate C_3; 35. Precipitate α_1; 36. Precipitate 1A1; 37. Precipitate α_2; 38. Precipitate 1A2; 39. Precipitate α_3; 40. Precipitate 1A3; 41. Precipitate α_4; 42. Precipitate 1A4; 43. 67% ammonium sulfate saturation.

Between the well containing the extract E_2 and the well containing serum 16 precipitation bands are observed (see Fig 3). However, keeping in mind the considerations which have been expressed concerning the complicated nature of the π band and the presence of several other zones seen on Figs 5 and 6, the existence of antigens giving 28 precipitation bands in an extract of P. pestis EV (NIIEG) can be claimed. On Fig 7 a schema of the antigenic composition of P. pestis EV (NIIEG) is presented.

During the course of this work we carried out a series of extractions of different portions of the bacterial mass of P. pestis EV (NIIEG) and performed a large number of tests with double diffusion in gel, with the use of these extracts. A study of the precipitation patterns completely confirmed the schema proposed of the antigenic composition of P. pestis EV (NIIEG) grown out on casein acid hydrolysate medium.

It should be noted that the very intense precipitation band π can sometimes hold up the diffusion of the antigens T, YФ and XII, and Ч. On Fig 8 it is seen that reduction of the quantity of extract of the bacterial mass in the wells leads to demonstration of zones YФ, I and Ч.

The study of the antigenic fractions of P. pestis EV (of the NIIEG) by the diffusion in gel method showed that they are inhomogeneous with respect to composition. Fractions 1A4, 1B4, δ_2, δ_3 and γ_3 give a precipitation band π without the admixture of other zones. Thereby, it should be noted that by the method of diffusion in gel fractions 1A and 1B cannot be distinguished from each other. Sera absorbed either by fraction 1A or by fraction 1B fail equally to give precipitation bands corresponding to the 1 Baker fraction with a bacterial extract. It has also been determined by a number of special experiments that serum absorbed by fraction 1A does not give precipitation bands with the substance of fraction 1B, and vice versa.

Fractions δ_1, U_1, U_2, K and γ_1 give a precipitation band π accompanied by one or several other zones, which is evidence of contamination of fraction 1 in these preparations with other antigens.

The diffusion in gel of the toxic and residual fractions is shown in Fig 9. It is seen that both the toxic and residual fractions contain the band π which corresponds to the 1 Baker fraction and others. This once again is evidence of the inhomogeneity of the substances making up the precipitation band corresponding to the 1 Baker fraction.

On Fig 9 it is seen that in the toxic fraction there is a very large number of antigens, whereby some bands are appreciably more intense than with the original bacterial extract.

In conclusion, it should be noted that the schema proposed for the arrangement of antigens is true only for the strain of plague microbe being studied (P. pestis EV of the NIIEG), grown out on a medium made of acid casein hydrolysate at 37°. Other plague microbe strains may have different antigen concentrations, which will have a great effect on the spatial interrelationship of the precipitation bands. The absence of some antigens or the presence of others will change the general picture. Therefore, for reliable identification of these antigens they must be isolated in the purified form; monoantigenic antisera must be prepared, and using these preparations, the work of interpreting the antigenic composition of avirulent and virulent strains of the plague microbe should be begun.

We adhere to the opinion that the modified method of double diffusion

in gel which we adopted (method B) makes it possible better to demonstrate the presence of existing antigens in an extract of a bacterial mass and does not contribute to the occurrence of the Liesegang phenomenon. Recent studies by Allison and Humphrey (1960), made with ordinary and labeled antigens, once again attest to the fact that the corresponding antigens do not pass through the visible precipitation zone, and only negligible quantities of antibodies pass through it. The patterns of formation of several precipitation zones with periodic addition of reactants to the wells, given by Oudin (1952) and other investigators, are, in our opinion, the result of the demonstration of several protein components in the antigens used (erroneously taken as homogeneous proteins). Thus, for example, chemical analysis showed that egg albumen is a mixture of several proteins (Rhodes, Azari and K. Feeney, 1958) and, naturally, a preparation of this substance can give several precipitation zones when a method is used which possesses greater resolving power.

Conclusions

1. A schema has been proposed for the arrangement of 28 precipitation zones conditioned by antigenic substances of the plague microbe (B. pestis EV of the NIIEG) grown out on casein acid hydrolysate medium.
2. A study has been made of the distribution of antigens in the fractions made of an extract of the plague microbe obtained by separation with ammonium sulfate.
3. The inhomogeneity of Baker's and others' fraction 1, which consists of at least six components, has been shown.
4. The purity of fractions 1A and 1B, isolated in accordance with Baker's and others' plan, from a mixture of other antigens, has been confirmed.

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The Effect of Terramycin on the Diffusion Properties of the Plague Microbe

I. G. Lalazarova
(Saratov)

Duran-Reynals' spreading factor (1933) was first found in the plague microbe by Jawets and Meyer (1944) and afterwards thoroughly studied by Ye. I. Korobkova (1947, 1950, 1951, 1958). The presence of hyaluronidase was

also confirmed by Z. Ye. Malinina (1953), who showed that streptomycin reduces the activity of the spreading factor of the plague microbe in the rabbit's skin. However, in a work published in 1960, I. V. Domaradskiy, G. A. Yaromyuk and Z. I. Vasil'yeva state that the enzyme hyaluronidase is absent from the plague microbe. As these authors point out, the main reason for discrepancies between their data and results obtained by Ye. I. Korobkova lies in the fact that in her experiments she used very dense microbial suspensions, as the result of which she ran into the phenomenon of nonspecific inhibition of clot formation. Taking this into consideration, we, in test-tube experiments for detection of hyaluronidase in the plague microbe culture, worked with diluted supernatant fluid (from 0.025 to 0.00004 cc per cc of volume) and in all cases obtained results which were in agreement with the data of Ye. I. Korobkova.

In Ye. I. Korobkova's opinion (1950), the plague microbe spreading factor is liberated during the course of mass destruction of the microbe: autolysis, lysis or other similar phenomena occurring in the living organism and under test-tube conditions. Thereby, the author finds no complete parallelism between the virulence of the microbes and the presence of hyaluronidase in them. For example, the avirulent EV strain contained the same quantity of hyaluronidase as the virulent strains of plague microbe.

For the purpose of studying the effect of terramycin on the spreading factor of the plague microbe, we performed experiments under test-tube conditions on rabbits also.

With the aim of obtaining hyaluronidase a two-day agar culture of the EV strain was used, which in a quantity of 1,000,000,000 microbes per cc was seeded on Hottinger's agar containing 0.4 percent glycine. According to Ye. I. Korobkova's data (1957), on this medium an increase of hyaluronidase production occurs in the plague microbe together with an increase in the production of hyaluronic acid. After 48 hours of growth at 28° the mass of microbes was triturated on the surface of the agar by means of a spatula and emulsified in condensation fluid. The brownish slimy mass obtained was aspirated with a pipet and put into centrifuge tubes for 24 hours in a refrigerator at 4°, and then in an incubator at 28° for 48 hours. Thereby, the mass of microbes lost its viscosity. The suspension treated in this way was centrifuged until the microbes had been completely precipitated. The opalescent yellowish supernatant fluid was used as the preparation which contained the spreading factor, the enzyme hyaluronidase.

It is well known that in the presence of hyaluronidase the hyaluronic acid of umbilical cords breaks up into glycuronic acid and glucosamine, as the result of which it loses its capacity of forming clots in an acid medium. In studying the effect of terramycin on the spreading factor of the plague microbe under test-tube conditions, we used umbilical cords of newborn children, treated by a method proposed by L. G. Smirnova (1951) and modified somewhat by Ye. I. Korobkova, as a preparation containing hyaluronic acid. The cords taken in a sterile manner were carefully washed free of blood with distilled water: the blood vessels were removed with a scissors, and the whole was cut into small pieces with scissors and ground up in a mortar containing sand until a homogeneous pulp was formed. The mass obtained was suspended, and an equal gravimetric quantity of distilled water was added to it. After careful mixing the mixture was put into centrifuge tubes in a refriger-

ator at 4° for 24 hours, after which it was centrifuged for 15-20 minutes. As a preparation containing hyaluronic acid slightly opalescent supernatant fluid (Wharton's jelly) was used.

Before performing the experiment the preparation obtained was titrated (see Table 1), that is, the lowest concentration of hyaluronic acid solution which gave a clear coagulate with two drops of 15 percent acetic acid solution after incubation at 37° for 15 minutes was determined. The minimum dose was doubled and used as the working dose.

Table 1

Titration of the Substrate

	(3) Номера пробирок					
	1	2	3	4	5	6
Субстрат, мл (1)	0,4	0,3	0,2	0,1	0,05	0,025
Дистиллированная вода, мл (2)	0,6	0,7	0,8	0,9	0,95	0,975

1. Substrate, cc; 2. Distilled water, cc; 3. Numbers of test tubes.

After the addition of acetic acid coagulation occurred in the test tubes containing 0.4-0.05 cc of the preparation; 0.1 cc was used as the working dose. The presence of hyaluronic acid in the preparation obtained was confirmed by the reaction of the preparation with rabbit testicular extract (known hyaluronidase).

For the purpose of studying the terramycin effect on the hyaluronidase of the plague microbe, 0.1 cc of Wharton's jelly and terramycin in 0.1 cc of distilled water were added in the following doses to a series of test tubes containing different quantities of supernatant fluid (0.025; 0.0125; 0.00625; 0.00312; 0.00156; 0.00078; 0.00039; 0.00018; 0.00009 and 0.00004 cc): in one series, 800 milligrams each; in another, 400 milligrams each; in the third and fourth, 100 milligrams each. The antibiotic was dissolved in a phosphate buffer diluted with distilled water according to the ratio of 1:4 (one part of buffer solution and four parts distilled water). The need for using diluted phosphate buffer arose in connection with the fact that high terramycin concentrations (800-400 milligrams per cc) in the distilled water gave a markedly acid reaction of the medium (pH of 3.0). When the experiment was performed under such conditions the protein of Wharton's jelly became denatured and came down in a precipitate without the addition of acetic acid. The attempt to perform the experiment in undiluted phosphate buffer also met with failure because of the fact that thereby the terramycin rapidly combined with the salts of the buffer and went into the precipitate in the form of a greenish-white powder. In addition, during the course of the work it was noted that in pure phosphate buffer no splitting of the

hyaluronic acid by hyaluronidase of the plague microbe occurs, as the result of which the clot appeared in all test tubes after the addition of acetic acid. In diluted phosphate buffer this enzyme showed its effect, but not so strongly as in distilled water.

Considering what has been stated above, we found it possible to dissolve terramycin in diluted phosphate buffer according to the calculation that 0.1 cc of the solution contains 200 milligrams of the antibiotic. The test was performed in a volume of 1 cc. For the purpose of obtaining this volume the missing quantity of fluid was made up with distilled water. Thereby, the pH of the medium in different test tubes ranged from 5.5 to 7.0. The experiment was performed with corresponding controls (hyaluronate + hyaluronidase + distilled water + buffer solution; hyaluronate + hyaluronidase + distilled water). The test tubes were put into an incubator at 37° for 15 minutes, after which two drops of a 15-percent acetic acid solution were dropped into each of them. In the absence of hyaluronidase in the supernatant fluid a protein clot appeared in the test tubes, and the fluid became clear; in the case of a positive test the test tube remained turbid and no clot formed.

In the test-tube experiments terramycin inhibits the effect of the spreading factor of the plague microbe, whereby this inhibitory effect is directly proportional to the antibiotic concentration. Thus, in the presence of 800 micrograms per cc of terramycin in the mixture, the action of hyaluronidase stopped even in the test tube containing supernatant fluid in a dose of 0.00625 cc and in the corresponding control (hyaluronate + hyaluronidase + distilled water + 0.4 cc of buffer solution) it stopped only with a dose of 0.00156 cc; the presence of 400 milligrams per cc of terramycin in the mixture stopped the action of the spreading factor in the test tube containing supernatant fluid in a quantity of 0.00312 cc, while in the control (hyaluronate + hyaluronidase + 0.2 cc buffer solution) this occurred at 0.00078 cc; in the presence of 100 micrograms per cc of the antibiotic in the mixture, the action of the spreading factor was inhibited in the test tube containing 0.00156 cc of supernatant fluid, while in the control (hyaluronate + hyaluronidase + 0.05 cc of buffer solution) this occurred at 0.00078 cc; 100 micrograms per cc of terramycin in pure distilled water caused partial inhibition of the hyaluronidase effect in 0.00156 cc of supernatant fluid and complete inhibition, in 0.00078 cc; in the corresponding control (hyaluronate + hyaluronidase + distilled water) it stopped at 0.00039 cc.

The effect of terramycin on the spreading factor of the plague microbe in the living organism was studied on five white male rabbits. As a spreading factor the supernatant fluid of the EV strain obtained in the previous experiment was used. A 1-percent trypan blue solution served as an indicator of the spreading factor.

All the rabbits were injected subcutaneously with a mixture consisting of 0.2 cc of supernatant fluid, 0.1 cc of trypan blue and 0.1 cc of physiological saline solution, into the right side. The animals were injected subcutaneously into the left side with 0.1 cc of trypan blue and 0.3 cc of physiological saline solution.

The first (experimental) rabbit was given an intramuscular injection of 50,000 milligrams of terramycin (a total of 100,000 milligrams in two

injections) two hours before injection of the mixture and 10 hours after this.

The second and third (experimental) rabbits received terramycin in a quantity of 500 milligrams subcutaneously on the right side in a mixture of supernatant fluid, trypan blue and physiological saline solution and subcutaneously on the left side in a mixture of physiological saline and trypan blue.

The fourth (experimental) and fifth (control) rabbits were treated according to the same system as the second and third. The difference lay in the fact that mixtures of terramycin with supernatant fluid and physiological saline (for the experimental rabbit) and of supernatant fluid with physiological saline (for the control rabbit) were first kept at 28° for 24 hours, and trypan blue was added to this mixture before the experiment was performed.

The results of the experiment were read one, two, five, and 12 hours after its performance, and then every 12 hours for three days, by means of measuring the spreading area of the dye and calculating the diffusion index of the trypan blue (for both control and the first experimental rabbits—the quotient from dividing the spreading area of the dye containing hyaluronidase by the spreading area of the dye containing physiological saline; for the second and fourth experimental rabbits, the quotient obtained from dividing the spreading area of the dye containing hyaluronidase and terramycin by the spreading area of the dye containing physiological saline and antibiotic).

The data given in Table 2 were obtained 36 hours after the experiment was performed. At this time, the spread of the trypan blue was the greatest. Later, the staining of the skin with the trypan blue began to become pale, and its boundaries lost their distinctness. After 36-48 hours, hyperemia, edema, and infiltration of the skin began to decrease.

From Table 2 it is seen that in experiments on animals the terramycin inhibits the effect of hyaluronidase of the plague microbe: the index of dye diffusion in the control rabbit was approximately twice as great as in the experimental rabbits. The inhibitory effect of terramycin, thereby, did not depend on the duration of its contact with hyaluronidase.

Conclusions

1. Under test-tube conditions terramycin inhibits the action of the spreading factor of the plague microbe. The latter is directly proportional to the antibiotic concentration per cc of fluid.

2. Terramycin inhibits the action of the spreading factor of the plague microbe in the rabbit's skin. The influence of it on this factor does not depend on the duration of contact between terramycin and the spreading factor.

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Table 2

Effect of Terramycin on the Spreading Factor of the Plague Microbe in the Bodies of Animals

№ кролика (1)	В какой бок вводили препарат (2)	Составные части вводимой смеси (3)			Терразицин, мг (7)	Результаты опыта (8)		
		надосадочная жидкость, мл (4)	триптановая синь, мл (5)	физиологический раствор, мл (6)		реакция на месте введения смеси (9)	площадь распространения сини, см² (10)	индекс диффузии сини (11)
		Сразу после получения смеси (14)						
1 (опытный) (12)	Правый (15)	0,2	0,1	0,1	100 000	Гиперемия, отек (18)	21,6	2,0
	Левый (16)		0,1	0,3			10,5	
2 (опытный)	Правый	0,2	0,1	0,1	500	Гиперемия, отек	59,6	2,3
	Левый		0,1	0,3			25,5	
3 (контрольный) (13)	Правый	0,2	0,1	0,1	500	Гиперемия, отек	160	4,1
	Левый		0,1	0,3			39	
		Через 24 часа контакта при 28° (17)						
4 (опытный)	Правый	0,2	0,1	0,1	500	Гиперемия, отек (18)	52	2,0
	Левый		0,1	0,3	500		25	
5 (контрольный)	Правый	0,2	0,1	0,1		Гиперемия, отек	144	4,1
	Левый		0,1	0,3			35	

1. Number of rabbit; 2. Side into which preparation was injected; 3. Constituents of the mixture injected; 4. Supernatant fluid, cc; 5. Trypan blue, cc; 6. Physiological saline, cc; 7. Terramycin, mg; 8. Results of the experiment; 9. Reaction at the injection site of the mixture; 10. Area of spread of the trypan blue, sq. cm; 11. Index of diffusion of trypan blue; 12. Experimental; 13. Control; 14. Immediately after obtaining the mixture; 15. Right; 16. Left; 17. After 24 hours of contact at 28°; 18. Hyperemia, edema.

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Study of Bone Marrow Changes in Vaccinal Processes

I. P. Zhdannikova
(Saratov)

At the present time, it is generally accepted that in phenomena of infection and immunity the reticulo-endothelial system (RES) is of great importance. However, the phenomena of immunity, particularly artificial immunity caused by living vaccines, have been inadequately studied by morphologists. There are a number of works pertaining to the study of the action of the living vaccines which have been used most widely (V. V. Donskov, 1944; I. A. Chalisov and A. S. Grudenkov, 1947; R. S. Kolesnik, 1951; P. A. Vershilova and I. N. Kokorin, 1954; I. N. Kokorin, 1956, 1957; Ya. L. Rapoport, 1956, 1957, and others).

As is well known, the cellular elements of the RES are concentrated chiefly in the lymph nodes, spleen, liver and bone marrow. In the literature available to us, given over to the study of the morphology of immunity, the changes in various organs have been described, but there is little information about the condition of the bone marrow.

V. I. Vitushinskiy and E. G. Tomberg (1951) described changes in the hemopoietic apparatus of guinea pigs after the action of a "slightly virulent culture of the plague microbe of the EV strain." On the study of bone marrow of femora and humeri, hyperplasia of myeloid tissue was found with marked congestion of the blood vessels, and in some animals there were foci of hemorrhages. In a number of cases an increase in the number of megakaryocytes was noted which, in the opinion of the authors, occurred because of division of them as well as the development of them from hemocytoblasts.

R. S. Kolesnik and N. D. Altareva (1957) studied the pathological

changes in various organs (including bone marrow) of animals infected with cultures of the brucella 793 strain and the vaccine BA strain in different doses. With the injection of low and medium doses of the vaccine strain the changes in the bone marrow tissue were either absent, or a slight hyperplasia was noted with an increase in the number of megakaryocytes.

Increase in the number of megakaryocytes in the bone marrow has also been noted in various acute and chronic diseases: in plague (M. B. Stanishevskaya, 1913; S. Damberg, 1926; Ye. I. Korobkova, 1956; R. S. Kolesnik and G. P. Pletnikova, 1959), cholera, typhoid, smallpox (M. B. Stanishevskaya, 1913), lingering cases of typhus (K. Ya. Krauklis, 1948), poliomyelitis (Askanazy, 1927), and lobar pneumonia (O. P. Bykova, 1944) and others.

The aim of our studies was the demonstration of the initial orientative data concerning changes in the bone marrow observed after the injection of experimental animals with avirulent strains of the plague microbe in different doses and the determination mainly of its megakaryocytic reaction.

A histological examination was made of the bone marrow of guinea pigs, on which the properties of avirulent strains of the plague microbe, 2, 3413R6 and 100R6, were studied with the aim of determining their suitability as vaccine strains.

For our investigation, animals were used which had been vaccinated with the 2 strain in doses of 3,000,000,000 and 15,000,000,000 and the strains 3413R6 and 100R6 in doses of 50, 500, 1000, 500,000,000, 1,000,000,000 and 15,000,000,000 microbes. The injection was subcutaneous.

Strain 2 received a satisfactory evaluation after testing, but it was not better than those which had been proposed previously.

Strains 3413R6 and 100R6 produced too much side effect, were inadequately stabilized, in connection with which they were not adopted for production. Changes in the body produced by these strains were similar to vaccinal changes but could not be considered such. For this reason we could expect quite intense changes in the bone marrow, approximating the changes in the infectious process.

Microscopically, we investigated the bone marrow of the sternum and ribs of 75 guinea pigs taken from different experiments.

The material was fixed in 10 percent formalin solution, decalcified in 3 percent aqueous nitric acid solution and embedded in paraffin by the usual method. From each animal two-three pieces of sternum and one-two ribs were taken, and three-four sections were prepared from each piece at different levels. The sections 3-6 microns in thickness were stained with hematoxylin-erythrosine and embedded in balsam. At the same time, a control study was made of five healthy animals, the bone marrow from which was treated in the same way as in vaccinated animals. Aside from this, smear-impressions of bone marrow were made, fixed with methyl alcohol and stained by the Giemsa and May-Grünwald methods and with hematoxylin-erothrosine.

We did not determine the changes in the cells occurring in erythro- and myelopoiesis. The use of this method of investigation made it possible to demonstrate such processes as hyperplasia of the myeloid tissue, change in the degree of blood-filling of blood vessels, hemorrhages, areas of necrosis, and determine the quantitative and qualitative changes of the megakaryocytes.

For the purpose of determining the numbers of the latter we counted

them in 32 fields in each preparation (magnification 280X) and calculated the average. In sections, the megakaryocytes were distributed unequally among the other myeloid tissue cells. In connection with this, we counted them in both the central and peripheral areas of the sections. The number of megakaryocytes in the vaccinated animals ranged from five to 24; in the controls, from one to 12 and amounted to six per field, on the average.

Table 1

Average Megakaryocyte Counts per Field in Bone Marrow of Vaccinated Animals

(1) Штамм чумного микроба	(2) Доза, микробные тела	(3) Количество животных	(4) Срок гибели животных, сутки	(5) Среднее число мегакариоцитов в поле зрения
2	3 мард.(8)	9	4—12 (забиты)(10)	9—13
2	3 »	5	25—35 »	9—12
2	15 »	9	15—25 »	8—12
2	15 »	10	30—40 »	7—10
(6) 3413P6 и 100P6	50 »	3	10—18 »	11—13
(7) То же	50 »	2	20—22 »	10—5
» »	50 »	2	16—27 (пали)(11)	1—4
» »	500 »	2	7—10 (забиты)	9—16
» »	500	1	15 »	8
» »	500 млн.(9)	1	4 »	11
» »	500 »	1	15 »	7
» »	1 мард.	5	5—10 »	11—14
» »	1 »	2	14—18 (пали)	4—7
» »	3 »	1	7 (забита)	7
» »	15 »	4	7—24 »	8—11
» »	15 »	2	30—35 »	9

1. Strains of the plague microbe; 2. Dose, microbes; 3. Number of animals; 4. Survival time of the animals, days; 5. Average megakaryocyte count per field; 6. 3413P6 and 100P6; 7. The same; 8. Billion; 9. Million; 10. Sacrifice; 11. Die.

As is seen from Table 1, the megakaryocyte count in the majority of immunized animals was much greater than in the controls; the count varied in accordance with the properties of the strains, doses and the periods elapsing after vaccination.

When the animals were immunized with the 2 strain, a gradual increase in the megakaryocyte count was observed for 8-10 days, and then until the 30th-35th day after vaccination it remained increased, and toward the end of the observation period (40th day) a slight reduction in their number was noted. No particular differences brought about by doses of 3,000,000,000 and 15,000,000,000 microbes were noted.

Simultaneously with the increase in the number of mature cells which developed, there was an increase in their breakdown, as the result of which

cells were found which were in various stages of degenerative change. The appearance of such megakaryocytes was noted at the end of 10 days after vaccination, and a number of them increased by the 25th-30th day, and only in some preparations was it unchanged. In the majority of cases, mature cells predominated at the 35th-40th day.

Increase in the megakaryocyte count occurred in parallel with hyperplasia of the entire myeloid tissue. After vaccination of the animals with the 2 strain, hyperplasia increased gradually and was moderate for as long as 40 days. In addition, there was an irregular, sometimes very much increased blood-filling of the sinuses, and in a number of cases there were small foci of hemorrhage in animals which had been inoculated with a large dose (15,000,000,000 microbes).

The degree and nature of the bone marrow changes after injecting the animals with a culture of the 100R6 and 3413R6 strains were similar to one another but were very much different from the changes described above caused by the No 2 strain, particularly when small doses were tested. Some of the animals of this group died. In the surviving animals and in the guinea pigs which were sacrificed at various periods a rapid increase was noted in the megakaryocyte count simultaneously with hyperplasia of the entire myeloid tissue, which reached its greatest degree by the fifth-seventh day. The appearance of degenerated cells was observed as early as on the fourth day, and by the 18th-20th day they were predominant in the majority of cases.

There were also considerable circulatory disorders, which was evidenced by the presence of multiple small hemorrhages and a slight inflammatory reaction.

In the animals which died histopathological changes were more intense and were close to being characteristic of plague infection.

The morphological structure of the bone marrow after the injection of a high dose of the 100R6 and 3413R6 strains was very much different from those mentioned above. It was similar to the changes noted after the testing of the No 2 strain. The difference lay only in the more pronounced hyperplasia, irregular stasis of blood and the presence of multiple small hemorrhages in the first two weeks after immunization.

The differences between morphologic changes caused by low and high doses of the 100R6 and 3413R6 strains can apparently be explained by the inhomogeneity of the cell composition of the strains. In them, together with cells which meet the requirements made on vaccine strains in their degree of virulence and immunogenic properties, some of the individuals had preserved virulence exceeding the degree of "residual virulence." Therefore, after the injection of low doses the number of vaccinating cells was inadequate for the development of a vaccinal process, and cells with increased virulence provided for the development of the histopathological changes characteristic of the virulent strains. After immunization with high doses, the total number of "vaccine" cells was adequate for rapid occurrence of an immunological reorganization. Therefore, in the testing of 100R6 and 3413R6 strains in high doses the so-called "survival phenomenon" (N. N. Ginsburg, 1947, 1960) was observed, which was provided for by the cell composition of the strain itself.

We put animals which were the carriers of extraneous microflora inoculated with the same strains into a separate group. Of 16 guinea pigs

seven were sacrificed at various periods and nine died between the 10th and 20th days after vaccination. In all cases, particularly in animals which died, degenerative and necrotic processes as well as circulatory disorders and an inflammatory reaction were more intense than in animals which were not carriers of any latent infection.

Therefore, our data confirm N. N. Ginsburg's statement that in the weakened organism or organism which is the carrier of some latent infection, when inadequately stabilized strains are used, a specific infectious process can develop. The severity of the latter, under otherwise equal conditions, depends on the "degree of attenuation" of the virulence of the pathogen used.

Therefore, the quantitative and qualitative changes in megakaryocytes found on histological examination of the bone marrow, together with other changes, can constitute one of the morphologic signs characterizing the vaccinal process.

An increased production of megakaryocytes apparently occurs frequently in infection and immunity phenomena. The removal from the blood stream of microbes injected into the body is secured by a number of nonspecific factors. Specifically, the initial formation of fibrin and accumulation of blood platelets around bacteria precedes phagocytosis of them by reticulo-endothelial cells (N. F. Gamaleya, 1951; Gostev, 1959). In connection with this, the blood coagulating system apparently plays a great part in the protective reactions of the macroorganism.

Conclusions

1. When guinea pigs are injected with avirulent strains of the plague microbe (No 2 strain) changes occur in the bone marrow which attest to the process of immunogenesis. They are expressed specifically in quantitative and qualitative changes of the megakaryocytes. The number of the latter is considerably increased. In the early periods after immunization the predominance of young cells and the presence of mitotic figures are characteristic. In the later periods, the total megakaryocyte count is also increased, but many of these are in a state of degeneration.

2. Aside from the megakaryocytic reaction a moderate hyperplasia of myeloid tissue is observed, with irregular congestion, and in various cases, small hemorrhages when a large dose of the culture is injected. Thirty-thirty-five days after vaccination these changes gradually lessen.

3. The 100R6 and 3413R6 strains, which proved to be unsuitable as vaccine strains and productive of excessive side effects, produce deep-seated changes in the bone marrow in low doses characteristic of an infectious more than a vaccinal process.

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Material on Production of Side Effects and Immunological Effectiveness when the Vaccine of the Kashintsev Biologicals Plant is Used for Vaccination and Revaccination against Brucellosis

M. G. Lokhov, G. Ya. Shkarlat, A. V. Tseluykin, N. I. Kovalev, T. V. Teryayeva and M. I. Berdnikova
(Saratov, Orenburg)

In recent years, a number of papers have been published in which the complete innocuousness and the high degree of epidemiological effectiveness of dry living brucellosis vaccine of the IEM [Institute of Experimental Medicine] of the Academy of Medical Sciences USSR have been shown (P. A. Vershilova and others, 1952; P. F. Zdrodovskiy, 1953; D. I. Frankin and A. A. Malyutina, 1955; M. G. Lokhov, 1957, 1960 and others). However, the extensive utilization of this vaccine for the immunization of the population by subcutaneous injection is complicated by the need for examining the inoculees by laboratory methods prior to giving the inoculations.

The aim of the present work was the study of the side-effect production and immunological effectiveness of the brucellosis vaccine of the Kashintsev Biologicals Plant when used percutaneously as well as the determination of the possibility of using it for mass vaccination without preliminary laboratory examination for brucellosis.

Three groups of people, different with respect to immunological states were put under systematic observation: 1) those showing a positive Burnet test; 2) those showing negative Burnet, Wright and Huddleson tests, and 3) revaccinated persons. The majority of the subjects were from 17 to 25 years old, were not permanently engaged in taking care of agricultural animals.

For the purpose of studying general and local reactions to the inoculations a regular observation was made of 392 vaccinated and 113 revaccinated persons.

The vaccination was conducted from 15 to 18 April and from 27 October to 17 November 1958; revaccination, from 13 to 19 March 1959. For the vaccination the brucellosis vaccine of the Kashintsev Biologicals Plant was used for the April 1958 vaccination, using series 693 with an expiration date of 20 August 1958; in October-November series 450 was used which was suitable for use until 10 April 1959. For the revaccination vaccine of series 1083 was prepared which had a life until 20 December 1959. The vaccine was diluted with half the dose (0.37 cc) of physiological saline solution indicated in the instructions for the use of this vaccine.

The vaccine was taken up from the ampule into a syringe and through a No 18-20 needle was applied to the skin of the arm in one drop. By this method the drops of the vaccine did not diffuse, as usually occurs when drops are applied with an eye dropper. For the first vaccination two drops were applied to two areas of skin; for the revaccination, one drop. Through each drop two longitudinal and two transverse scratches were made with a vaccination needle. The vaccine was rubbed into the skin slightly until edema appeared in the direction of the scratches. The vaccinated and revaccinated persons were observed for six-10 days.

The degree of local reaction was determined by the degree of redness,

and the degree of the infiltrate at the site of application of the vaccine 24 hours after inoculation. A local reaction with slight redness and slight edema was considered first degree; marked redness and edema, second degree; marked redness and edema and the presence of papules, vesicles with subsequent formation of small scabs along the courses of the scratches, third degree; finally, local reaction with marked redness and edema around the scratches with the formation of vesicles and turbid contents changing into solid scabs, fourth degree. In persons in whom local third and fourth degree reactions were observed the scabs fell off after three-four days. The side-effect production of the vaccine in the inoculees is shown in Table 1.

Table 1

Side-Effect Production in Those Inoculated with the Vaccine of the Kashintsev Biologicals Plant

(1) Контингент привитых	(2) Число привитых	(3) Из них с положительными прививочными реакциями	(4) Из них с: (5) температурной реакцией свыше 37,2°	(6) недомоганием, головными болями	(7) Число привитых со степенью местной реакции: I	II	III	IV
(8) Положительно реагирующие по Бюрне	98	98	3	4	52	27	19	
					53,1	27,6	19,3	
(9) Отрицательно реагирующие по иммунологическим реакциям на бруцеллез	294	294	3	—	191	90	13	
					64,9	30,8	4,3	
(10) Ревакцинированные	113	113	9	12	30	23	29	31
					26,6	20,4	25,7	27,3

1. group of inoculees; 2. number of inoculees; 3. of these the number with positive inoculation reactions; 4. of these, the number with:; 5. temperature reaction above 37.2°; 6. malaise and headaches; 7. number of inoculees with the degree of the local reaction (the numerator is an absolute figure; the denominator, in %); 8. those showing a positive Burnet test; 9. those showing negative immunological tests for brucellosis; 10. revaccinated.

From Table 1 it is seen that inoculation reactions of various degrees of intensity occurred in all inoculees. No loss of the ability to work was seen in the vaccinated or revaccinated persons.

Malaise and headache were noted in four inoculees (4.1 percent) of the group of those reacting positively in the Burnet test and in 12 (10.6 percent, of revaccinated persons. A temperature rise above 37.2° was noted in three inoculees (3.1 percent) of the group of those who reacted positively in the Burnet test, in three (1.1 percent) of those who reacted negatively in the Burnet, Wright and Huddleson tests, and in nine (8 percent) of revaccinated persons. In all the inoculees the elevated temperature remained no more

than two-three days. In addition, of the group of revaccinated persons five had a chill and one had an enlargement of the lymph nodes to the size of a walnut in the axilla of the opposite arm the night after the inoculation. Lymphadenitis was preceded by the sensation of some pain in the axilla and elevation of the temperature to 38.1°. In the next few days the lymphadenitis underwent complete resolution, and after 10 days all its signs disappeared.

The results of our observations showed that in those inoculated percutaneously with dry living brucellosis vaccine of the Kashintsev Biologicals Plant the general reactive signs were poorly expressed.

In the inoculees from the group of those who showed a negative reaction in the immunological Burnet, Wright and Huddleson tests the local reactions were slight. In this group of subjects local reactions of third degree were noted only in 4.3 percent of the cases, whereas in persons who showed a positive reaction by the Burnet test prior to the inoculation, in 19.3 percent; in revaccinated persons, 25 percent; fourth degree local reactions were seen only in the revaccinated persons (27.3 percent). The general and local reactions to the injection of the vaccine among the revaccinated persons were most pronounced in those who showed a positive reaction in the immunological tests before revaccination, particularly those who were positive for all three; then came those who were positive for two of them (Table 2).

Table 2

Side-Effect Production in Persons (Inoculated a Year Before for the First Time) Revaccinated Percutaneously with the Brucellosis Vaccine of the Kashintev Biologicals Plant

До ревакцинации положительно реагировали на реакции (1)	Число привитых (2)	Из них с (3): температурной реакцией (4)	Из них с (3): недомоганием и головной болью (5)	Число привитых со степенью местной реакции (6): I	II	III	IV	Всего (7)
(8) Райта, Хеддльсона, Бюрне	62	8	10	11	9	18	24	62
(9) Райта, Хеддльсона	23	—	—	11	6	5	1	23
(8) Райта, Хеддльсона, Бюрне	1	—	—	—	1	—	—	1
(10) Бюрне	15	—	1	4	3	4	4	15
(11) Райта	—	—	—	—	—	—	—	—
(12) Хеддльсона	3	—	—	1	1	1	—	3
(10) Бюрне	9	1	1	3	3	1	2	9
Всего . . . (Total)	113	9	12	30	23	29	31	113

1. those who showed a positive reaction in the following tests before revaccination; 2. number of inoculees; 3, 4, 5. same as Table 1. 6. number of inoculees with the degree of local reaction; 7. total; 8. Wright, Huddleson and Burnet; 9. Wright, Huddleson; 10. Burnet; 11. Wright; 12. Huddleson.

The immunological effectiveness of the vaccine was checked on other population groups which were under the same conditions of possible contact

with animals: 1) among persons who showed positive reactions by the Burnet test before the inoculations, and 2) among persons who reacted negatively by the Wright, Huddleson and Burnet tests before the inoculations. In the first group of inoculees, that is, those who reacted positively to brucellin, the study of the immunological state after inoculation was conducted dynamically in 113 persons after 10 days, one, three, six months and after a year (Table 3).

Table 3

Immunological State of the Organism in Those Inoculated from the Group of Those Reacting Positively by the Burnet Test before Vaccination

(1) Время, истекшее после вакцинации	(2) Число привитых	(3) Из них положительно реагировали после вакцинации по реакциям						
		(4) Райта, Хеддльсона, Бюрне	(5) Райта, Хеддльсона	(6) Райта, Бюрне	(7) Хеддльсона, Бюрне	(8) только по реакции		
						(9) Райта	(10) Хеддльсона, Бюрне	(11) Бюрне
10 дней (12)	12	2	—	1	—	—	—	9
1 месяц (13)	5	3	—	—	1	—	—	1
3 месяца (13)	30	22	—	—	5	—	—	3
6 месяцев (13)	28	14	—	—	9	—	—	5
1 год (14)	38	25	—	—	8	—	—	5
(15) Всего . . .	113	66	—	1	23	—	—	23

1. time after vaccination; 2. number of inoculees; 3. of these the number who reacted positively after vaccination by the following tests:; 4. Wright, Huddleson, Burnet; 5. Wright, Huddleson; 6. Wright, Burnet; 7. Huddleson, Burnet; 8. only in the following tests:; 9. Wright; 10. Huddleson, Burnet; 11. Burnet; 12. days; 13. month(s); 14. year; 15. total.

From Table 3 it is seen that the Burnet test is positive in all inoculees in all cases. The serological tests (Huddleson and Wright) are positive in the inoculees 10 days after inoculation in three out of 12; after a month, in four out of five; after three months, in 27 out of 30; after six months, 23 out of 28; after a year, 33 out of 38. If it is permissible to derive the percentages from such comparatively low absolute numbers, we obtain, respectively, 25, 80, 90, 82.2 and 86.8 percent. These data show that specific antibodies in the inoculees appear beginning with the 10th day after inoculation; by the third-sixth month they are present in the great majority of inoculees, and are maintained for a year in 87 percent of the cases. The agglutinin test titer in the inoculees increases considerably as early as the third month after vaccination (Table 4).

Thus, the agglutinin titer of the Wright test of from 1:100 to 1:400 was found at the third month in 72.4 percent of the inoculees; after six months, in 57.1 percent of the inoculees and at one year, in 76 percent of the inoculees.

Table 4

Agglutinin Titers of Wright and Huddleson Tests in Those Vaccinated from the Number of those Reacting Positively by the Burnet Test before the Inoculation

Время, истекшее после вакцинации (1)	Число обследованных (2)	Из них с положительной реакцией Райта (3)	Из них реакция положительна в титре (4) 1:80	1:100	1:200	1:400	1:800	Число обследованных с положительной реакцией Хеддльсона (5)	Из них со степенью реакции (6) сомнительной (7)	положительной (8)	резко положительной (9)
10 дней (10)	12	3	2	—	1	—	—	2	1	1	—
1 месяц (11)	5	3	1	—	1	1	—	4	1	1	2
3 месяца	30	22	6	7	5	3	1	27	4	6	17
6 месяцев	28	14	6	6	1	1	—	23	1	12	10
1 год (12)	38	25	6	7	11	1	—	33	5	18	10
Всего (13)	113	67	21	20	19	6	1	89	12	38	39

1. time after vaccination; 2. number examined; 3. of these, the number who had a positive Wright test; 4. of these, the test was positive in a titer of:; 5. the number of those examined with a positive Huddleson test; 6. of these, the number with the following degree of reaction; 7. doubtful; 8. positive; 9. markedly positive; 10. days; 11. month(s); 12. year; 13. total.

In the inoculees the Huddleson test was positive and markedly positive three months after vaccination in 85.2 percent of the cases; six months after, in 95.6 percent of the cases; a year after vaccination, in 85.8 percent of the cases.

After vaccination the intensity of the Burnet test increased somewhat in the inoculees (Table 5).

Table 5

The Intensity of the Burnet Test in Inoculees of Those Reacting Positively in this Test before Vaccination

Время, истекшее после вакцинации (1)	Число обследованных (2)	Из них с величиной папулы, см (3) 1–3: до вакцинации (4)	1–3: после вакцинации (5)	3–5: до вакцинации (4)	3–5: после вакцинации (5)	5–8: до вакцинации (4)	5–8: после вакцинации (5)	8–10: до вакцинации (4)	8–10: после вакцинации (5)
10 дней (6)	12	7	6	4	5	1	1	—	—
1 месяц (7)	5	2	2	3	2	—	1	—	—
3 месяца	30	8	4	17	20	5	6	—	—
6 месяцев	28	12	8	14	12	2	8	—	—
1 год (8)	38	18	13	17	21	3	4	—	—
Всего (9)	113	47	33	55	60	11	20	—	—

1. time after vaccination; 2. number examined; 3. of these, the number with a papule of the following size, cm; 4. before vaccination; 5. after vaccination; 6. days; 7. month(s); 8. year; 9. total.

In the second group of persons, who had shown a negative reaction in the immunological tests before the inoculations (Wright, Huddleson and Burnet), the immunological effectiveness was checked in 308 inoculees.

The immunological effectiveness of the inoculations against brucellosis was also checked dynamically: after 10 and 20 days, one, three, six months and after a year (Table 6).

Table 6

The Immunological State of Inoculees of the Group Reacting Negatively for Brucellosis before the Inoculations

Время, истекшее после вакцинации (1)	Число привитых (2)	Из них (3) с положительными прививочными реакциями	Из них по реакциям (4)						
			Райта, Хеддльсона, Бюрне (5)	Райта, Хеддльсона (6)	Райта, Бюрне (7)	Хеддльсона, Бюрне (8)	Райта (9)	Хеддльсона (10)	Бюрне (11)
10 дней (12)	34	16	2	4	1	—	2	—	7
20 „	16	12	11	—	—	—	—	1	—
1 месяц (13)	60	60	21	31	1	2	3	1	1
3 месяца	80	77	44	25	—	4	—	3	1
6 месяцев	35	29	8	8	2	6	—	4	1
1 год (14)	83	78	36	23	1	—	2	13	3
Всего (15)	308	272	122	91	5	12	7	22	13

1. time after vaccination; 2. number of inoculees; 3. of these, the number with positive inoculation reactions; 4. of these, the number positive in the following tests:; 5. Wright, Huddleson and Burnet; 6. Wright and Huddleson; 7. Wright and Burnet; 8. Huddleson and Burnet; 9. Wright; 10. Huddleson; 11. Burnet; 12. days; 13. month(s); 14. year; 15. total.

As is seen from Table 6, in this group of inoculees immunological reactions for brucellosis were positive 10 days after vaccination in 47 percent of the cases; 20 days after, in 35 percent; one month after, in 100 percent; three months, in 96.3 percent; six months after, in 82.9 percent; one year after, in 94 percent of the cases. Of the 308 inoculees 272 showed a positive reaction in the immunological tests for brucellosis.

It is interesting to note that immunological rearrangement as the result of vaccination is demonstrated in a large number of cases by all three immunobiological tests (in 112 out of 308 inoculees); next in frequency, by two serological tests (in 91 inoculees). The presence of an immunological reorganization was found by the Wright test in 225 inoculees; by the Huddleson test in 247; by the Burnet test, in 152 inoculees.

In evaluating the immunological state after vaccination the highest figures are shown by the Huddleson and Wright serological tests (positive in, respectively, 80.2 and 73.1 percent of the cases). The allergic Burnet test gives positive results in only 50.6 percent of the inoculees.

As in the previous experiment, the agglutinin titers of the Wright test

and the degree of the Huddleson test reached the highest figures in the third month after vaccination. Thus, a month after the inoculations the Wright test titer of 1:100 to 1:400 was found in 76.8 percent of the cases. Three months after the vaccination it ranged from 1:800 to 1:1600 in five inoculees; from 1:100 to 1:1600 in 78.3 percent; after six months, from 1:100 to 1:400 in 66.7 percent; after a year, in 66.1 percent of the cases.

Positive and markedly positive Huddleson tests in the inoculees were found one month after vaccination in 81.8 percent of the cases; three months after vaccination in 92.1 percent; six months after vaccination in 90 percent; one year after vaccination, in 76.3 percent of the cases.

The Burnet test with erythema and edema of 1x3 centimeters was found in 48.7 percent; of 3x5 centimeters in 49.2 percent; of 5x8 centimeters, in 2.1 percent of the cases.

Conclusions

1. The results of our observations showed that in those inoculated percutaneously with dry living brucellosis vaccine of the Kashintsev Biologicals Plant local and general side effects were slight. They were most pronounced in persons (revaccinated persons) who had reacted positively before the inoculations in three immunological tests (Wright, Huddleson and Burnet) and in persons with a positive Burnet test in combination with a single positive serological test. However, in these persons the side effects were not so great as to serve as a contraindication to the inoculations.

2. Immunological reorganization as the result of vaccination and revaccination is, for the most part, demonstrated by all three tests; then, by two serological tests (in those reacting negatively before the inoculations) and by the Huddleson and Burnet tests (in revaccinated persons). In persons who had shown a negative reaction before the inoculations positive results by the Huddleson test were observed in 80.2 percent; by the Wright test, in 73.1 percent; by the Burnet allergic test, in 50.6 percent; in persons who had reacted positively by the Burnet test before the inoculations, this test remained positive in 100 percent of the cases for a year.

3. Serological tests (Wright and Huddleson) become positive beginning with the 10th day after inoculation; by the third-sixth month they are positive in the great majority of inoculees and remain positive for a year in a high percentage of cases (87 percent). The Wright agglutinin test titer and the degree of the Huddleson test showed the highest indices in the third month.

4. The vaccine of the Kashintsev Biologicals Plant for percutaneous use can be recommended for mass vaccination of the population against brucellosis without a preliminary check of the immunological state of the inoculees. However, in the population group which is subject to frequent revaccination (workers in meat-packing houses, persons taking care of animals on farms unfavorable for brucellosis), it is desirable to check the immunological state by the Burnet test alone before the inoculation.

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Vaccination of Guinea Pigs and Rabbits with Dry Living Brucellosis Vaccine by Means of the N. R. Ivanov Apparatus

N. R. Ivanov, M. G. Lokhov and N. B. Igonina
(Saratov)

The creation of a high degree of immunity to brucellosis depends largely on the methods with which the vaccine is introduced into the body. The problem of the immunological effectiveness and strength of immunity depending on the method of administration of vaccine strains of living brucellas (subcutaneously, percutaneously, intradermally, intranasally) has been studied by many authors (P. A. Vershilova, 1947, 1950, 1958; N. F. Zen'kova, 1956; V. G. Pilipenko, A. M. Polyakova and T. A. Shchekina, 1956; Ye. A. Gubina, 1957; K. N. Shlygina, 1958; N. K. Vereninova and others, 1958).

In 1957, at the interinstitute conference on problems of natural focalization and epidemiology of the particularly dangerous infectious diseases ("Mikrob" Institute), N. R. Ivanov suggested a new method of vaccination, replacing the intradermal method, with a special instrument which he had designed.

The Ivanov instrument consists of a metal cylinder 6 centimeters long, in which there is a movable plunger with a spring (Figs 1, 2 and 3). Nine ordinary sewing needles, the ends of which project 2 millimeters from the plunger, are set in the base of the plunger. The technique of vaccination with this instrument is simple: the inoculation site is treated as usual, and a drop of vaccine is applied to the skin with an eye dropper or syringe needle, and by means of this instrument needle punctures are made in the skin in a manner similar to the puncture with the Francke spring lancet. Intradermal injection of the vaccine is carried out with this instrument in a manner similar to tattooing.

In experiments on guinea pigs and rabbits we studied the immunological reorganization after the administration of brucellosis vaccine with the N. R. Ivanov instrument.

Vaccination was carried out with living dry brucellosis vaccine prepared from the B. abortus 19-BA strain for percutaneous use. The vaccine was diluted as follows: 0.1 cc of physiological saline solution was added to the ampule containing 10 doses of the vaccine and a dense suspension was obtained. A drop of suspension was applied to the upper part of the leg on the inside in guinea pigs; near the base of the ear, in rabbits. At the site of the applied drop the skin was punctured with the needles of the instrument. All manipulations were carried out under strictly sterile conditions.

In this method of vaccination no general reaction was observed in the animals, as a rule. The local reaction was expressed as a very slight hyperemia of the skin at the puncture site.

Experiments on Guinea Pigs

The immunological reorganization of guinea pigs vaccinated once in one place with the Ivanov instrument was studied by means of a determination of the agglutinin accumulation in the serum (by performing the Wright and Huddleson tests). In this experiment 14 guinea pigs were used. Studies were made on the 12th, 19th, 38th and 69th days and 245 days after vaccination. Prior to vaccination all guinea pigs had shown a negative reaction in the immunological tests for brucellosis (Table 1).

Table 1

Serological Tests in Animals after Intradermal Vaccination with the N. R. Ivanov Instrument

Дни исследования после вакцинации (1)	Число животных (2)	Из них реагировали по реакции Райта (3)		Число животных, давших положительную реакцию Райта в титре (4)						Число животных, у которых реакция Хеддльсона была (7)			
		положительно (5)	отрицательно (6)	1:50	1:100	1:200	1:400	1:800	1:1600	рез. положительная (8)	положительная (9)	слабо положительная или сомнительная (10)	отрицательная (11)
(12) Опыт на свинках													
12-й	14	14	—	1	3	5	5	—	—	14	—	—	—
19-й	14	14	—	—	1	7	5	1	—	14	—	—	—
38-й	12	12	—	—	1	4	5	2	—	12	—	—	—
69-й	2	2	—	—	2	—	—	—	—	2	—	—	—
245-й	4	3	1	1	2	—	—	—	—	2	—	1	1
(13) Опыт на кроликах													
27-й	12	12	—	2	—	4	4	2	—	12	—	—	—
60-й	8	8	—	2	2	2	1	1	—	7	1	—	—
180-й	4	4	1	1	2	—	—	—	—	3	—	1	—

1. day of examination following vaccination; 2. number of animals; 3. of these, the number which reacted positively in the Wright test; 4. the number of animals showing a positive Wright test in a titer of; 5. positive; 6. negative; 7. number of animals in which the Huddlesontest was:; 8. markedly positive; 9. positive; 10. slightly positive or doubtful; 11. negative; 12. experiment on guinea pigs; 13. experiment on rabbits.

As is seen from Table 1, agglutinins were found in the sera of guinea pigs 12 days after vaccination in all cases, whereby in 10 out of the 14 guinea pigs the Wright test was obtained in a titer of 1:200 to 1:400. From the 19th to the 38th day the agglutinin titers increased, and on the 69th day a reduction of them was noted. Two hundred forty-five days after vaccination the Wright test was positive in three of the animals investigated. Similar results were obtained in the Huddleson test: up to the 69th day after vaccination it was markedly positive in all animals: after 245 days, it was markedly positive in two out of four guines pigs, slightly positive in one and negative in one.

For the purpose of solving the problem of the acceptance of the vaccine strain by the guinea pig organs, 20 animals weighing from 300 to 350 grams were used in the experiment. For the purpose of obtaining the cultures from the organs of the animals we began to sacrifice them on the second day after injection of the vaccine (Table 2).

Table 2

Acceptance of Vaccine Strain in Guinea Pigs

(1) День вскрытия свинок после вакцинации	(2) Число свинок	(3) Число свинок, от которых культура бруцелл высеяна из						
		(4) лимфатических узлов	(5) печени	(6) селезенки	(7) крови	(8) костного мозга	(9) мочи	(10) тестикул
1—5-й	4	1	1	1	—	--	1	1
6—27-й	10	10	4	2	1	3	2	2
28—69-й	6	—	—	--	—	--	--	--

1. day of dissecting guinea pigs after vaccination; 2. number of guinea pigs; 3. number of guinea pigs from which a culture of brucellas was plated out from the; 4. lymph nodes; 5. liver; 6. spleen; 7. blood; 8. bone marrow; 9. urine; 10. testicles.

From Table 2 it is seen that the 19-BA strain developed in the bodies of guinea pigs for 27 days by the vaccination method used. Brucellas of the vaccine strain taken from the organs of animals began to be isolated as early as the first day. Beginning with the sixth day, in all animals they were isolated from lymph nodes; in some of them, from the internal organs; in one case, from the blood also. Beginning with the 28th day after vaccination brucellas could not be isolated at all from the internal organs.

The strength of the immunity which developed was studied in 27 guinea pigs. All the animals were divided into three groups. The animals of the first group (15 guinea pigs) were infected subcutaneously 35 days after vaccination with two infecting doses of a virulent strain of B. melitensis 487. Guinea pigs of the second group were vaccinated, like the animals of the first group, but were not infected (first control). The animals of the third group (nine guinea pigs) were infected but were not vaccinated (second control). The killed animals of groups one and three were dissected 20, 26 and 33 days after infection; animals of the first and second groups, 56, 62 and

69 days after vaccination (Table 3).

Table 3

Strength of Immunity in Guinea Pigs

Группа животных (1)	Число животных (2)	Число свинок, у которых не наблюдалось инфекции по (3)	Число свинок с генерализованной инфекцией (4)
(5) Вакцинированные и зараженные	15	7	8
(6) Вакцинированные, незараженные (1-й контроль)	3	3	—
(7) Невакцинированные, зараженные (2-й контроль)	9	—	9

1. group of animals; 2. number of animals; 3. number of guinea pigs in which no dissemination of the infection was noted; 4. number of guinea pigs with dissemination of the infection; 5. vaccinated and infected; 6. vaccinated, not infected (first control); 7. unvaccinated, infected (second control).

As is seen from Table 3, among guines pigs infected on the 35th day after vaccination seven out of 15 were found to be uninfected. In eight guinea pigs the culture of brucellas of the melitensis type was isolated as single colonies but from all organs. In nine control (unvaccinated) guinea pigs the infectious process was disseminated in 100 percent of the cases. The culture of brucellas in this group of animals was plated out in abundance from all organs.

The organs of guinea pigs which had been vaccinated but were not infected were found to be uninfected.

Experiments on Rabbits

The immunological reorganization in rabbits in reponse to the single injection of living brucellosis vaccine into one place by means of the Ivanov instrument was studied by means of Wright tests, Huddleson tests and the complement-fixation test (by the classical method and by methods modified by Grigor'yev-Rapoport and Vaynshteyn-Reznikova). The Wright and Huddleson tests were performed after 27 days, two and five months; the complement-fixation test, one month after vaccination.

The Wright and Huddleson tests were positive for five months in 100 percent of the cases (see Table 1). The agglutinin test titers were high: 27 days and two months after vaccination, from 1:100 to 1:1600; after five months the titers dropped to 1:200.

After 27 days the Huddleson test was markedly positive in all rabbits. By the fifth month the intensity of this reaction decreased.

Five months after vaccination two rabbits were revaccinated. As a

result, after two months an increase in the agglutinin test titer was found in one rabbit from 1:100 to 1:400; in another, from 1:50 to 1:800. In two rabbits vaccinated seven months before the test was performed (the control), the test titer, conversely, dropped from 1:200 to 1:100.

On examination of 12 rabbits by the complement-fixation test positive results were obtained in all animals.

For the purpose of studying the strength of immunity in the rabbits three rabbits were used in the experiment. One and a half months after the vaccination two rabbits were infected subcutaneously with a culture of brucellas of the melitensis type, using 10 infecting doses for guinea pigs (10⁶ microbes). Simultaneously, one unvaccinated rabbit (control) was infected with the same doses. After 25 days, the rabbits were killed and dissected.

On liver agar plates made from lymph nodes, spleen, liver, blood, bone marrow, urine and testicles, no brucellas were found in two vaccinated rabbits. In the control rabbit the solid growth of brucellas of the B. melitensis type was found in the urine.

Conclusions

1. The 19-BA brucellosis vaccine strain, when injected once into one place in guinea pigs and rabbits by means of the Ivanov instrument, does not cause either general or local reaction.

2. In the animals agglutinins were formed in the first few days and were demonstrated for a long time. The agglutinin titers in revaccinated rabbits increased.

3. The 19-BA vaccine strain injected into guinea pigs by means of the Ivanov instrument, multiplies in their organisms, producing a vaccinal process. The guinea pig organisms eliminated the vaccine brucellas 27 days after injection.

4. For the purpose of creation of immunity of high strength in the animals the dose of vaccine, when used once and injected into a single place, was apparently inadequate. Either the number of needles in the Ivanov apparatus have to be increased or the vaccine has to be injected in two or three places.

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Quantitative Analysis of Antigens of Anthrax Bacilli by the Method of Specific Diffusion in Agar

V. V. Akimovich, N. S. Goncharova, L. ". Samoylova and I. N. Zaytseva
(Saratov)

The systematic study of antigens of the anthrax pathogen is connected with the investigations of Tomcsik, Ivanovics and others. In 1932, Tomcsik and Szongott reported on their isolation of a polysaccharide from a culture of anthrax bacilli, the chemical composition and serological activity of which was the same, regardless of whether it was isolated from virulent or attenuated anthrax pathogens. A detailed chemical analysis of the anthrax polysaccharide was made by Ivanovics (1940), Smith and Zwartow (1956). From various strains of the anthrax bacillus Tomcsik and Szongott (1932, 1933) and later Tomcsik and Bodon (1934) isolated the capsular substance of this microbe. In the organs and blood of animals infected with anthrax bacilli Tomcsik and Bodon (1934, 1935) found the capsular substance in large quantities.

In 1937, Ivanovics and Erdös obtained the capsular substance of the anthrax bacilli in an almost purified state. In their subsequent studies Ivanovics and Bruckner (1937, 1937a, 1938) established the fact that the capsular haptene of this microbe is a high molecular polypeptide made up of alpha-glutamic acid esters.

Aside from polysaccharide (the C substance) and the capsular polypeptide of glutamic acid (the P substance), Tomcsik and Szongott (1933), Tomcsik and Bodon (1934) found a nucleoprotein which was very active serologically in the antigenic complex of anthrax bacilli. Aside from these antigens, Cromartie, Bloom and Watson (1947) determined that the anthrax pathogen has the power of producing a substance which injures tissue in the animal organism. By electrophoretic and chemical means, Watson, Cromartie and others (1947) isolated a fraction with toxic properties from the edema fluid of rabbits infected with anthrax bacilli. The toxic effect on guinea pigs and white

mice, as shown by Smith and Keppie (1954) is also found in blood plasma of guinea pigs which have died of anthrax. According to the data of Smith, Keppie and Stanley (1953), the anthrax toxin is not connected with capsular polyglutamic acid. Smith, Tempest and others (1956) came to the same conclusion. According to the data of these authors, the toxin of anthrax microbes consists of two different components, which form the specific toxic mixture. The toxin present in the blood plasma of animals infected with anthrax bacilli cannot be obtained in cultures of the pathogens grown out on synthetic nutrient media (Smith, Keppie and Stanley, 1953). Attempts by Smith, Tempest and others (1956) to detect the toxin on cultivation of anthrax bacilli under conditions as identical as possible, created in the body of the infected animal, were also unsuccessful. However, in contrast to data presented by the authors, Evans and Shoesmith (1956) found a toxin in filtrates of anthrax cultures grown out on a well aerated special medium which produced edema and necrosis of the skin after intradermal injection in rabbits. The chemical nature of the toxin was not determined; it is specifically neutralized by anti-anthrax serum (Smith and Keppie, 1954; Smith, Keppie and Stanley, 1953).

Under certain conditions anthrax bacilli produce an immunizing substance, which is not connected with any of the antigens known for this microbe (Smith, Keppie, Stanley, 1953).

The problem of the immunizing antigen of anthrax bacilli has attracted particularly concentrated attention in recent years. Gruber and A. Staub (1946) showed that guinea pigs can be immunized with edem fluid of guinea pigs which have died of anthrax. According to the data of Cromartie, Watson and others (1947), aqueous extracts of edematous tissues of rabbits infected with anthrax bacilli also possess immunizing power. Watson, Cromartie and others (1947) called the immunizing substance of the anthrax bacilli a protective antigen. According to the studies of Keppie, Smith and Harris-Smith (1953), this antigen is also present in blood plasma and in the fluid of the body cavities of guinea pigs infected with anthrax bacilli. In 1946, Gladstone succeeded in obtaining an immunizing antigen by cultivation of anthrax bacteria on plasma and serum of animals. These observations were corroborated by Heckly and Golwasser (1949). Boor (1955) found the protective antigen in filtrates of anthrax cultures grown out on a special medium containing serum albumin. Wright, Hedberg and Hein (1954) proved the capacity of anthrax bacilli for producing the protective antigen on media of a defined chemical composition. The attempt to find a protective antigen in extracts of anthrax bacilli grown out on synthetic nutrient media proved unsuccessful (Gladstone, 1946); this also occurred with the attempt to detect the protective antigen in extracts of anthrax bacilli cultured on the bodies of animals (Keppie, Smith, Harris-Smith, 1953; Smith, Zwartow, Harris-Smith, 1956). In Gladstone's opinion (1946), the immunizing anthrax substance is not preexistent in the microbe but is formed on the surface of it during the course of its development on suitable nutrient media. Smith and Gallop (1956) came to the same conclusion. A certain evaluation of the chemical structure of the anthrax protective antigen made it possible to substantiate the use of chemical and physical methods of investigation. According to data of Watson, Cromartie and others (1947), this antigen consists of α-, β- and γ-globulins.

Grabar and Staub (1946) showed that the immunizing substance contained in the edema fluid of the animals infected with the anthrax bacillus is a glycoprotein. According to the data of Smith and Gallop (1956), the purified protective antigen of the anthrax bacillus is a lipoprotein containing a small quantity of carbohydrate. Boor and Tresselt (1955) obtained the protective antigen in the form of the α-globulin product.

For the purpose of studying the quantitative composition of anthrax antigens we made use of the method of specific diffusion in agar proposed by Ouchterlony (1948). This method has some advantages over the other methods of diffusion in gell (Oudin, 1957; Oakley and Fulthorpe, 1953); specifically, it is very simple and makes it possible to investigate various strains and species of bacteria simultaneously.

For the diffusion in agar test 1 percent clarified agar containing 0.85 percent salt, merthiolate (1:10,000) with a neutral reaction were used. The fused agar was poured out into Petri dishes in a 3-millimeter layer. In the agar which had gelled wells 6 or 8 millimeters in diameter were cut out with a corkscrew punch; a drop of fused agar was applied to the bottom of the well so as to prevent penetration of the fluid being investigated under the layer of agar. The spatial interrelationship of wells containing antigen and immune serum was changed in accordance with the specific conditions of the experiment; the distance between them was kept constant, 10 millimeters. In all cases 18-20-hour agar cultures were used; the bacterial concentration was determined by the optical standard after careful shaking of the bacterial suspension. The suspension of microbes in physiological saline solution was introduced into the well in a volume of 0.05 cc; the sera were also used in this volume. The cultures were incubated in a drier at 37° for six days. The precipitin test was recorded daily for 10 days (for four days the cultures were kept at room temperature or in a refrigerator, which in a number of cases contributed to better contrast of the various precipitation zones).

In all, 15 strains of anthrax bacilli were studied; of these 12 were typical and virulent strains and three were vaccinal strains (Tsenskovskiy's first and second vaccines and the STI vaccine). The virulent anthrax strains were obtained from the museum of the "Mikrob" Institute; the bacteria of the vaccine strains were isolated from the corresponding vaccines. For the precipitin test horse precipitating anthrax sera and sera obtained by means of hyperimmunization of rabbits with cultures isolated from Tsenskovskiy's first and second vaccines were used. For the purpose of excluding random artefacts the experiments were performed in three-five variants.

First of all, we attempted to determine the nature, number and sequence of appearance of precipitation bands. In this experiment 3,000,000,000 microbes were put into each of the wells for the antigens; whole horse and rabbit immune sera were used. The wells containing the microbe suspension and the serum were arranged in parallel rows, whereby the wells containing serum occupied a central position.

As the experiment showed, the number of precipitation zones in different strains of anthrax bacilli ranged from two to four. The first precipitation band appeared in all of the strains investigated after 24-30 hours; it was broad, compact, came close to or directly touched the well containing serum; its boundaries were not always distinctly outlined. The

second band could be found after 48-72 hours. This precipitation band was comparatively fine, compact with clear-cut boundaries and was located nearer to the well containing antigen. The finer and less distinct third line appeared after 96 hours and was located between the first and second lines. A fine and distinct fourth precipitation line was found four-five days after the beginning of the experiment and was located between the second and third bands. The number of precipitation zones in different strains of anthrax bacilli is shown in Table 1.

Table 1

The Results of the Diffusion in Gel Test with Various Strains of Anthrax Bacilli and Precipitating Sera

Штамм	Номера и число линий преципитата в реакции преципитации с сыворотками			
	иммунной		нормальной	
	лошадиной	кроличьей	лошадиной	кроличьей
1	1, 2, 3, 4	1, 2, 3, 4	0	0
2	1, 2, 3	1, 2, 3, 4	0	0
3	1, 2	1, 2, 3	0	0
4	1, 2, 3	1, 2, 3	0	0
5	1, 2	1, 2, 3	0	0
9	1, 2	1, 2, 3	0	0
10	1, 2, 3	1, 2, 3, 4	0	0
11	1, 2, 3	1, 2, 3	0	0
12	1, 2, 3	1, 2, 3	0	0
13	1, 2, 3, 4	1, 2, 3, 4	0	0
14	1, 2	1, 2	0	0
15	1, 2	1, 2, 3	0	0
Вакцина Ценковского 1	1*, 2	1*, 2, 3	0	0
Вакцина Ценковского 2	1, 2, 3	1, 2, 3	0	0
Вакцина СТИ	1, 2, 3	1, 2, 3	0	0

Note. 0--diffusion test negative; *--not found constantly.

Key: 1. strain; 2. Nos and number of precipitatation bands in the diffusion in gel test with the following sera:; 3. immune; 4. normal; 5. horse; 6. rabbit; 7. Tsenskovskiy's first vaccine; 8. STI vaccine.

In the majority of strains of virulent anthrax bacilli three precipitation bands were found with the horse precipitating sera, which correspond to the three different partial antigens. An exception is constituted by strains 1 and 13, which form a fourth precipitation band, and strains 3, 5, 14, and 15, which have only two antigens which cause precipitation in gel. Therefore, the serological differences between the strains deal only with the antigenic components, from which the third and fourth precipitation bands are created. Conversely, the antigens causing the first and second bands are regularly encountered in all of the strains of anthrax bacilli studied. The antigenic structure of the bacilli vaccine strains is not much different from that of the virulent bacilli. It can be noted only that bacteria of

Tsenskovskiy's first vaccine form the first precipitation band with a certain degree of inconstancy.

When rabbit immune serum is used the number of separate antigens found for the anthrax bacilli increases; however, this phenomenon was not observed in all strains. The number of partial antigens in this experiment also did not exceed four, although the number of strains with this number of antigens increased somewhat. It is very likely that the discrepancy between the results of experiments with horse and rabbit immune sera is conditioned, on the one hand, by the relatively low content of antigens in the microbes of some strains inducing the formation of the third and fourth precipitation zones and, on the other hand, this phenomenon apparently depends on the inadequate quantity of antibodies to these antigens in the horse serum.

With respect to the time of appearance of the precipitation bands and their location with respect to the wells containing serum and antigen, in all probability it is possible to draw a conclusion concerning the size of the antigen molecule and its territorial distribution in the bacterial cell. Apparently, the antigen which forms the first precipitating zone with its corresponding antibody has a molecule of comparatively small size and is located on the surface of the bacterial cell and is very loosely connected with it, which is responsible for the early appearance of this zone and its attraction to the well containing serum. As far as antigens which induce the formation of the other precipitation bands are concerned, they, as might be supposed, are connected with the cell cytoplasm and are released as the cells are destroyed. The studies of Crumpton and Davies (1956), who determined the fact that the antigens released by the bacteria more slowly induced the formation of precipitation bands located nearer to the well containing the antigen can serve as confirmation of what has been stated.

The number of precipitation bands in various experiments with the same strains of anthrax bacilli and the same immune serum was not always the same. This discrepancy in the results of unitypical experiments affected the third and fourth precipitation bands only; they could be absent in one experiment and present in the next. Such a phenomenon is apparently conditioned by several rather than a single cause. First of all, even in parallel unitypical experiments it is difficult to create absolutely identical conditions for the precipitation reaction (G. N. Chistovich, 1955). A certain variation in the number of precipitation bands might depend on the different antigenic compositions of various cells in different populations of the same strain.

With the aim of substantiating this idea a culture of anthrax bacilli was seeded on an agar plate so as to obtain well isolated colonies. Each of the 10-20 colonies of approximately the same size was suspended in 0.05 cc of physiological saline solution contained in wells on the agar plates.

It was found that in some of the strains of anthrax bacilli (11) the bacteria of each colony possessed three antigens; conversely, in other strains (10) bacteria of different colonies gave rise to the appearance of one, two or three precipitation bands. In an analysis of the antigenic composition of the various cells made in this way particularly interesting data were obtained from the study of Tsenskovskiy's vaccine strain. As has already been mentioned, this strain in various experiments at times induced

and at times failed to induce the appearance of the first precipitation band. It was found that at the time of the first seeding the antigen responsible for the first precipitation band was found in only 20 percent of the colonies; at the time of the second seeding, this antigen was found in 70 percent of the colonies. Therefore, the number of precipitation bands can vary in accordance with the antigenic composition of various cells in the bacterial population. Using very large quantities of microbes it is possible to avoid the effect of inhomogeneity of the cell composition on the results of the precipitin test. Thus, in various experiments with immune horse serum, in strain No 1 the appearance of four precipitation bands could be observed regularly if the number of bacilli in the well for the antigen was brought up to 10,000,000,000.

In connection with the results of this experiment studies were made with the aim of determining the quantity of various antigens in the anthrax bacilli of different strains. The precipitin test was performed with whole immune horse serum using different numbers of microbes: 3,000,000,000, 2,500,000,000, 1,000,000,000, 750,000,000, 500,000,000, 200,000,000, 100,000,000, 50,000,000, 25,000,000 and 10,000,000.

Table 2

The Quantity of Different Antigens According to the Data of the Diffusion in Gell Test with Immune Horse Serum

(1) Штаммы сибиреязвенной бациллы	(2) Минимальные количества бактерий (в млрд.), образующих линии преципитата			
	(3) первую	(4) вторую	(5) третью	(6) четвертую
1	0,05	0,20	0,75	1,50
2	0,01	0,20	1,50	
3	0,01	0,20		
4	0,01	0,01	0,50	
5	0,01	0,10		
9	0,025	0,50		
10	0,01	0,10	0,50	
11	0,01	0,20	0,50	
12	0,01	0,025	0,50	
13	0,01	0,75	3,00	3,00
14	0,05	0,75		
15	0,01	0,75		
(7) Вакцина Ценковского 1	0,50	0,20		
Вакцина Ценковского 2	0,025	0,05	3,00	
(8) Вакцина СТИ	0,05	0,05	3,00	

1. strains of anthrax bacilli; 2. minimum numbers of bacteria (in billions) forming the precipitation bands; 3. first; 4. second; 5. third; 6. fourth; 7. Tsenskovskiy's first vaccine; 8. STI vaccine.

From the data given in Table 2 it follows, first of all, that in the same strain different antigens are present in unequal quantities and, secondly, a considerable difference is observed in the quantity of the same antigen in different anthrax bacilli strains. However, it follows regularly for all

strains that the higher the ordinal number of the precipitation band, the smaller the quantity of antigen needed to produce this band. While the antigen of the first precipitation band is found in approximately the same quantity in different strains, the content of antigens inducing the second and third precipitation zones undergoes very pronounced variation in different strains. The microbes of Tsenskovskiy's second and the STI vaccines contain the same antigens in almost the same quantity.

According to the data of Oudin (1946), Oakley and Fulthorpe (1953), the two precipitation zones can mask each other.

According to Karngold's data (1956), when there are two different antigen-antibody systems applied to each other, a slight change in the concentration of one of the antigens always shows up the heterogeneity of these systems. Since in our experiments reduction of the antigen concentration did not interfere with the homogeneity of the first precipitation zone, we can consider that this zone, despite its considerable width and compactness, is the result of the reaction of only one antigen with its corresponding antibody and, therefore, it does not mask the other precipitation bands located near it. According to Oudin's statement (1946), this problem can be solved also by means of diluting the immune serum. We used precipitating anthrax serum in a dilution of 1/2, 1/4 and 1/8. In this manner of performing the experiment the nature and location of the first precipitation zone with serum dilutions of 1/2 and 1/4 did not change essentially, which again confirmed its homogeneity. In parallel, it was made clear that when the serum was diluted 1/2 the third and fourth precipitation bands disappear, and with a dilution of 1/8 the precipitin test becomes negative. In addition, it was made clear that Chen's and Meyer's (1955) statement that very small quantities of the antigen cannot be detected because of an excess of antibodies cannot be applicable to the results of our studies. The use of diluted sera in our experiments did not lead to the appearance of new precipitation bands, in any case, none which were not present in the precipitin test with whole immune serum.

Some variations in the number of various antigens in the same strains may also depend on the quality of the nutrient media. In accordance with this idea, parallel experiments were performed with four strains of the anthrax bacillus (3, 5, 9, 11), grown out on Hottinger's agar, Hottinger's agar containing defibrinated blood (5 percent), on Martin's agar and Martin's agar containing blood (5 percent). It was found that with cultivation on media containing blood strains 3 and 5 very constantly showed an antigen which was responsible for the third precipitation band. In the next experiment it was made clear that the temperature of cultivation (20, 28, 37°) of the anthrax bacilli exerts no appreciable effect on the number and quantity of the antigens recorded in the diffusion in agar test.

Conclusion

The number of precipitation zones ranges from two to four in various strains of anthrax bacilli. The first and second zones are observed in all strains; conversely, the third and particularly the fourth precipitation band are characteristic only of separate strains. The bands conditioned by one antigen or another have different external appearances, different times of

formation and different spatial relationships to the wells containing antigen and serum. This phenomenon is apparently induced by the chemical nature of the antigens, the size of their molecules, their territorial distribution in the cell and the strength of their connection with the cell. The antigenic structure of virulent anthrax bacilli is the same in principle as the antigenic structure of bacilli of the vaccine strains. It may be noted that in some virulent strains an antigen is found, particularly in experiments with rabbit immune serum which induces the formation of the fourth precipitation band, which under all circumstances is absent from vaccine strains. However, this antigen is not evidence of the virulence of the anthrax bacilli, because it may be absent in strains with a high degree of virulence. By means of the diffusion in agar test it is possible with a certain degree of accuracy to gain an idea of the content of various antigens. It was determined that in the same strain various antigens are present in different quantities. In precisely the same way a considerable difference is observed in the quantity of the same antigen in different strains of anthrax bacilli. The following is a rule for all strains: the higher the ordinal number of the precipitation band, the smaller the quantity of antigen in the bacterial cell needed to produce this band. By means of a change in the quality of the nutrient medium it is possible to demonstrate the antigen inducing the fourth precipitation zone in some strains. This phenomenon is not associated with the new formation of the given antigen but with an increase in its content in bacterial cells grown out under optimum conditions. The cell composition of the anthrax population is inhomogeneous in its antigenic composition. Apparently, by means of selection it is possible to obtain strains of anthrax microbes which are most complete in an antigenic respect.

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BIOCHEMISTRY

Changes in the Lactic Acid Content of White Mice with Plague

M. N. Dzhaparidze and N. K. Sidorova
(Saratov)

Only isolated papers have been given over to the study of carbohydrate metabolism experimentally in plague. In our previous communications (N. N. Ivanovskiy and M. N. Dzhaparidze, 1959; M. N. Dzhaparidze and V. L. Kulikova, 1958; M. N. Dzhaparidze and N. K. Sidorova, 1959), we pointed to a reduction in the quantity of pyruvic and citric acids in the tissues of animals sick with plague, a disorder in the synthesis of citric acid in plague and the effect of inhibitors of the Krebs cycle increasing intoxication with the plague toxin. These data permit us to believe that oxidation of pyruvic acid in the Krebs cycle, that is, aerobic respiration, is impaired in plague. As is well known, the reduction of pyruvic acid under anaerobic conditions leads to the production of lactic acid. Normally, in internal organs, when there is an adequate supply of oxygen, lactic acid is found in comparatively small quantities. The accumulation of lactic acid is observed in different pathological conditions, where the consumption of carbohydrates occurs under conditions of inadequate oxygen supply to the tissues (an exception is constituted only by some tissues: vigorously working muscle, seminiferous cells, in which lactic acid is formed in large quantities under aerobic conditions, but they are not the object of the present study). At the same time, it is well known that in experimental plague, as has been shown by K. M. Mokhin (1959), oxygen deficiency develops in animals: the oxygen content of the arterial and venous blood begins to drop as early as one day after infection.

The present communication is given over to the study of the effect of plague and intoxication on lactic acid metabolism under test-tube conditions and in experiments on animals.

Material and Method of Investigation

The experiments were performed on male white mice weighing 18-20 grams. The animals were infected by subcutaneous injection of 10 CLD of the 708 strain of the plague microbe (500 microbes). The plague microbe toxin was prepared from the same strain by the method of A. I. Zheltenkov (1946) and injected into the mice intraperitoneally in a dose of 3 CLD (0.3 cc).

Mice of one group were injected with the toxin or, depending on the experimental conditions, with a plague microbe culture; a group of healthy mice constituted the control. All animals were kept under the same conditions.

Lactic acid was determined by the method of Barker and Summerson (1941) in the liver, kidneys, spleen, lungs, heart and brains of the animal by the color test with p-hydroxydiphenyl prepared by the V. N. Okunov method (1958).

Five white mice were used for each determination of lactic acid. The animals were killed by means of decapitation; the organs to be studied were

rapidly extracted and ground up in cooled trichloroacetic acid solution. The lactic acid determination was made after centrifugation, using a non-protein filtrate in a photoelectric microcolorimeter (FEK-M-5% model). The effect of the plague microbe toxin on the tissues in test-tube experiments was determined in the following way: 250 milligrams of the tissue of healthy animals was ground up in a test tube with powdered glass and suspended in 3.6 cc of a phosphate buffer (pH, 7.1). Depending on the experimental conditions, 0.4 cc of the toxin or physiological saline solution was added to the suspension. In parallel, a determination was made in the sample without the addition of tissue and using the same toxin. Trichloroacetic acid in a quantity of 1 cc of the 20-percent solution was added to the experimental mixture immediately or two hours after incubation in a water bath at 37° with constant mixing. The precipitate was filtered off, and the lactic acid was determined in 1 cc of the filtrate obtained.

Personal Observations

Changes in the lactic acid content in white mice caused by the injection of the plague microbe toxin are shown in Table 1.

Table 1

Changes in the Lactic Acid Content of White Mice Three Hours after Intraperitoneal Injection of 3 CLD of the Plague Microbe Toxin

(1) Объект исследования	(2) Показатели количества молочной кислоты у животных				
	(3) здоровых		(4) больных		T
	M_1	±m	M_2	±m	
(5) Печень	44,2	1,57	19,0	1,99	9,96
(6) Мозг	46,2	5,47	23,4	1,69	4,00
(7) Селезенка	19,0	1,68	32,4	3,09	5,32
(8) Почки	19,1	1,01	27,8	0,8	7,43
(9) Сердце	42,3	2,62	35,0	3,33	1,71
(10) Легкие	18,4	0,67	18,2	1,06	0,15

Note. M. The arithmetic mean of five determinations of the quantity of lactic acid (in milligrams) per 100 grams of tissues; m. The standard deviation (root-mean-square deviation of the mean.); T. Index of significance of the difference.

Key: 1. Object examined; 2. Indices of the quantity of lactic acid in the animal; 3. Healthy; 4. Sick; 5. Liver; 6. Brain; 7. Spleen; 8. Kidney; 9. Heart; 10. Lungs.

It should be noted that the destructive effect of the toxin on white mice under conditions of this experiment was quite strong: death of all the animals occurred six-eight hours after injection of the toxin. We

considered this manner of performing the experiment advisable, because in this case--investigation three hours after injection of the toxin--the effect of extraneous stimuli on the animals (diet, etc.) was ruled out to a considerable degree.

Changes in the lactic acid content in various tissues were not equivalent. Thus, three hours after injection of the toxin the quantity of lactic acid in the liver and brain of the animals decreased by more than two times, whereas in the spleen the opposite relationships were observed: the lactic acid in sick animals was two times greater than in healthy animals; in the kidneys this increase was less pronounced, and in the lungs and heart the differences were not significant statistically.

Reduction in the lactic acid content in the brain and liver, the carbohydrate metabolism of which is closely connected with the condition of the nervous system, represents a reflection of involvement of the nervous systems of animals with plague intoxication. It was determined that with the death of dogs, whose central nervous systems are in a state of pronounced inhibition (drug-induced sleep), the lactic acid content in their brain decreases by comparison with animals which die in the waking state. Adynamia in the animals [adynamia in Russian is more apt to mean lack of movement than weakness], blindness, a disorder of defecation and urination along with convulsions in the agonal state, that is, signs always observed with intoxication by the plague toxin, undoubtedly attest to deep-seated involvement of the nervous system.

Under the influence of plague infection, as has been mentioned above, there is a reduction in the glycogen content of the heart muscle of animals, which is explained by anoxia of the tissue (K. M. Mokhin and others, 1957). The absence of lactic acid accumulation which we found in the heart muscle does not contradict this explanation, because Bugard and others (1957), studying the effect of anaerobic conditions on the glycogen and lactic acid content of heart sections of rats and finding a marked reduction in the glycogen content in them, did not find any changes in the lactic acid content, in connection with which they expressed the idea that there is a change in the normal course of carbohydrate metabolism in anoxia.

The data which we obtained attest undoubtedly to the unique effect of the plague microbe toxin on carbohydrate metabolism of the susceptible animal. As was determined by Kun and Abood (1949), the endotoxin obtained from salmonellas and meningococcus by the Boivin and Mesrobeanu method (whole antigen) stops glycogen synthesis in the livers of animals, considerably increasing lactic acid production in them.

At the same time, the obtaining of a whole antigen, by the Boivin method, from plague microbe is impossible because of the characteristics of its chemical structure (Ye. I. Korobkova and Ye. E. Bakhrakh, 1951). Probably for these reasons its effect is different also.

Changes in the lactic acid content in the tissues of white mice sick with plague are shown in Table 2. The studies were made on the fifth day after infection of the mice; by this time about 60 percent of the animals had died of plague. Before determining the lactic acid, the plague microbe was isolated from all the organs studied and from the blood by making a culture.

The quantity of lactic acid in various tissues changed in different

Table 2

Changes in the Lactic Acid Content of White Mice Five Days after Infection with 10 CLD of the 708 Strain of Plague Microbe

(1) Объект исследования	(2) Показатели количества молочной кислоты у животных				
	(3) здоровых		(4) больных		T
	M_1	±m	M_2	±m	
(5) Печень	39,0	1,25	40,0	1,42	0,52
(6) Мозг	47,0	1,42	51,7	1,35	2,99
(7) Селезенка	19,0	1,70	37,8	3,13	5,28
(8) Почки	20,7	0,69	39,0	1,25	12,10
(9) Сердце	37,0	0,48	43,0	3,09	1,92
(10) Легкие	17,0	0,82	31,9	4,28	9,80

Note. M. The arithmetic mean of three determinations of lactic acid (in milligrams %); m. and T.—see the note to Table 1.

Key: 1-10. Same as Table 1.

ways, just as under the influence of the toxin. However, the nature of these changes was somewhat different. Thus, we failed to notice any reduction in the lactic acid content of the liver and brain of sick animals. In the presence of a general tendency toward an increase in the lactic acid content in the tissues of sick mice, the differences were very slight in the liver and brain (not statistically significant). In the hearts of animals sick with plague, as in those affected by toxin, the quantity of lactic acid also failed to change; in the spleen and kidneys it was like that noted after intoxication. In the lungs of mice sick with plague the lactic acid content increased by two times.

Some differences in the lactic acid content in tissues of animals sick with plague and affected by the toxin are explained, probably, by the presence of the plague microbe itself in the body, in which, as is well known, there are not only toxic substances which are soluble only in water and weak salt solutions but also insoluble toxic substances (Baker and others, 1952).

With the aim of studying the direct effect of the plague microbe toxin on the tissues of white mice, experiments were performed under test-tube conditions according to the method described above (Table 3).

From Table 3 it is seen that in these experiments the toxin did not exert any appreciable effect on lactic acid production in the tissue of liver or kidneys.

Summing up the data obtained briefly, it may be considered an established fact that in plague infection a general increase in the quantity of lactic acid is observed in the tissues of white mice, which attests to a disorder of aerobic respiration in them.

When mice are affected by the plague microbe toxin, changes occur in

Table 3

Lactic Acid Content in Tissues of White Mice in Test-Tube Experiments

(1) Объект исследования	(2) Количество молочной кислоты (в мг %) в опытах			
	1		2	
	(3) начальное	(4) через 2 часа инкубации	(3) начальное	(4) через 2 часа инкубации
(5) Печень	38,8	50,0	43,0	58,2
(6) Печень + токсин	42,0	53,0	45,8	60,8
(7) Почки	21,0	30,0	19,1	28,2
(8) Почки + токсин	23,2	32,4	22,3	29,6
(9) Токсин	3,1	3,2	3,4	3,4

1. Object of the examination; 2. Quantity of lactic acid (in milligrams %) in the experiments; 3. Initial; 4. After two hours of incubation; 5. Liver; 6. Liver plus toxin; 7. Kidneys; 8. Kidneys plus toxin; 9. Toxin.

the quantity of lactic acid in the organs which are different from those in plague, which is probably connected with the characteristics of the infectious process itself. The lactic acid content in the tissues, changing considerably under the influence of injection of the toxin into the animals, did not change in experiments performed in vitro.

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The Effect of Some Cultivation Conditions on the Synthesis of Polysaccharide by the Plague Microbe

Ye. E. Bakhrakh and V. D. Yegorova
(Saratov)

Study of the polysaccharide-polypeptide complex which we isolated from the plague microbe showed that it can be used as an allergen for determining the immunological rearrangement of the bodies of animals vaccinated against plague or surviving an experimental infection (Ye. E. Bakhrakh, Ye. I. Korobkova and others, 1960).

The present study had as its aim the demonstration of conditions contributing to the synthesis of polysaccharide by the plague microbe. Aside from theoretical interest, the clarification of this problem will make it possible to increase the yield of the allergenic preparation when it is obtained from plague microbe cells.

As the basic medium bouillon and agar from Hottinger's beef hydrolysate and agar from an acid casein hydrolysate were used. In order to exclude the possible effect of the nonstandard nature of the agar media on the synthesis of polysaccharide by the plague microbe, they were first dried in a quantity necessary for performing all experiments (V. M. Tumanskiy, Ye. E. Bakhrakh and others, 1959).

A certain weight of dry agar was moistened in tap water and dissolved with heating. It was filtered, the pH was established (7.2), poured out into bottles in 30-cc quantities and sterilized for 30 minutes at 110°. The sugars and amino acids were sterilized separately and added aseptically to the fused agar before culture.

All the experiments were performed with the vaccine strain of the EV plague microbe. The bottles were seeded with a measured quantity of a suspension of a two-day culture (500,000,000 microbes) in physiological saline solution. After incubation at the appropriate temperature, the cells which grew out were washed off with physiological saline solution, carefully filtered through several layers of gauze for the purpose of eliminating pieces of agar, centrifuged and washed off with physiological saline solution.

Polysaccharide and protein were determined directly in the cells. For the purpose of determining polysaccharide a test was used with anthrone reagent, which had been applied by Gary and others (1957) to the analysis of polysaccharide in the cells of brucellas. Test tubes containing 5,000,000,000-10,000,000,000 microbes in 3 cc were put into an ice bath, and 7 cc of the anthrone reagent was added to the samples with constant mixing (200 milligrams of anthrone dissolved in 100 cc of concentrated sulfuric acid), after which the samples were transferred to a boiling water bath for

10 minutes. After cooling the test tubes in ice water the intensity of the color was measured in an FEK N-54 colorimeter at a wavelength of 620 millimicrons, and the polysaccharide content was determined from a standard curve calculated for glucose.

The protein content was determined by the intensity of the biuret test (Stickland, 1951). The quantity of polysaccharide and protein was calculated in milligrams per 100,000,000,000 microbes (the content of the latter was determined nephelometrically), and the percentage ratio of polysaccharide to protein was calculated. The ratio of polysaccharide to protein in the cells of the plague microbe grown out at 28° on Hottinger's agar served as a control. In our experiments it ranged from 2.4 to 3.4 percent.

The effect of some cultivation conditions on the yield of microbes per cc of the medium and on the synthesis of polysaccharide by the plague microbe is shown in Tables 1 and 2.

Table 1

The Effect of Age of the Culture and Some Cultivation Conditions on the Synthesis of Polysaccharide by the Plague Microbe

(1) Возраст культуры, сутки	(2) Урожай микробов в 1 мл среды, 10^9	(3) ПС/Б, %	(4) Температура выращивания, °	(2) Урожай микробов в 1 мл среды, 10^9	(3) ПС/Б, %	(5) Концентрация аммиачного азота в 1 мл среды, мг %	(2) Урожай микробов в 1 мл среды, 10^9	(3) ПС/Б, %
1	1.1	6,3	28	3,6	2,9	50	1,5	2,6
2	3.2	3,0	35	2,0	3,9	100	3,0	3,0
3	3,6	2,5	37	0,6	4,8	150	3,8	2,5
4	4,4	3,1						
5	4,5	3,1						

1. Age of the culture, days; 2. Microbe yield per cc of the medium, 10^9; 3. Polysaccharide/protein, %; 4. Cultivation temperature, degrees; 5. Concentration of amine nitrogen per cc of the medium, milligrams %.

From the data of Tables 1 and 2 it is seen that the formation of polysaccharide by the plague microbe depends on the physiological condition of the culture and the conditions under which it is cultivated.

In a one-day culture much more polysaccharide is found than in two- five-day cultures. With increase in the cultivation temperature the production of polysaccharide by the plague microbe is increased. Increase in the quantity of polysaccharide in the culture of the plague microbe is observed as the result of addition of certain amino acids to the medium (tyrosine, serine, threonine, and particularly glycine). Of the sugars studied only the addition of 1 percent glucose to the medium caused a marked increase in the synthesis of polysaccharide by the plague microbe. A considerable increase in polysaccharide production by the plague microbe was

Table 2

The Effect of Addition of Some Substances to the Medium on the Synthesis of Polysaccharide by the Plague Microbe

(1) Аминокислота	Урожай микробов в 1 мл среды, 10^9	(3) ПС/Б, %	(4) Сахар	Урожай микробов в 1 мл среды, 10^9	(3) ПС/Б, %	(5) Стимулятор роста	Урожай микробов в 1 мл среды, 10^9	(3) ПС/Б, %
(6) Фенилаланин	3,3	3,6	Глюкоза (15)	0,4	27,0	Сульфит натрия (0,05%) (18)	4,8	2,6
(7) Глицин	2,0	8,9	Галактоза (16)	4,0	3,4	Кровь (0,1%) (19)	5,2	3,0
(8) Тирозин	5,5	5,0	Ксилоза (17)	5,0	2,9	Перевар Филдса (0,1%) (20)	5,0	3,0
(9) Триптофан	4,8	4,4	Контроль (14)	3,1	2,7	Аутолизат дрожжей (5,0%) (21)	5,4	3,5
(10) Глутаминовая кислота	5,5	3,8				Контроль (14)	3,6	2,6
(11) Аспарагиновая кислота	5,8	3,7						
(12) Серин	6,0	5,0						
(13) Треонин	5,5	5,2						
(14) Контроль	3,4	3,4						

Note. Amino acids were added in a concentration of 0.5 percent; sugars, in a concentration of 1 percent. The addition of these substances in a concentration of 0.1 percent had no appreciable effect on the production of polysaccharide by the plague microbe.

Key: 1. Amino acid; 2. Yield of microbes per cc of the medium, 10^9; 3. Polysaccharide/protein, %; 4. Sugar; 5. Growth stimulant; 6. Phenylalanine; 7. Glycine; 8. Tyrosine; 9. Tryptophane; 10. Glutamic acid; 11. Aspartic acid; 12. Serine; 13. Threonine; 14. Control; 15. Glucose; 16. Galactose; 17. Xylose; 18. Sodium sulfite; 19. Blood; 20. Field's digest; 21. Yeast autolysate.

also noted when it was cultivated in an atmosphere of carbon dioxide.

Increased polysaccharide production was frequently observed under conditions which were not very favorable for active growth of the microbe (elevated temperature, addition of glucose to the agar, addition of glycine, etc.). However, the addition of certain growth stimulants of the plague microbe to the medium—blood, Field's digest, sodium sulfite, yeast autolysate—had no effect on the power of polysaccharide synthesis by the plague microbe. Change of the content of nitrogenous substances in the medium had no effect on the polysaccharide content in the cells of the plague microbe either, although increase in their concentration considerably increased the microbe yield. Therefore, no direct relationship is observed between the growth rate of the plague microbe and its power of synthesizing polysaccharide.

Statistical treatment of the results obtained showed the significance

of the fact that cultures grown out on agar media at 37° and when glycine is added to the medium contain, on the average, one and a half-two times more polysaccharide than cultures grown out at 28°. On bouillon under aeration conditions the plague microbe produces approximately one and a half times less polysaccharide than when it grows on agar media at 28°. It was noted that in the plague microbe cells grown out on casein agar there is 30-40 percent more polysaccharide than in cells grown out on Hottinger's agar (Table 3).

Table 3

The Ratio of Polysaccharide to Protein in the Cells of the Plague Microbe (in %) as a Function of the Cultivation Conditions

(1) Среда	(2) Температура выращивания, °	M	m	T
(3) Агар Хоттингера	28	2,9	±0.35	
	37	4,6	±0.74	4,0
(4) Агар Хоттингера с глицином	28	5,9	±0,80	3,4
(5) Казеиновый агар	28	3,8	±0,26	2,2
(6) Бульон Хоттингера	28	1,8	±0,12	3,0

Note. M is the arithmetic mean of eight determinations of the ratio of polysaccharide to protein (in %); m is the root-mean-square deviation of the mean; T, the index of significance of the difference. The calculation was made by the following formula:

$$T = \frac{M_{experimental} - M_{control}}{\sqrt{m_1^2 + m_2^2}}.$$

The ratio of polysaccharide to protein in plague microbe culture grown out on Hottinger's agar at 28° served as a control.

Key: 1. Medium; 2. Cultivation temperature, degrees; 3. Hottinger's agar; 4. Hottinger's agar with glycine; 5. Casein agar; 6. Hottinger's bouillon.

In order to determine the part of the cell—capsule or body—in which polysaccharide synthesis occurs, we performed the following experiment. From the plague microbe cells capsular substance was extracted, and a determination was made of the polysaccharide and protein content in the entire cell and in the capsular extract. For the purpose of obtaining the capsular substance, 100,000,000,000 cells were suspended in 5 cc of physiological saline solution (pH of 7.4). After inactivation of the cells at 56°-58° for an hour, they were put into a refrigerator for seven days, mixing

periodically. The capsular substance was separated from the cells by centrifugation and filtration through a candle [Chamberland filter].

It was found that approximately one seventh of the cell protein goes into the capsular extract. As far as the polysaccharide is concerned, simultaneously with an increase of its content in the cell there is an increase in the quantity of it in the capsular substance (Table 4).

Table 4

Quantity of Polysaccharide in Whole Cells and Capsular Extract of Plague Microbe

Среда (1)	Температура выращивания° (2)	Результаты анализа (3)			
		целые клетки (4)		капсульный экстракт (5)	
		полисахарид (6)	белок (7)	полисахарид (6)	белок (7)
(8) Агар Хоттингера	28	0,33	15,7	0,20	2,15
	37	0,88	16,8	0,57	2,24
(9) Агар Хоттингера с глюкозой	28	1,21	21,2	1,05	2,70
(10) Агар Хоттингера с глюкококоллом (0,5%)	28	0,96	17,7	0,68	2,30

1. Medium; 2. Cultivation temperature, degrees; 3. Results of the analysis; 4. Whole cell; 5. Capsular extract; 6. Polysaccharide; 7. Protein; 8. Hottinger's agar; 9. Hottinger's agar with glucose; 10. Hottinger's agar with glycocol (0.5%).

In recent years, many investigators have made an immunochemical study of the capsular substance of the plague microbe and have isolated several fractions from it, of which the immunogenic fraction 1A, which is a carbohydrate-protein complex (Baker and others, 1952), is of undoubted interest. The probability that the quantity of polysaccharide which we determined in the cell and which varies in accordance with the cultivation conditions reflects the change in the power of the plague microbe for synthesis of the immunogenic complex (fraction 1A) occurring under the influence of the same factors and, therefore, that it can characterize the change in the immunogenic properties of the strain depending on its cultivation conditions has not been ruled out.

Confirmation of this idea is the fact that increase in polysaccharide production by the plague microbe is stimulated by precisely those factors which, according to data in the literature, contribute to an increase in the immunogenic properties of the plague pathogen, namely, elevation of the cultivation temperature to 37°, cultivation in an atmosphere of carbon dioxide, addition of glycine to the medium, cultivation on a medium made of acid casein hydrolysate (Schütze, 1932; Ye. I. Korobkova and others, 1938; A. I. Zheltenkov and S. V. Anokhina, 1951; Ye. I. Korobkova, 1951; Seal

and Mukerjee, 1950; A. D. Garmazova, 1959).

Conclusions

1. The polysaccharide content in the plague microbe cells varies in accordance with its age and cultivation conditions.

2. In cultures of the plague microbe grown out on Hottinger's bouillon with aeration there is less polysaccharide than in cultures grown out on agar media. Increase in the cultivation temperature contributes to the synthesis of polysaccharide by the plague microbe. The production of polysaccharide by the plague microbe is also increased when certain amino acids are added to the medium (particularly glycine), with the addition of glucose and when the plague microbe is cultivated in an atmosphere of CO_2.

3. The correlation noted between factors stimulating the synthesis of polysaccharide by the plague microbe and the conditions contributing to an increase in the immunogenic properties of the culture, according to the data in the literature.

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Chemical Characterization of the Thermostable Antigens of the Plague Microbe

Ye. P. Denisova and V. D. Yegorova
(Saratov)

In 1932, Schütze established the fact that in the plague microbe there are thermostable antigens, along with the thermolabile antigens, which possess slight immunogenic and antigenic properties.

In preliminary studies by one of the authors (Ye. P. Denisova) it was found that the thermostable antigens possess marked allergic properties. The latter are demonstrated with reproduction of the Sanarelli-Schwartzmann phenomenon in rabbits and with the performance of the intradermal allergic test on guinea pigs immunized with living plague vaccine and immune guinea pigs surviving a control infection. The thermolabile antigens (capsular antigen) did not cause either the Sanarelli-Schwartzmann phenomenon or the allergic skin reaction.

In the present work the results of the study of the chemical composition of the thermostable complexes isolated from the plague microbe are presented.

In this work three strains of the plague microbe were used: two virulent: the continental No 708, oceanic No 751 and the EV vaccine strain. The thermostable antigens were extracted from bacterial suspensions of the plague microbe with physiological saline solution by boiling them for an hour. (The method of obtaining the thermostable unpurified antigens is given in the work by Ye. P. Denisova. Trudy Instituta "Mikrob" [Transactions of the "Mikrob" Institute], 1959.) The suspension of microbes, containing 35,000,000,000 cells per cc, was put into the refrigerator after boiling in sealed ampules, and kept at a temperature of 5°-7° for one-two months. Thereby, the microbes settled to the bottom of the ampule. The absolutely clear supernatant fluid of a slightly yellowish color was the object of the investigation.

When the antigens were obtained in a dry state the bacterial suspension was centrifuged, cooled to 5°-7° and precipitated with six volumes of cooled 96-percent alcohol. After settling of the dense particles, light clumps, some of which floated to the surface of the fluid, remained in the suspended state. This was observed to an even greater degree in the virulent strains than in the vaccine strain (EV). The antigen precipitate was separated off by centrifugation, washed four times with cold alcohol, once with ether, and dried in a drier over calcium chloride.

In the preparations obtained the ash content, moisture content, nitrogen (by the method of isothermal distillation in Conway diffusion cell), phosphorus (by the modified Fiske-Subbarow method with ascorbic acid), reducing substances (by the method of Hagedorn and Jensen), and glucosamine (by the method of Elson and Morgan) were determined.

For the purpose of identifying the amino acids and monosaccharides, the method of unidimensional ascending paper chromatography was used.

For the purpose of determining the amino acids, the preparation was hydrolysed with 6 N hydrochloric acid, evaporated to dryness, and the amino acids were extracted with isopropyl alcohol (Block and Lestrani). The solvent was a mixture of N-butanol and acetic acid and water (in a proportion of 4:1:5); the developer was 1 percent ninhydrin solution in acetone.

For the purpose of determining the monosaccharides, the preparation was hydrolysed in 1 N sulfuric acid. The hydrolysate was neutralized with barium hydroxide; the precipitate of barium sulfate was separated off by centrifugation and washed several times with small portions of hot water, which were added to the centrifugate. The entire volume of fluid was filtered, and the filtrate was passed through a column containing SBS cationite (the resin was obtained from the Chemical-Technological Institute imeni Mendeleyev) for 10 minutes. The resin absorbed amino sugars, amino acids and other substances. The sugars were carefully washed out of the column with water into the evaporating dish. The solution was evaporated to the necessary concentration and applied to paper.

For the purpose of separating the sugars, the solvent used was a mixture of pyridine, benzene, N-butanol, and water (in a proportion of 3:1:5:3); the developer was aniline phthalate in water-saturated butanol.

The preparations of thermostable antigens isolated in the crude form are loose powders of a slightly yellowish color, readily soluble in water and physiological saline solution with a slight opalescence. In the test with 0.2 percent trichloroacetic acid the solutions produce an abundant precipitate. The chemical characterization of the antigenic complexes obtained from different strains of the plague microbe is given in Table 1 (air-dried preparations were analyzed).

From the data of Table 1 it is seen that all the preparations are characterized by a high content of inorganic impurities, quite high degree of moisture absorption (in the air-dried state they contain about 9 percent water), are rich in phosphorus, whereby more than half of it is from inorganic phosphorus; they contain 20-22 percent reducing substances and a considerable quantity of nitrogen. In the calculation the quantity of protein in the non-residue preparation amounts to 60-63 percent.

Chromatographic analysis showed that the same 12 amino acids (cystine/cysteine, lysine, arginine, aspartic acid, glycine, glutamic acid, threonine, alanine, tyrosine, valine, phenylalanine and leucine) are contained in the protein component of all three antigens. In the polysaccharide fraction five sugars were identified (galactose, glucose, arabinose, xylose and ribose).

An attempt to purify the antigens by reprecipitation with alcohol led to a deterioration of the solubility of the preparation, which could have been the result of a change in its physicochemical properties during the treatment.

Table 1

Chemical Characterization of Thermostable Antigens

Штамм (1)	Содержание в препарате, % (2)							Содержание в беззольном препарате, % (10)	
	воды (3)	золы (4)	общего азота (5)	неорганического фосфора (6)	общего фосфора (7)	редуцирующих веществ (8)	глюкозамина (9)	белка (11)	редуцирующего сахара (12)
(13) EB	9,28	25,16	7,31	3,46	5,54	16,6	1,44	61,0	22,2
708	8,45	23,2	7,58	3,24	5,16	14,97	1,35	61,7	19,5
751	8,74	25,0	7,56	2,95	5,61	15,79	1,32	63,0	21,0

1. Strain; 2. Content in preparation, %; 3. Water; 4. Ash; 5. Total nitrogen; 6. Inorganic phosphorus; 7. Total phosphorus; 8. Reducing substances; 9. Glucosamine; 10. Content in non-residue preparation, %; 11. Protein; 12. Reducing sugar; 13. EV.

After reprecipitation with alcohol twice the antigen was hydrolysed with 0.1 N acetic acid on a boiling water bath for four hours.

After 24 hours of standing in a refrigerator the hydrolysate was divided into precipitate and clear fluid. No lipoid layer was found on the surface of the fluid.

The precipitate which had been separated off by centrifugation (the "protein" fraction) was washed three times with alcohol and ether. Three volumes of acetone were added to the transparent fluid, as the result of which a white loose precipitate came down ("the polysaccharide fraction"), which was washed three times with acetone. Both fractions were dried in a vacuum drier, after which they were subjected to chemical analysis. The results of the chemical analysis of the fractions obtained from the antigen of the No 708 strain are shown in Table 2.

From Table 2 it is evident that reprecipitation of the antigen with alcohol did not change its composition. The fractions isolated were appreciably different from one another. By comparison with the readily soluble "polysaccharide" fraction, the "protein" fraction dissolved poorly in water, contained half as much reducing sugar and glucosamine and twice as much nitrogenous substance (protein). A comparison of the results of the analysis of the "polysaccharide" fraction obtained, which had been isolated from the thermostable antigen of the plague microbe, with previously obtained data on the analysis of the polysaccharide-containing fraction of the plague microbe (Ye. E. Bakhrakh, Ye. I. Korobkova and others, 1960), isolated directly from the cells by means of hydrolysis with 0.1 N acetic acid solution, shows the similarity in their chemical composition.

Table 2

Chemical Characterization of Fractions Obtained from Antigen of the No 708 Strain

Препарат (1)	(2) Содержание, %					(8) Содержание белка в беззольном препарате, %	(9) Содержание редуцирующих веществ в беззольном препарате, %
	(3) золы	(4) общего азота	(5) общего фосфора	(6) редуцирующих веществ	(7) глюкозамина		
(10) Термостабильный антиген штамма № 708 (дважды переосажденный)	25,8	6,89	5,4	16,15	1,24	58,1	21,75
(11) „Полисахаридная" фракция	26,6	3,43	7,4	20,79	1,46	46,1	28,3
(12) „Белковая" фракция	8,3	11,23	1,55	13,02	0,6	76,5	14,1

1. Preparation; 2. Content, %; 3. Ash; 4. Total nitrogen; 5. Total phosphorus; 6. Reducing substances; 7. Glucosamine; 8. Protein content in non-residue preparation, %; 9. Content of reducing substances in non-residue preparation, %; 10. Thermostable antigen of No 708 strain (reprecipitated twice); 11. "Polysaccharide" fraction; 12. "Protein" fraction.

Conclusions

1. Thermostable antigens isolated by extraction with physiological saline solution and boiling from the cells of the plague microbe are protein-polysaccharide complexes. They contain 61-63 percent protein and 20-22 percent reducing sugar.

2. On hydrolysis with 0.1 N acetic acid solution for four hours on a boiling water bath the thermostable antigenic complex breaks up into two fractions, which are different in their content of protein and reducing substances.

3. The protein component of the antigen consists of 12 amino acids: cystine (cysteine), lysine, arginine, aspartic acid, glycine, glutamic acid, threonine, alanine, tyrosine, valine, phenylalanine, and leucine. In the polysaccharide portion, five sugars were identified (glucose, galactose, arabinose, xylose, and ribose).

The Rates of Oxygen Consumption and Glucose Oxidation by Plague and Rodent Pseudotuberculosis Microbes

F. K. Drozdovskaya
(Saratov)

In the present work we had the aim of giving the quantitative characteristics of the rate of respiration of the plague and pseudotuberculosis microbes by their power of absorbing oxygen and oxidizing glucose as well as clarifying the effect of certain enzyme toxins on the carbohydrate metabolism of these microbes.

Method of Work

For the experiments two-day cultures of the vaccine strain of the EV plague microbe and strain No 6 of rodent pseudotuberculosis which had been washed three times with physiological saline solution were used. The rate of oxygen uptake by the bacteria was determined in a Warburg apparatus. The experimental mixture consisted of 1 cc ($12.5 \cdot 10^9$) of a suspension of microbes, 0.5 cc of a 2-percent glucose solution and 2 cc of a phosphate buffer at a pH of 7.2 (M/7.5). Into the central flask 0.1 cc of a 20-percent solution of potassium hydroxide was placed for absorption of the carbon dioxide given off during the process of cell metabolism. The total volume of the mixture was brought up to 4 cc with physiological saline solution. In the control, instead of glucose, distilled water was added. The respiration of the bacteria was observed for an hour, after which the enzymatic processes were stopped by the addition of 0.5 cc of a 20-percent solution of trichloroacetic acid. Along with consideration of the oxygen consumed, in the non-protein trichloroacetic acid filtrate glucose was determined by the method of Hagedorn and Jensen. For the purpose of determining the quantity of glucose fermented under relatively anaerobic conditions, the experimental mixture was put into test tubes and poured over with vaseline oil. The mixture was incubated for an hour at 37°. The rate of fermentation was judged by the loss of glucose.

In the study of the effect of the inhibitors the latter were added to the experimental mixtures in different concentrations.

The rate of cell respiration in the presence of a substrate (glucose) was determined by means of subtraction of the value for endogenous respiration from the total quantity of oxygen taken up. In resting cells of plague and pseudotuberculosis microbes grown out on agar media, the latter was relatively high, 1.2-2 micromoles, which amounts to approximately 24 percent of the quantity of oxygen used by the cells in the presence of glucose (Table 1).

Results of the Experiments

The data given in Table 1 show that both species of microorganisms actively oxidize glucose, accompanying this process by the active consumption of oxygen, which ranged within comparatively broad limits (6.4-11 micromoles

Table 1

Rate of Oxygen Consumption and Glucose Oxidation by Plague and Pseudotuberculosis Microbes

	(1) Потреблено кислорода			(2) Окислено глюкозы			(3) Потреблено кислорода на 1 μM глюкозы		
	M	±m	T	M	±m	T	M	±m	T
(4) Чумной микроб	8,4	0,64	2,6	5,0	0,3	3,2	1,7	0,22	4,4
(5) Псевдотуберкулезный микроб	5,8	0,75		9,3	1,2		0,68	0,14	

Note. M, the arithmetic mean of five-six determinations expressed in micromoles; m, the root-mean-square deviation of the arithmetic mean; T., index of significance of the difference. The difference is significant if T. is greater than or equal to 2.5.

Key: 1. Oxygen consumed; 2. Glucose oxidized; 3. Oxygen consumed per micromole of glucose; 4. Plague microbe; 5. Pseudotuberculosis microbe.

of oxygen for plague microbes and 4.5-8.4 micromoles of oxygen for pseudotuberculosis microbes) in various experiments. On oxidation of glucose the resting cells of the plague microbe consumed oxygen more actively than did the cells of the pseudotuberculosis microbe. The average figures were equal, respectively, to 8.4 and 6.2 micromoles of oxygen. At the same time, the plague microbe oxidizes glucose less actively than the pseudotuberculosis microbe (5 and 9 micromoles, respectively). In calculating the quantity of oxygen consumed per mole of oxidized glucose, the significance of the difference between the respiration of these microorganisms was quite high (4.4). The data obtained are in agreement with the principle of the more aerobic nature of respiration in the plague microbe than in the pseudotuberculosis microbe (N. N. Ivanovskiy, 1951; M. N. Dzhaparidze, 1953).

For a more complete idea of the nature of the enzyme system involved in the oxidation of glucose, we made a study of the effect of potassium cyanide and sodium fluoride on this process. First, we checked the effect of potassium cyanide on endogenous respiration. It was found that in a concentration of 1/5000 M the cyanide reduces oxygen consumption by almost two times; in a concentration of 1/1000 M the rate of respiration amounts to approximately 30-35 percent of the control for both species of bacteria. However, potassium cyanide exerts a particularly strong effect on oxygen consumption by the cells in the presence of glucose (Table 2).

Thereby, with increase in the concentration of the poison the degree of respiratory inhibition increases, and in a concentration of 1/1000 M, potassium cyanide almost completely suppresses the respiration of both plague and pseudotuberculosis microbes. In contrast to the respiratory system of

Table 2

The Effect of Potassium Cyanide on the Carbohydrate Metabolism of the Plague and Pseudotuberculosis Microbes

Условия инкубирования	Показатели углеводного обмена микробов при концентрации цианистого калия					
	0		M/5000		M/1000	
	окислено глюкозы, μM	потреблено кислорода, μM	окислено глюкозы, μM	потреблено кислорода, μM	окислено глюкозы, μM	потреблено кислорода, μM
Чумной микроб						
Аэробные	4,0	7,5	12,5	1,2	9,6	0,12
Анаэробные	7,4	—	7,8	—	7,9	—
Псевдотуберкулезный микроб						
Аэробные	7,4	5,9	14,7	1,09	12,8	0,19
Анаэробные	8,3	—	11,0	—	9,0	—

Note. In Table 2 the average data of three experiments are included.

Key: 1. Incubation conditions; 2. Indices of carbohydrate metabolism of microbes with a potassium cyanide concentration of:; 3. Glucose oxidized, micromoles; 4. Oxygen consumed, micromoles; 5. Plague microbe; 6. Aerobic; 7. Anaerobic; 8. Pseudotuberculosis microbe.

the cells, the glycolytic system is markedly activated under the influence of cyanide under aerobic conditions. In the presence of potassium cyanide the rate of aerobic oxidation of glucose exceeds that of anaerobic oxidation of glucose for the plague microbes by 21-60 percent; for pseudotuberculosis microbes, by 34-47 percent. It is interesting to note that under the influence of cyanide the differences between plague and pseudotuberculosis microbes with respect to aerobic oxidation of glucose are leveled off to a considerable degree.

Increase in the rate of glucose utilization along with marked inhibition of oxygen uptake observed in the presence of potassium cyanide permits us to suppose that potassium cyanide, inhibiting the cytochrome respiratory system of plague and pseudotuberculosis microbes, converts these microorganisms to a more anaerobic type of metabolism.

It is well known that potassium cyanide exerts an effect not only on respiration but also on fermentation in bacteria, changing it from heterofermentative to homofermentative lactic acid fermentation (Kubovitz, 1934; Pappenheimer and Shaskan, 1944). In connection with this, we performed experiments to determine the effect of potassium cyanide on the composition of products of glucose oxidation by plague and pseudotuberculosis microbes. After incubation of a suspension of microbes with glucose for one day at 37° in the presence of the cyanide salt (1/1000 M), the fermentation products were investigated by chromatography. The results of the analysis show that

in the presence of the cyanide, cells of the plague and pseudotuberculosis microbes break down glucose only to lactic acid, whereas with incubation of the plague microbe with glucose but without potassium cyanide, pyruvic, succinic, citric and malic acids are found among the fermentation products in addition to lactic acid (Santer and Ajl, 1954). On the basis of the st s presented it may be supposed that the presence of iron-containing enzymes in the cell is necessary for the normal fermentation of glucose by plague and pseudotuberculosis microbes. The effect of sodium fluoride on fermentation of carbohydrates by plague and pseudotuberculosis microbes is shown in Table 3.

Table 3

The Effect of Sodium Fluoride on Carbohydrate Metabolism of Plague and Pseudotuberculosis Microbes (Average Data from Three Experiments)

(1) Условия инкубирования	(2) Показатели углеводного обмена при концентрации фторида натрия								
	0	M/200		M/100		M/20		M/10	
	(3) окислено глюкозы, μM	(3) окислено глюкозы, μM	(4) торможение, %	(3) окислено глюкозы, μM	(4) торможение, %	(3) окислено глюкозы, μM	(4) торможение, %	(3) окислено глюкозы μM	(4) торможение, %
	(5) *Чумной микроб*								
(6) Аэробные	5.9	5.9	0	5,2	12	4,2	29	3,9	34
(7) Анаэробные	11,0	9,6	13,0	7.2	35	3,1	72	2,8	75
	(8) *Псевдотуберкулезный микроб*								
Аэробные	11,3	10,5	7	10	12	4,9	57	3,9	66
Анаэробные	15,5	9,7	37	6	62	3,3	79	1.4	91

1-3. Same as Table 2; 4. Inhibition, %; 5-8. Same as Table 2.

From Table 3 it is seen that cells of the pseudotuberculosis microbe are more sensitive to fluoride. In the presence of even small quantities of the poison (1/200 M, 1/100 M) there is an appreciable reduction of the activity of glucose assimilation, particularly under anaerobic conditions (37-60 percent), and when there is a high fluoride content a considerable suppression of cell activity (80-90 percent) is observed. The cells of the plague microbe are less sensitive to the effect of sodium fluoride. Low concentrations of it under aerobic conditions do not exert any effect on the activity of the cells, and under anaerobic conditions they reduce it very slightly. Even when the sodium fluoride content is high (1/20 M and 1/10 M) the activity of the cells is not completely suppressed. Thus, under aerobic conditions the oxidation of glucose is approximately 30-35 percent inhibited,

and only under anaerobic conditions does the degree of inhibition rise to 70-75 percent.

Conclusions

1. It has been determined that plague microbe cells consume oxygen more actively than pseudotuberculosis microbe cells in the oxidation of glucose.

2. Potassium cyanide markedly inhibits the respiratory activity of both species of microorganisms. The glycolytic system of the cells is not inactivated thereby.

3. Sodium fluoride reduces the rate of carbohydrate metabolism in the pseudotuberculosis microbe to a greater degree than in plague microbes.

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THE PRODUCTION OF BACTERIALS

The Effect of the Temperature of Preliminary Freezing on the Number of Viable Microbes in Dry EV Plague Vaccine

A. I. Tinker

(Stavropol')

In the literature there are quite controversial data on the effect of the freezing temperature on the viability of various microorganisms. Thus, Kurylo-Borowska and Pitekowna (1955), in freezing cell suspensions of Staphylococcus aureus Oxford, Escherichia coli and Shigella flexneri in a protein solution, noted the better survival at a temperature of -75° than at -9° or -23°.

Ch'en Chen-jen and others (1957) point to the better survival of the plague microbe when frozen to -40°.

Yu. L. Subbotina (1958), after freezing BCG vaccine, concluded that a temperature of from -15 to 35° is less harmful than one from -45 to -65°.

Horter (1958), studying 32 species of bacteria, also concluded that slow freezing from +20 to -20° for 20-25 minutes is better than fast freezing to -70° for two-three seconds.

K. Ye. Dolinov (1947) noted that observations of pneumococci and smallpox vaccine frozen at -20°, -40° and -78° showed the complete equivalence of the dry preparations.

M. V. Pelevina (1950), freezing typhoid, paratyphoid (A and B), dysentery (Shiga, Sonne, Stutzer and Flexner), E. coli and paracholera microbes at -20°, -30° and -78°, found no difference in the degree to which the microbes died after drying.

L. K. Arzhelas (1952) was unable to find any changes in the survival of pneumococci, streptococci or other microbes when frozen to -78° or -180°.

M. Strumia (1954) showed that preliminary freezing of blood plasma can be accomplished at such different temperatures as -12° and -72° without any essential difference in the quality of the product obtained.

S. L. Stepanova (1959), like M. V. Pelevina, found no difference in the survival of typhoid bacteria when frozen to -20°, -35° or -75°.

Freezing is classified by the speed of the process. O. Smith (1954) distinguishes superfast, fast, slow and very slow freezing. The rate of freezing depends on the properties of the coolant used and the degree of contact of the material with it. In the literature there are numerous data

on the end temperatures to which the suspension was cooled in freezing. Only in the work of Yu. L. Subbotina (1959) was the relationship between the survival of a BCG culture and the rate of cooling shown in detail during freezing to different temperatures.

In connection with this, we attempted to determine the rate of cooling of a suspension being frozen to -30 and -78° in a mixture of dry ice and alcohol and to -30° in a refrigerator chamber of the NS 270 (20-60) brand for the production of dry EV plague vaccine. For experiments on the study of the cooling rate a suspension of two cc volume was used so that the thermometer bulb be completely immersed in the fluid. Thereby we partially avoided reading errors. Experiments on the study of the viability of the microbes were made in a volume of one cc of suspension (Tables 1 and 2).

From Table 1 it is seen that cooling two cc of a suspension of plague vaccine in a refrigerator chamber to -30° takes place five times more slowly than in a mixture of dry ice and alcohol. When the temperature of the mixture of dry ice and alcohol is -78° the suspension is cooled (to -70°) in three minutes and 20 seconds; at -30°, in four minutes and 10 seconds.

In the study of the cooling rate of a suspension of plague vaccine microbes we paid special attention to the temperature range between -10° and -20°, where, according to the data of M. M. Faybich (1947), the eutectic point of freezing is located. In freezing a suspension in a refrigerator chamber the cooling rate was least -- 0.02-0.1° per second. In the case of freezing in a mixture of dry ice and alcohol with a temperature of -30° the cooling rate was equal to 0.1-0.4° per second. In both cases the cooling rate decreased from the beginning to the end of the process. The cooling rate of a suspension in a mixture at a temperature of -78° changed in a somewhat different manner: at the beginning and end of the process it was somewhat less (0.1-1° per second), and between -10° and -40° it reached 1.3-1.4° per second. Therefore, at the eutectic point the rate of cooling of the vaccine suspension under conditions of a refrigerator chamber at -30° was five times less than in a mixture of dry ice and alcohol at a temperature of -30° and 50 times less than in a mixture at a temperature of -78°.

Subsequently, we attempted to determine the effect of the cooling rate on the number of viable microbes in the vaccine suspension and to determine what mode of freezing is more effective for preparation of dry living EV plague vaccine. For this purpose part of the vaccine suspension of the same series was frozen in a mixture of dry ice and alcohol at a temperature of -78°; the other, in a refrigerator chamber at -30°. Suspensions frozen by both methods were dried in the same chamber of a drier. In other experiments the survival of the microbes

Table 1

Duration of Cooling EV Vaccine Suspension with Freezing in a Refrigerator Chamber and in a Mixture of Dry Ice and Alcohol to -30° and -70°

(1) Условия замораживания	(2) Температура, °	(3) Количество суспензии, мл	(4) Среднее время охлаждения от температуры 22–23° до									(5) Число опытов
			10°	0°	−10°	−20°	−30°	−50°	−60°	−70°		
(6) Холодильная камера	−30	2	1 мин. 35 сек. (9)	3 мин. 39 сек.	9 мин. 50 сек.	16 мин. 16 сек.	21 мин. 48 сек.	—	—	—	—	5
(7) Смесь твердой углекислоты и спирта	−30	2	30 сек. (10)	60 сек.	1 мин. 7 сек.	3 мин. 7 сек.	4 мин. 10 сек.	—	—	—	—	5
(8) То же	−78	2	22 сек.	32 сек.	40,5 сек.	48 сек.	55,5 сек.	1 мин. 2 сек. (11)	1 мин. 20 сек.	2 мин.	3 мин. 20 сек. (12)	5

1. freezing conditions; 2. temperature, degrees; 3. quantity of suspension, cc; 4. average cooling time from a temperature of 22-23° to:; 5. number of experiments; 6. refrigerator chamber; 7. mixture of dry ice and alcohol; 8. the same; 9. one minute and 35 seconds; 10. seconds; 11. one minute and ~~two~~ seconds; 12. three minutes and 20 seconds.

Table 2

Rate of Cooling of EV Vaccine Suspension with Freezing in a Refrigerator Chamber and in a Mixture of Dry Ice and Alcohol

(1) Условия замораживания	(2) Температура,°	(3) Количество суспензий, мл	(4) Скорость охлаждения (град/сек) от 22–23° до													(5) Число опытов
			10°	0°	−10°	−12°	−14°	−16°	−18°	−20°	−30°	−40°	−50°	−60°	−70°	
(6) Холодильная камера	−30	2	0,1	0,08	0,03	0,02	0,02	0,03	0,04	0,05	0,03	—	—	—	—	5
(7) Смесь твердой углекислоты и спирта	−30	2	0,4	0,33	0,33	0,2	0,1	0,1	0,1	0,1	0,15	—	—	—	—	5
(8) То же	−78	2	0,5	1,0	1,2	1,3	1,3	1,3	1,3	1,3	1,3	1,4	0,5	0,2	0,1	5

1-8. Same as for Table 1.

was determined in the vaccine with rapid freezing of the suspension in a mixture of dry ice and alcohol (with a temperature of -30°) and slow freezing in a refrigerator chamber. The number of living microbes was determined four days after drying by the generally accepted method on agar plates. In all cases the same agar series was used.

In addition, we calculated the average figures for the survival of microbes in industrial series of EV plague vaccine produced in 1960. Of the series 53 had been frozen in dry ice (-78°); the next 53 series, in a refrigerator chamber (-30°).

The results of these experiments are shown in Tables 3, 4 and 5.

Table 3

The Effect of the Temperature of Preliminary Freezing on the Number of Living Microbe Cells in Frozen-Dried Plague Vaccine (Experimental Series)

(1) Сроки исследования	(2) Температура замораживания, °	(3) Число серий	(4) Число микробных клеток в исходной взвеси по оптическому стандарту, в 1 мл, 10^9	(5) Выживаемость, %	(6) Процентное соотношение
(7) До высушивания	—	23	78,2	51,0	—
(8) После высушивания	—30 (М)*	25	63,7	39,3	100
	—78	25	65,7	32,1	81,7
(9) При —30° (М) больше живых микробных тел, чем при —78,° на:				7,2	18,3

* (M) -- slow freezing

1. time of examination; 2. freezing temperature; 3. number of series; 4. number of microbes in the original suspension by the optical standard in cc, 10^9; 5. survival, percent; 6. percentage; 7. before drying; 8. after drying; 9. at -30° (M) there were more living microbes than at -78° to the extent of:

As is seen from Table 3, with slow freezing in a refrigerator chamber to -30° there were 18.3 percent more living microbes in the vaccine suspension than on freezing in a mixture of dry ice and alcohol to -78°. Statistical analysis of the data on survival of microbes in the EV vaccine at different freezing temperatures showed the significance of the results obtained.

The data of Table 4 show that despite the same end temperature to which the suspension of the plague vaccine was frozen, with slow freezing

Table 4

The Effect of the Freezing Rate on the Survival of Microbes in Dry Living EV Plague Vaccine

(1) Температура замораживания,*	(2) Число опытов	(3) Средний процент микробных тел к оптическому стандарту	(4) Процентное соотношение
−30 (M)	6	36,2	100
−30	6	30,9	85,3
(5) При −30° (M) больше живых микробных тел, чем при −30°, на:		5,3	14,7

1. freezing temperature; 2. number of experiments; 3. average number of microbes in percent of the optical standard; 4. percentage relationship; 5. at -30° (M) there were more living microbes than at -30° to the extent of:

Table 5

Survival of Microbes in Frozen-Dried EV Plague Vaccine After Preliminary Freezing at a Temperature of -30° and -78° (Production Series)

(1) Сроки исследования	(2) Температура замораживания,*	(3) Число серий	(4) Число микробных тел в исходной взвеси по оптическому стандарту, в 1 мл, 10^9	(5) Выживаемость, %	(6) Процентное соотношение
(7) До высушивания	—	83	79,5	49,2	—
(8) После высушивания	−30°	53	63,0	39,0	100
	−78	53	64,6	32,6	83,6

* slow freezing.

1-8. same as Table 3.

(refrigerator chamber) the survival of the microbes was 14.7 percent more than with rapid freezing (mixture of dry ice and alcohol).

The average survival of the microbes in 53 production series, frozen in a refrigerator to -30°, was 16.4 percent greater than in 53 series frozen in dry ice to -78° (Table 5).

In the freeze-drying method part of the microorganisms dies during preliminary freezing, but we cannot determine the number of microbes which die in the suspension this way, because the thawing process also causes death of some of the microbes. In connection with this it is difficult to say when more cells die: during the preliminary freezing or during the drying. However, comparing some data, it may be supposed that during preliminary freezing a considerable percentage of the microorganisms dies.

If we take the number of living microbes in the suspension prior to drying as 100 percent, with slow freezing at a temperature of -30° and subsequent drying 23 percent of the microbes died in our experiments. With rapid freezing at a temperature of -78° and similar drying conditions the mortality amounted to 37 percent. These results were obtained from a study of 53 production series of EV plague vaccine frozen at a temperature of -30° and the same number of series frozen at a temperature of -78°. We obtained similar data from the freezing of 25 experimental vaccine series under corresponding temperature conditions: in the former case the mortality amounted to 20 percent; in the latter case, 32 percent. If we take the death of the microbes during the course of rapid freezing at -78° as 100 percent, the number of microorganisms which died at -30° will amount to 62 percent. The difference in the number of microbes which died during freezing to -30° and -78° amounts to 38 percent. Therefore, it may be considered that a considerable number of the microbes dies in the preparation of dry vaccine during the preliminary freezing.

Conclusions

1. Survival of microbes in the EV plague vaccine during slow freezing in a refrigerator chamber to -30° is 16-18 percent greater than during freezing in a mixture of dry ice and alcohol to -78°.

2. Survival of microbes in the EV plague vaccine depends on the freezing rate. With slow freezing (22 minutes) to -30° the number of living microbes in the EV vaccine is 14.7 percent greater than with fast freezing (in four minutes) to the same temperature.

3. During the course of preparation of dry living plague EV vaccine a considerable number of the microbes dies during the preliminary freezing.

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The Effect of Glucose on the Growth of the Plague Microbe under Aeration Conditions

F. K. Drozdovskaya, N. K. Murav'yeva and A. N. Kraynova

(Saratov)

The role of glucose in the nutrition of the plague microbe began to be studied only recently, particularly in connection with its cultivation under conditions of aeration on media made of acid hydrolysates of casein. With this aim in view, the work of Higuchi and Carlin (1957, 1958) is of interest; they showed the important role of carbohydrate components in the nutrition of the plague microbe, and Wessman and coauthors (1958) determined the relationship between the effect of glucose on the growth of the plague microbe and the cultivation temperature.

The aim of our work was a study of the effect of glucose on the growth of the plague microbe under aeration conditions on different nutrient media.

Material and Methods

The effect of glucose on the growth of the plague microbe was studied on Hottinger's bouillon, ordinarily used in the production of plague vaccine, and bouillon made of an acid casein hydrolysate. The latter was prepared from food acid casein (Saratov Dairy No 1) containing 12.25 percent total nitrogen by two methods.

In the first case the casein was hydrolyzed with a mixture of sulfuric and hydrochloric acids for six hours at 115° with subsequent decolorization with five percent activated charcoal and elimination of the sulfuric acid residues by means of barium hydroxide and the hydrochloric acid residues by neutralization with two percent sodium hydroxide solution (Ye. E. Bakhrakh and others, 1960). In the second case the casein was hydrolyzed with 6.5 percent hydrochloric acid for three hours at 120°.

The demineralization was carried out by the method of Ye. E. Bakhrakh and others using an EDE-10P anion-exchange resin.

For preparation of the bouillon the casein hydrolysate was neutralized with two percent sodium hydroxide solution to a pH of 7.2-7.4 and diluted with tap water until it contained 200 milligrams percent of amine nitrogen. In the bouillon of the first hydrolysate the quantity of chloride amounted to 0.5-0.6 percent; in the second hydrolysate, 0.3 percent.

In the study of the effect of inorganic substances on the growth of the plague microbe, the following salts were added to the neutral casein bouillon prior to sterilization: $K_2HPO_4 \cdot 3H_2O$, 0.25 percent; $MgSO_4 \cdot 7H_2O$, 0.06 percent; $MnSO_4 \cdot 7H_2O$, 0.0003 percent; $FeSO_4 \cdot 7H_2O$, 0.003 percent; sodium gluconate, 0.2 percent; sodium citrate, 0.036 percent. Sodium thiosulfate and glucose were sterilized separately and added to the medium before culture in a concentration of 0.6 and 0.1 percent respectively. The basis for the introduction of the corresponding salts into the medium was constituted by the work of Higuchi and Carlin (1957) and Ye. E. Bakhrakh and others.

The media were sterilized for 30 minutes at 110°. When an abundant precipitate of phosphates came down it was filtered off, and the bouillon was sterilized again.

In cultivation of the plague microbe the aeration was carried out by shaking the cultures in test tubes in a water incubator of a Warburg apparatus or in small bottles, on a rocker. The bouillon was poured out into test tubes in quantities of 2.5 cc; into small bottles, in quantities of 25 cc. The seeding was made with a two-day culture of the plague microbe (EV vaccine strain) in a quantity of 100,000,000 per cc. The experimental samples were grown out at 28°.

The total concentration of microbes was determined nephelometrically in an FEK-N-54 colorimeter-nephelometer; the number of living microbes was determined by plating out on blood agar plates; phosphates, by the modified Fiske-Subbarow method with ascorbic acid; chlorides, by the Volhard method; amine nitrogen, by formol titration by the Gavriolov-Sorensen method; total nitrogen, by formol titration after calcining the sample with sulfuric acid by the method of Ye. E. Bakhrakh and Viktorova (see the present collection); the pH was determined potentiometrically; reducing substances were determined by the Hagedorn-Jensen method. The purity of the cultures during cultivation was checked on by microscopy of smears of the culture and by plating out on agar plates.

Results of the Investigation

The experiments were begun with the determination of the optimum glucose concentration needed for the growth of the plague microbe on Hottinger's bouillon. The following carbohydrate concentrations were studied: 0.05, 0.1, 0.2, 0.3 and 0.5 percent. The cultures were grown out for 12 hours in test tubes in the water incubator of the Warburg apparatus.

The results of the experiments on the determination of the effect of different contents of glucose on the total yield of cells are shown in Table 1.

Table 1

The Effect of Different Glucose Concentrations on the Growth of the Plague Microbe on Hottinger's Bouillon

Концентрация глюкозы, % (1)	Число микробных тел в 1 мл, 10^9 (2)	pH	Число микробных тел в 1 мл, 10^9	pH
	6 часов (3)		12 часов	
0	1,25	7,36	3,0	7,55
0,05	1,25	7,07	3,25	7,12
0,1	1,7	7,01	4,25	6,8
0,2	1,7	7,0	4,25	6,7
0,3	2,0	6,88	4,8	5,64
0,5	1,5	6,85	3,3	5,0

1. glucose concentration, percent; 2. number of microbes per cc, 10^9; 3. hours

From Table 1 it is seen that the addition of glucose to the medium improves the growth of the plague microbe.

The maximum effect was observed when the glucose content was equal to 0.1-0.3 percent. However, increasing the carbohydrate concentration to 0.3 percent led to a marked acidification of the medium (pH 5.62) through the formation of acid breakdown products of glucose. Therefore, it was more advisable to utilize lower glucose concentrations (0.1-0.2 percent) which did not change the reaction of the medium much.

Repeated experiments on the study of the effect of glucose on the growth of the plague microbe on bouillons made of different series of

Hottinger's hydrolysate showed that the addition of glucose to the medium in a quantity not exceeding 0.1 percent in many cases actually contributes to the growth of the plague microbe. Thereby, no sharp reduction of the pH in the medium was noted.

In various experiments glucose did not improve the growth of the plague microbe but sometimes even suppressed it. The latter can be explained primarily by the accumulation of acidification products of glucose (pH equals 5.5-6.1), further utilization of which was difficult (Table 2).

Table 2

The Effect of Glucose on the Growth of the Plague Microbe on Various Series of Hottinger's Bouillon

Серия бульона Хоттингера (1)	Число микробных тел в 1 мл, 10^3 (2)	pH	Число микробных тел в 1 мл, 10^3 (2)	pH
	(3) с глюкозой (0,1%)		(4) без глюкозы	
15	7,0	7,8	4,0	8,0
13	10,4	7,7	11,8	8,2
13	2,5	5,8	5,5	8,0
6	9,4	8,0	7,8	8,4
6	9,0	8,0	7,5	8,5
21	5,0	7,1	2,3	7,5
21	3,0	6,1	13,2	8,2
21	4,8	5,5	5,0	8,1
10	11,5	7,6	9,5	8,2

Note. Duration of growth 24 hours.

Key: 1. series of Hottinger's bouillon; 2. number of microbes per cc, 10^3; 3. glucose (0.1 percent); 4. without glucose.

The results of chemical analysis of Hottinger's bouillon and bouillon made of an acid casein hydrolysate are shown in Table 3. They show that the latter contains much less phosphate.

Subsequently, we studied the effect of phosphates and some other salt components of the medium on the growth of the plague microbe on casein bouillon in the presence of glucose. For the experiments bouillon which had been demineralized with barium hydroxide was used.

The inorganic phosphorus content in the casein bouillon was brought up to 43-50 milligrams, which was equivalent to 0.25 percent of K_2HPO_4.

It was determined that in the absence of glucose growth on a medium containing phosphates was almost the same as growth on a medium

Table 3

Results of Chemical Analysis of Hottinger's Bouillon and Bouillon Made of Acid Casein Hydrolysate

Среда	Содержание в среде (в мг%)					
	азота		фосфора		хлоридов	редуцирующих веществ
	общего	аминного	общего	неорганического		
Бульон Хоттингера	340	180	250—260	22—30	260—300	70—95
Бульон из гидролизата казеина	300	200	10—20	5—10	400—600	70—95

1. medium; 2. content of the following in the medium (in milligrams percent); 3. nitrogen; 4. phosphorus; 5. total; 6. amine; 7. inorganic; 8. chlorides; 9. reducing substances; 10. Hottinger's bouillon; 11. bouillon of casein hydrolysate.

without phosphates. However, in the presence of glucose the presence of phosphates appreciably increased the yield of the bacterial mass.

Undoubtedly, the role of phosphates is not limited to increasing the buffering capacity of the casein medium. Phosphoric acid and its salts take a direct part in the carbohydrate metabolism of microbes. In the absence of phosphates the rate of carbohydrate metabolism is reduced, and growth of the culture slows down.

The results of experiments on the study of the effect of some other saline components of the medium on the growth of the plague microbe are shown in Table 4.

From Table 4 it follows that in the absence of glucose these salts did not stimulate the growth of the plague microbe. In the presence of glucose the addition of them contributed to greater accumulation of the bacterial mass. A particularly favorable effect on the effect of the plague microbe was exerted by potassium phosphate (medium 2) and sodium thiosulfate (medium 5). These rules and regulations were observed in the study of growth of the plague microbe on casein hydrolysate demineralized with the EDE-10P anion-exchange resins (Ye. E. Bakhrakh and others).

We also made a study of the effect of sodium gluconate and sodium citrate on the growth of the plague microbe in casein bouillon. As is well known, sodium citrate is added for the purpose of preventing the precipitation of phosphates, and sodium gluconate can be an intermediate

Table 4

The Effect of Glucose on the Growth of the Plague Microbe on Casein Bouillon ir the Presence of Different Salts

Номер среды (1)	Соли, введенные в бульон (2)	Число микробных тел в 1 мл, 10^9 (3)	pH	Число микробных тел в 1 мл, 10^9 (3)	pH
		с глюкозой (4)		без глюкозы (5)	
1	—	1,4	6,1	3,7	6,8
2	K_2HPO_4	4,5	6,6	4,2	7,3
3	$K_2HPO_4 + MgSO_4 + MnSO_4$	4,8	6,7	3,8	7,1
4	$K_2HPO_4 + MgSO_4 + MnSO_4 + FeSO_4$	4,8	6,7	4,5	7,3
5	$K_2HPO_4 + MgSO_4 + MnSO_4 + FeSO_4 + Na_2S_2O_3$	6,5	7,3	4,5	7 4

Note. Duration of growth 12 hours.

Key: 1. number of the series; 2. salts introduced into the bouillon; 3. number of microbes per cc, 10^9; 4. with glucose; 5. without glucose.

product in the breakdown of glucose (under conditions in which the enzymes of the hexose monophosphate series function in the cells of the plague microbe) and as the result of this can be utilized by the plague microbe.

The addition of sodium citrate and gluconate to basic medium 4 did not improve the growth of the plague microbe (media 6 and 7) (Table 5).

If to the medium containing gluconate or sodium gluconate and sodium citrate sodium thiosulfate is added, however, the yield of the microbial mass was considerably increased, particularly in the presence of glucose. The favorable effect which we noted on the growth of the plague microbe by sodium thiosulfate confirms the special part of sulfur-containing compounds in the metabolism of the plague microbe.

According to data obtained in the works of a number of authors (Rao, 1940); Rockenmacher and others (1950); I. V. Domaradskiy, 1955; I. V. Domaradskiy and V. A. Ivanov, 1957 and others, sulfur-containing amino acids (methionine, cystine) are essential for the growth of the plague microbe. At the same time, Doudoroff (1943) and Englesberg (1952) showed the interreplaceability of some sulfur-containing amino acids with inorganic compounds which contain sulfur. Specifically, for growth of the plague microbe it was possible to replace the cystine in the medium with sodium thiosulfate.

If we consider the low content of cystine in casein and its partial

Table 5

The Effect of Glucose and Sodium Thiosulfate on the Growth of the Plague Microbe in Casein Bouillon

Номер среды	Соли, введенные в среду 4	Число микробных тел в 1 мл, 10^9	pH	Число микробных тел в 1 мл, 10^9	pH
		с глюкозой		без глюкозы	
4	—	7,5	7,0	6,0	7,6
5	Тиосульфат натрия	22,0	8,2	10,6	8,2
6	Глюконат натрия	7,8	6,9	7,8	7,1
9	Глюконат + тиосульфат	19,4	8,1	10,5	8,1
7	Цитрат натрия	7,7	6,7	6,7	7,7
8	Глюконат+цитрат+тиосульфат	27,0	8,3	11,0	8,1
	Бульон Хоттингера серия 13	10,4	7,7	11,8	8,2
	(11) . . . 6	9,4	8,0	7,8	8,4

Note. The duration of the growth was 24 hours.

Key: 1. number of medium; 2. salts introduced into medium 4; 3. number of microbes per cc, 10^9; 4. with glucose; 5. without glucose; 6. sodium thiosulfate; 7. sodium gluconate; 8. gluconate plus thiosulfate; 9. sodium citrate; 10. gluconate plus citrate plus thiosulfate; 11. Hottinger's bouillon, series 13.

destruction through hydrolysis with inorganic acids, the need of the plague microbe for sodium thiosulfate when it grows on media composed of acid casein hydrolysate becomes understandable. At the same time, sodium thiosulfate is a factor reducing the redox potential of the medium, which is particularly important in the first few hours of growth of the plague microbe culture (Ye. E. Bakhrakh, 1950).

Englesberg (1952) notes the favorable effect of the combination of thiosulfate with methionine on growth of the plague microbe. In our experiments the addition of methionine to media containing sodium thiosulfate did not improve the growth of the culture.

In subsequent experiments we decided to clarify the nature of the change in the reaction of the medium during the course of growth of the plague microbe on various media under aeration conditions.

For this work Hottinger's bouillon and casein bouillon were used demineralized with an ion-exchange resin, with inorganic salts and without

them (for the purpose of judging the effect of buffering in maintaining the pH at a definite level). The results of the experiments are shown in Figs. 1 and 2.

With the growth of the plague microbe under aeration conditions on all bouillons investigated a certain reduction of the pH of the medium was noted during the first few hours of growth with a gradual alkalinization with subsequent growth of the culture. Similar rules and regulations had been determined by Ye. I. Korobkova (1929) during the incubation of the plague microbe under hospital conditions. However, on different series of Hottinger's bouillon and with different durations of growth of the plague microbe the absolute pH readings are different. Thus, in the 21st series of bouillon slight pH changes were observed and the beginning of alkalinization of the medium was noted only in a 16-hour culture. In the 10th series the increase in the pH became noticeable in a seven-hour culture. After the addition of glucose to the 21st series of bouillon the pH of the medium dropped to 5.4 and no alkalinization of the medium was noted even after 24 hours of growth (pH 6.1). The growth of the plague microbe stopped. Introduction of carbohydrate into bouillon of the 10th series caused no marked change in the reaction of the medium toward the acid side and somewhat delayed alkalinization of the medium, which contributed to an increase in the yield of bacterial mass.

From Fig. 2 it is seen that the addition of salts to the casein bouillon reduces the variations of pH during the growth process, while the presence of glucose in the medium contributes to a more appreciable reduction in the pH and a prolongation of the period of alkalinization of the medium.

Considering the alkalinization of the medium occurring with prolonged cultivation of the plague microbe and the unfavorable effect of this alkalinization on the viability of the cells, we decided to maintain the pH at a certain level (7.2-7.3) during the growth process by the introduction of glucose in fractional portions and to establish the effect of this method of adding glucose on the accumulation of the bacterial mass for 24 hours (Table 6).

The data given in Table 6 attest to the fact that it is best to add glucose to the casein bouillon before streaking in a quantity of 0.1 percent with subsequent additions to 0.05 percent at the time of alkalinization of the medium. With growth of the plague microbe on Hottinger's bouillon the glucose should be added only at the time that alkalinization begins and to a final concentration of no more than 0.1 percent.

Therefore, fractional addition of glucose during the growth process of the plague microbe not only contributes to maintaining the pH at a certain level but also assures an increase in the yield of the bacterial mass.

Table 6

The Effect of Addition of Glucose on the Growth of the Plague Microbe during the Course of Growth of the Culture

Бульон	Время внесения глюкозы в среду	Конечная концентрация глюкозы, %	Величина pH при продолжительности роста, часы				Количество микробных тел в 1 мл, 10^9
			16	18	21	24	
Казеиновый	—	0	7,3	—	7,5	7,5	5,0
"	До посева	0,1	7,0	7,1	7,2	7,8	9,0
"	Через 16 часов	0,1	7,3	—	6,9	7,0	6,8
"	До посева	0,1					
	Через 18 часов	0,1	7,0	7,1	7,2	7,3	9,3
	До посева	0,1					
	Через 18 часов	0,05	7,1	7,2	7,2	7,4	12,4
Хоттингера	—	0	7,5	—	—	8,1	5,0
	До посева	0,1	5,8	—	—	5,5	4,8
	Через 16 часов	0,1	7,5	—	—	6,2	8,0
	" 16 "	0,05					
	" 18 "	0,05	7,5	6,9	—	6,9	9,4

1. bouillon; 2. time of introduction of the glucose into the medium; 3. final glucose concentration, percent; 4. value of the pH with growth duration of the following number of hours:; 5. number of microbes per cc, 10^9; 6. casein; 7. Hottinger's; 8. before streaking; 9. after 16 (18) hours.

Conclusions

1. The addition of glucose to the bouillon improves the growth of the plague microbe under aeration conditions.

2. The optimum glucose concentration for growth of the plague microbe depends on the buffering capacity of the medium; as a rule, the quantity of glucose added should not exceed 0.1 percent.

3. Taking into consideration the lack of standardization of Hottinger's bouillon with respect to buffering capacity or the presence of carbohydrates in the original bouillon, it is recommended that glucose be added to it only at the time of beginning of alkalinization of the medium in fractional portions, to a final concentration of no greater than 0.1 percent.

4. Glucose may be added to bouillon made of acid casein hydrolysate before streaking in a quantity which does not exceed 0.1 percent and then during the growth process, beginning with the time of alkalinization of the culture, fractionally, to 0.05 percent, maintaining the pH of the medium on a level of 7.2-7.3.

5. The favorable effect of sodium thiosulfate on growth of the plague microbe on casein bouillon containing certain inorganic salts and glucose has been noted.

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The Problem of Conditions Affecting the Determination of the Number of Living Microbes in Dry I-17 Plague Vaccine

A. N. Kraynova and I. V Liskina

(Saratov)

The number of living microbes contained in dry I-17 vaccine is one of the basic indices of its immunogenic properties, although not the only one.

It is well known that prior to 1953 living dry plague vaccine was produced with a content of no less than five percent living microbes. As far as the inoculation dose is concerned, regardless of the percentage of living microbes (five percent or over), in all cases this dose was equal to 1,500,000,000 microbes.

At the present time, according to existing instructions on the preparation and utilization of dry living plague vaccine, one man-dose for inoculations is determined not only by the concentration of microbes (in accordance with the optical standard) but also by an obligatory count of the number of living microbes, which should amount to 300,000,000 microbes for subcutaneous and intradermal methods and 3,000,000,000 microbes for percutaneous method, which amounts to 20 percent living microbes in the total number in the vaccine, that is, 1,500,000,000 for subcutaneous and 15,000,000,000 for the percutaneous method.

Because a man-dose of vaccine is calculated by the number of living microbes, a check on the survival of the vaccine is very important. An error in the determination of the number of living microbes sometimes leads to an incorrect calculation of the dose of vaccine, which can cause an undesirable, stronger reaction in the inoculees.

Determination of the number of living cells in living vaccines has been made to date exclusively by the culture method. However, it is well known that the culture method suffers from serious shortcomings: aside from the time needed for the determination (five days), its accuracy is not always satisfactory and depends on many factors: the quality of the nutrient medium, the cultivation conditions, the accuracy with which the suspensions are diluted, the nature of distribution of the cells in the material being investigated, the temperature conditions of the control,

et cetera. In connection with this, many investigators have made attempts to replace the culture method of determination of the number of living cells with various other methods: 1) cytochemical (V. G. Drobot'ko, 1934; M. A. Peshkov, 1955 and others); 2) biochemical (Yu. K. Veysfeller and A. M. Yengalycheva, 1955; I. V. Liskina, 1960 and others); 3) cultural-morphological (Valentine and Bradfield, 1953); V. V. Akimovich and T. I. Dudova (1960); 4) microrefractometric (B. A. Fikhman, 1959) and others. However, none of these methods has been extensively used in practice. The culture method at the present time is the main method of determining the number of living microbes in a vaccine, although it is not standardized nor absolutely accurate and needs improvement with respect to the selection of better-quality media as well as in standardization of control conditions (meaning the laboratory temperature). It is well known that the survival of plague bacteria in the form of a suspension and in the dry state depends on the temperature at which it is kept. In a suspension of the plague microbe kept at 28-37° the number of living bacteria decreases considerably by comparison with a suspension kept at a temperature of 4-8°.

Chinese authors, Ch'en Chen-jen, Lou Shih-liang and Liu Te-chen, believe that when dry vaccine is dissolved in physiological saline solution at a temperature of less than 19° the percentage of living microbes obtained is higher than when this vaccine is dissolved in physiological saline at a temperature of 28-37°.

In the present study the aim was to check the effect of the ambient temperature on the results of the control for the microbe survival in the vaccine.

With this aim in view, we used controls for 90 different series of dry plague 1-17 vaccine. In each ampule of vaccine the total number of microbes was determined in accordance with the optical standard, and two series of parallel tenfold dilutions were prepared from it in physiological saline solution cooled to 4-8° in the first series (control) and left at room temperature (20-28°) in the second (experiment). Thereby, we adhered to the following sequence. The contents of the ampules were dissolved in physiological saline solution at room temperature (20-24-28°). From the solution obtained the dilutions of the first series were obtained. Then the ampule containing vaccine was put into a cooled water bath, after which work was done on preparing dilutions of the second series. Therefore, the preparation of the vaccine dilutions in the cooled physiological saline was always carried out 15-20 minutes later. Otherwise, there were no deviations from the generally accepted method proposed by Ye. I. Korobkova.

We divided all the vaccine series studied into three groups (with 30 series in each): the first group included vaccine series in which from

zero to 10 percent of the microbes survived (under ordinary control conditions, that is, at room temperature); the second group, with a survival of 10 to 20 percent; the third group, 20 percent or more (Table 1).

Table 1

Comparative Data for the Determination of the Survival of Microbes in Dry Living Plague 1-17 Vaccine as a Function of the Temperature of the Physiological Saline Solution in the Test-Tubes

Номер группы	Количество серий вакцины	Выживаемость микробов, %	Средняя выживаемость вакцины, % при температуре		Среднее количество доз в вакцине при температуре	
			25–28°	4–8°	25–28°	4–8°
1 Первая	30	0 - 10	5,1 (2,7 - 6,1)	11,6 (9,1 – 13,3)	—	19
2 Вторая	30	10—20	15,1 (10,2 – 17,3)	24,7 (20,1 – 27,1)	34	56
3 Третья	30	20 и выше	29,6 (24,1—32,4)	38,7 (33,4—41,1)	68	89

1. number of the group; 2. number of vaccine series; 3. survival of microbes, percent; 4. average survival of the vaccine, percent; 5. average number of doses in the vaccine; 6. at a temperature of; 7. first; 8. second; 9. third; 10. 20 or more.

In all groups the survival rate of the microbes increased slightly when the control was carried out at 4-8°. Thus, in the first group the survival of vaccine at the ordinary control temperature was equal to 5.1, as the result of which the vaccine was rejected, whereas at 4-8° the survival of the vaccine was equal to 11.6 percent; in this case the vaccine is suitable for use and contains 19 man-doses. In the second group at the usual control temperature the survival of the vaccine was equal to 15.1 percent, which amounts to 34 man-doses; at 4-8°, 24.7 percent, which amounts to 56 man-doses. Finally, in the third group, at the usual control temperature we obtained a survival of 29.6 percent, which amounts to 68 man-doses; at 4-8°, 38.7 percent, equal, respectively, to 89 man-doses.

Therefore, in all 90 vaccine series investigated a higher percentage of living microbes was obtained when the control was carried out in the cold, that is, at 4-8°. With increase in the percentage of living microbes there was a corresponding increase in the number of man-doses in the vaccine. In the determination of the survival of microbes in the vaccine diluted in physiological saline at room temperature the percentage of living microbes drops and, therefore, an error is made in the vaccine dosage. In the same vaccine ampule, depending on temperature conditions

under which the control test is performed for survival of microbes, a different number of man-doses is determined (68-89).

On the basis of what has been presented above, we suggest carrying out the control for determination of the number of living microbes in the vaccine at a strictly constant temperature, namely at 4-8°, considering that the plague microbe is susceptible to physical effects, including temperature effects. The plague microbe is preserved on nutrient media as well as in a dry state at low temperatures; all phases of the work of vaccine production (sedimentation, dilution, pouring, et cetera) are carried out at low temperatures. Therefore, the control for determination of the number of living microbes, associated with dilution of the vaccine by millions of times in a medium less favorable for the microbe (physiological saline), should also be carried out at a low temperature.

In practice we repeatedly came up against the fact that in dried vaccine kept in the storeroom, at the time of a repeat check of it after six months storage the percentage of living microbes exceeded that which had been determined at the time the vaccine was released for utilization, or when a repeat check was made after one year of preservation the percentage of living microbes was higher than at the time of the first recheck (although the nutrient medium was the same). This can be explained by the fact that repeated checks on the vaccine were made at different times of the year, and the laboratory temperature was different in connection with this. This error can be excluded by means of establishing a constant temperature when the control is carried out for survival of the vaccine.

In addition, we came across a phenomenon in which the vaccine which had been prepared in the summer showed an inadmissible survival (less than 10 percent) and had to be discarded; however, when it was rechecked for survival in the autumn months a permissible survival was found, and the vaccine was considered suitable for use. This once again confirms the need for establishing constant temperature conditions in the vaccine survival control.

Therefore, without denying the prime significance of the nutrient medium for determining the number of living microbes in 1-17 vaccine, we have concluded that the temperature at which the given control test is carried out also exerts an effect on the results of the determination of the number of living microbes.

Conclusions

1. The temperature conditions under which the vaccine is diluted for the purpose of determining the percentage of living microbes in 1-17 plague vaccine exert an influence on the control results obtained.

2. When the vaccine is diluted with physiological saline which has been cooled to 4-8° the percentage of living microbes in the vaccine obtained is much higher than when the control is performed with physiological saline whose temperature is equal to 25-28°.

3. For the purpose of a more accurate determination of the number of living microbes in dry 1-17 vaccine it is essential to standardize the control conditions.

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The Use of Casein Media in Vaccine Production

N. K. Murav'yeva, A. N. Kraynova and O. P. Maslova

(Saratov)

At the present time, in the majority of laboratories in which living plague vaccine is prepared by the subsurface method, beef media are used. Along with their positive features, the latter have negative qualities: they are costly, nonstandard, complicated and require painstaking work in their preparation. Therefore, a search for a cheap simple media which will assure the full-scale development of microbes with preservation of their immunogenic activity is a problem of current importance.

In the production of bacterials every year casein media are acquiring progressively greater importance. By comparison with beef, casein is a more homogeneous protein, and for this reason its utilization as a source of protein makes the production media more like semisynthetic media. Casein is different from the other sources of protein raw material in having a higher protein content as well as in its lower content of impurities and extraneous matter when its types and variety are correctly selected. The casein includes all amino acids necessary for the growth of pathogenic bacteria and it is easily hydrolyzed; finally, which is very important for production, it is available, cheap, and can be preserved in the dry form.

The industry of the Soviet Union is putting out various types and varieties of casein: TU 153-54 food acid casein, technical acid casein GOST [All-Union State Standard] 1211-41 of the highest, first, second and third varieties, rennet casein and others, which are different in their properties, technical preparation and purposes.

Until recently the majority of institutes used any casein and did not make an definite requirements on the quality of the latter, in connection with which various kinds of difficulties were encountered (poor filtration, large precipitate of undigested protein in the hydrolysate, et cetera). In a number of institutes and abroad only technical acid casein is considered suitable for preparation of media. I. N. Vinogradova, F. F. Tsurikov, A. A. Tret'yakova and N. A. Palkina (1958) recommended food acid casein on which definite requirements are to be made for the preparation of nutrient media in the production of bacterials.

Through the studies of a number of authors it has been determined that casein media prepared from casein by different methods: hydrolysis of acid and alkali, by pancreatic digestion) are perfectly suitable for cultivation of the plague microbe. Casein media obtained by the method

of acid hydrolysis of casein have been used for more than 10 years at the Haffkine Institute in India for the purpose of obtaining plague and cholera vaccines (Sokhey, Habbu, Bharucha, 1950); Higuchi and Carlin (1957). Vaccines prepared on these media have proved to be quite effective.

The method of preparing an enzymatic hydrolysate of casein was worked out by Z. I. Sosina (1947). In 1955, it was modified somewhat for cultivation of the plague microbe by Z. A. Yurgina (1960), and casein agar from an enzymatic hydrolysate of casein was recommended for diagnostic purposes. S. I. Zaplatina and coauthors (1959), after testing a casein-hydrolysate agar according to Z. A. Yurgina's recipe, considered it possible to recommend casein media prepared by tne enzymatic hydrolysis of casein for the production of living dry plague vaccine.

Under production conditions the methods of splitting protein based on the use of acids or alkalis rather than enzymes are of great interest. Unfortunately, we did not have media made of acid hydrolysates at our disposal, but since the need for mastering the use of casein media under production conditions was obvious, we set before ourselves the task of studying the possibility of utilizing bouillon made of an enzymatic casein hydrolysate for the production of dry living plague vaccine under conditions of subsurface cultivation. For this purpose casein hydrolysates were prepared from dry casein obtained from the Saratov Dairy No 1, by enzymatic digestion of it with pancreas using Z. I. Sosina's method, but with the addition of a large quantity of pancreas.

In the present work we studied the following: the rate of growth of vaccine strains of the plague microbe, 1 and 17, on casein bouillon, their morphological, biochemical and biological characteristics when cultivated and kept for a long time on media made of an enzymatic casein hydrolysate, the capacity of the mass of microbes for settling after subsurface growth and the immunogenic properties of the vaccines cultivated on this medium.

For this purpose we prepared six different series of enzymatic casein hydrolysates (1, 2, 3, 8, 10, and 12), on which the casein bouillon was subsequently prepared. Hottinger's bouillon, usually used for production, was tne control medium. Both the experimental and control media contained 150 milligrams percent of amine nitrogen; the pH was 7.2. The initial production culture, subcultured directly from a standard culture, was seeded in small bottles containing 0.5 liter of bouillon and grown out at 28°. The bouillon in bottles set up for cultivation by the aeration method for 20-22 hours was seeded with a culture which developed. After this, the culture was poured out in a sterile fashion into the separate bottles and put into a refrigerator for settling of the mass of microbes.

Simultaneously, the pH, density of the microbial suspension, the purity of the culture which grew out were determined by means of performing bacteriological controls and determining the number of living microbes. After 24 hours of settling in the refrigerator the presence of a sediment was noted; then, the supernatant fluid was aspirated, and bivalent 1-17 vaccine was prepared from the microbial suspension in accordance with the requirements of the existing instructions for the preparation and control of living dry plague vaccine. The vaccine was dried on a header.

As the studies showed, cultivation of vaccine strains of the plague microbe on casein bouillon exerts no effect on their morphology, biochemical characteristics, capacity for capsule formation or agglutinating properties. When kept for a long time (about a year) in the dry state the morphological and biochemical properties of the plague microbe, strains 1 and 17, grown out on casein bouillon did not change.

Both strains grew out somewhat better on casein bouillon than on Hottinger's bouillon. The same should be noted with respect to settling of the microbial suspension and the yield of the mass of microbes per liter of medium (Table 1).

Although the concentration of microbes in casein bouillon is very slightly different, on the average, from that in Hottinger's bouillon, the yield of microbial mass per liter of casein medium is greater, which can be explained by the better settling of microbes in this medium. However, our task included, aside from obtaining a higher yield of the microbial mass, the preparation of the vaccine and study of its characteristics under conditions of long preservation. With this aim in view, we dried six experimental series of monovalent and bivalent vaccines made of 1-17 strains. Simultaneously, as controls mono- and bivalent vaccines of these strains which had been grown out on Hottinger's bouillon under the same conditions were dried.

Analyzing the qualitative indices of the dry vaccine obtained on casein bouillon, we concluded that it perfectly satisfies the basic requirements of existing instructions for the preparation and control of plague living dry vaccines.

The dried vaccine represents a bacterial mass which is white, of honeycombed structure, easily separated from the walls of the ampule. It dissolves readily in physiological saline solution, producing a homogeneous emulsion. The bacterial morphology in the smears made of the vaccine is typical; the cultures show the growth of colonies typical of the plague microbe. When checked on guinea pigs the vaccine was innocuous. All six experimental series of vaccine were bacteriologically pure and showed the permissible survival of bacteria. Despite the fact that the percentage of living microbes in various series of vaccine ranges from 10.8 to 39.9 percent from the very beginning of preparation, nevertheless,

Table 1

Comparative Characteristics of Growth Rates of Vaccine Strains of Plague Microbe 1-17 on Casein Bouillon and Hottinger's Bouillon by the Subsurface Method of Cultivation

Серия гидролизата (1)	Штамм (2)	Число посевов (3)	(4) Среднее содержание микробов (в млрд.), выращенных на бульоне		(5) Выход микробной массы с 1 л среды, 10^9 микробных тел	
			(6) казеиновом	(7) Хоттингера	(8) казеинового бульона	(9) бульона Хоттингер
1	1	3	4,6	4,0	943	285
2		1	6,0	3,5	3 058	1 800
3		3	5,3	4,3	23 868	280
8		4	2,9	3,1	290	84
10		6	4,7	4,0	2 881	413
12		1	5,5	1,5	850	88
1	17	2	4,7	3,7	105	499
2		1	6,0	6,0	539	452
3		2	4,2	5,5	1 007	254
8		5	3,3	3,5	1 540	446
10		6	5,0	4,1	2 982	1 373
12		2	4,0	1,7	2 891	176

1. series of hydrolysate; 2. strain; 3. number of cultures; 4. average content of microbes (in billions) grown out on the following bouillon:; 5. yield of microbial mass per liter of medium, 10^9 microbes; 6. casein; 7. Hottinger's; 8. casein bouillon; 9. Hottinger's bouillon.

when kept for three-nine months no considerable reduction of it occurred (Table 2).

The immunogenic properties of vaccines prepared on casein media were tested on 60 guinea pigs.

The guinea pigs were divided into six equal groups. A comparison was made of the immunogenic properties of vaccines which had been dried and freshly prepared on liquid and solid casein media and Hottinger's media. The vaccine was injected once subcutaneously in a dose of 1,000,000,000 microbes in a volume of one cc. The immunity was tested 21 days after vaccination by means of infecting the animal subcutaneously with a virulent strain of plague microbe 708 (200 MLD).

As the result of the experiment all the immunized animals survived more than 35 days; all the controls died. The experiment permits concluding that vaccines prepared on casein media and on Hottinger's medium ordinarily used in production possess the same immunogenic

Table 2

Survival of Microbes in Dry [illegible] ng 1-17 Plague Vaccine Grown Out on [illegible] Casein Bouill[illegible] and Kept for a Long Time

Серия	До сушки		В момент изготовления		После сушки		
	содержание микробных тел, 10^9	выживаемость, %	содержание микробных тел, 10^9	выживаемость, %	выживаемость (в %) при хранении в месяцы		
					3	6	9
1	80	52,8	60	39,9	36,0	30,9	30,0
2	20	63,5	14	26,7	25,0	25,3	24,9
3	100	62,4	65	10,8	10,7	10,3	10,5
4	55	55,3	45	23,8	22,7	20,1	20,4
5	60	45,4	40	19,1	19,1	18,6	17,9
6	10	71,3	8	12,3	12,0	10,0	10,1

1. series; 2. before drying; 3. at the time of preparation; 4. after drying; 5. content of microbes, 10^9; 6. survival, percent; 7. survival (in percent) when kept for: ; 8. months.

properties.

Agar media prepared from casein hydrolysates by the usual method, containing amine nitrogen in a quantity of 200-250 milligrams percent, are transparent, produce a typical plague microbe growth, and when hemolyzed rabbit blood is added (one percent) they may be used as media for determination of the number of living microbes in the vaccine.

Conclusions

1. A comparative study of the growth rate of plague microbe on bouillons made of enzymatic casein and beef hydrolysates by the Hottinger method showed that casein bouillons can be utilized for the production of plague vaccine together with beef bouillon.

2. With cultivation (in bottles) of vaccine strains of plague microbe 1 and 17 on casein bouillon by the method of continuous aeration for 20-22 hours the growth of the latter reaches the same concentrations and at the same time as on media made of Hottinger's beef broth.

3. The survival of microbes in vaccine prepared on casein bouillon is not inferior to that of microbes in vaccine prepared on Hottinger's bouillon either before or after drying and with observation for as long as nine months.

4. The immunogenic properties of the plague vaccines prepared on casein media satisfy the requirements of instruction.

5. Casein agar can be used for determining the number of living microbes in the vaccine if one percent fresh hemolyzed blood is added to it; it can be used this way on a par with media made of blood digests.

6. The use of casein media in the production of plague vaccine simplifies the production technology of hydrolysates and considerably reduces the cost of the vaccine.

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LABORATORY DIAGNOSIS AND METHODS OF INVESTIGATION

Obtaining Fluorescent Sera and Determination of the Possibility of Using Them for Rapid Identification of Plague and Cholera Pathogens

Ye. V. Chibrikova, L. P. Bazunova, P. K. Tabakov, I. I. Shurkina and Ye. I. Vel'ner
(Saratov)

The fluorescent antibody used for the first time by Coons and others (1942, 1950) for the detection of a bacterial antigen in the tissues of an animal has been used by investigators in recent years for the solution of various theoretical problems of microbiology and practical problems of laboratory diagnosis.

As is well known, a fluorescent antibody is an immune serum globulin combined chemically with a specially selected fluorochrome. Such globulin does not lose its power of reacting specifically with homologous antigen and, at the same time, acquires the property of fluorescing brightly in ultraviolet or blue-violet rays. Because of this, the stained or, as they are called, labeled antibodies form fluorescent complexes by reacting with the corresponding antigens and these are readily detected by fluorescence microscopy. The fluorescent antibody method, possessing a high degree of sensitivity, makes it possible to detect single microbes which have adsorbed the fluorescent antibody. Because specificity of the immune reactions underlies this adsorption, they can be identified directly in microscope preparations, that is, in as short a time as possible, by the presence of luminescence of the cells.

The fluorescent antibodies were used by a whole series of investigators for the demonstration and rapid identification of bacterial cells. Hobson and Mann (1957) report their utilization for the detection of enterococci and streptococci in smears of intestinal content. Moody, Goldman and Thomason (1956) as well as Thomason and others (1956) used the fluorescent antibody method for rapid identification of pseudoglanders [B. pseudomallei] bacteria in smears of artificially infected soil and from animals.

The possibility of successful utilization of corresponding fluorescent sera for rapid identification of the typhoid pathogen has been pointed out by I. O. Dashkevich and I. F. Mikhaylov (1957), I. F. Mikhaylov and Li-Li (1958); the pathogen of anthrax, by Ye. N. Levina (1958); dysentery bacteria, by Ye. A. Kabanova and A. I. Glubokina (1958); pathogenic group A streptococci, by Halpern and others (1958).

The aim of our work was to obtain fluorescent sera against plague and cholera pathogens and to determine the possibility of utilizing them for the rapid identification of homologous bacteria in smears of pure cultures and bacterial mixtures.

As the starting material for obtaining fluorescent antibodies we used diagnostic agglutinating sera of the "Mikrob" Institute obtained from horses (anticholera series 16, 11, 22 with a titer of 1:3200; antiplague series 444 and from a horse named "Pobeda" with a titer of 1:1000) as well as rabbit sera which we obtained from the immunization of animals, with titers from

1:800 to 1:2000.

The globulin fractions from these sera were obtained either by means of reprecipitation with equal volumes of saturated ammonium sulfite solution three times or by means of salting-out with dry sodium sulfate. In the former case, the reprecipitation and centrifugation were performed in the cold at a temperature of about 4°; in the latter case, at room temperature. The globulin solution obtained was dialyzed against buffered physiological saline solution (pH, 9.0) after the last precipitation until the ammonium sulfate or sodium sulfate was completely removed. Then, the washed globulin fractions were studied for their protein content by the Kjeldahl method and for serological activity in a full-scale agglutinin test by the generally accepted method. The globulins were labeled with the fluorochrome in those cases where they showed an agglutinin test titer no less than the original serum.

The main mass of globulin fractions which we obtained, as the study of them by the method of paper electrophoresis showed, were made up of gamma-globulins; alpha- and beta-globulins were present in small quantities; there were no albumins.

As the fluorochrome die, for marking the globulins we made use of fluorescein isocyanate (series 19, 20 and 21), prepared at the All-Union Scientific Research Institute of Chemical Reagents under the direction of G. I. Mikhaylov. (We should like to express our sincere appreciation to G. I. Mikhaylov and to science fellows of the Institute imeni N. F. Gamaleya, Ye. N. Levina and Ye. A. Kabanova, for putting the fluorescein isocyanate at our disposal.) Combination of the serum globulin fractions (conjugation) was carried out by the Coons and Kaplan method (1950). First, a mixture was prepared from solutions of globulin (1-2 percent), dioxan (15 percent), acetone (7 percent), carbonate-bicarbonate buffer at a pH of 9.0 (14 percent) and 0.15 M sodium chloride solution (to 100 percent). The mixture was cooled to 0°, and the solution of fluorescein isocyanate was added to it, drop by drop, with constant mixing with a mechanical mixer, calculating 5 milligrams per 100 milligrams of protein. The fine yellow precipitate which came down thereby was gradually partially dissolved over the course of 18 hours in the cold with constant mixing.

The nonreacting isocyanate was removed by means of dialysis against a 0.15 M saline solution containing 0.01 carbonate-bicarbonate buffer (pH of 9). The dialysis was continued until the fluorescence of the dialyzing fluid became hardly noticeable when illuminated with an ultraviolet lamp (usually five-six days). Then, the labeled globulin was reprecipitated three-four times with ammonium sulfate (or sodium sulfate) and again dialyzed until the SO_4 ions were completely removed.

The serological activity of the fluorescent sera prepared was tested by the agglutinin test and, principally, by their capacity of causing specific fluorescence of bacteria in microscopic preparations treated with these sera. The latter was performed in the following way.

A suspension of bacteria was applied drop by drop or with a loop to defatted glass slides; the smears were dried at room temperature, fixed with ethyl alcohol for 20-30 minutes and dried in air. A drop of fluorescent serum was applied to the smears placed in a wet chamber (Petri dishes with a wad of cotton moistened in water) and they were kept at room temper-

ature for 20-30 minutes. The smears were washed for 15 minutes in buffered physiological saline solution which was changed several times, dried and embedded in a buffered mixture of glycerin and physiological saline solution under a cover glass, along the margins of which a thin layer of melted paraffin was applied with a brush (one part of physiological saline solution at a pH of 7.0 for nine parts of glycerin). In this way, the entire procedure for preparation and treatment of the smear takes about one hour.

The study of the preparations was conducted under an MBI-1 microscope (objective 90x, ocular 7x) furnished with a fluorescent opaque OI-17 condenser. As a source of light we used an OI-18 illuminator with an SVD-120-A mercury vapor-quartz lamp into which an FS-1 blue light filter was put. For the purpose of absorbing the excess of rays exciting luminescence a yellow ZhS-18 light filter was set on the microscope ocular. Ordinary immersion oil was used for the examination under oil immersion, to three parts of which one part of nitrobenzol was added for the purpose of eliminating the natural luminescence of the oil (Yu. N. Zubzhitskiy, 1957). At the same time, the preparations were studied in a phase-contrast microscope under ordinary illumination with the aim of detecting those bacteria which are not seen on fluorescence microscopy; for this purpose the microscope was equipped with a phase-contrast KF-1 attachment.

Results of Testing Anticholera Fluorescent Serum

The original anticholera sera which we used gave a marked agglutination of cholera vibriones to the serum titer, and in low dilutions (1:50-1:100-1:200) agglutinated certain strains of Proteus vulgaris, the colon bacillus and dysentery pathogens. A positive test was observed with cholera-like vibriones [V. paracholerae] in dilutions up to 1:100-1:200 and with various strains, to 1:400.

The globulin fractions obtained from these sera are more specific, and agglutinated, also in low dilutions, a smaller number of strains of the cholera-like vibriones and other bacteria. After marking with fluorescein isocyanate, the globulins reduced the agglutinin test titers of homologous cultures by two-four times and gave practically no nonspecific reactions (with the exception of the V. paracholerae).

A test of the capacity of fluorescent anticholera sera for producing luminescence of bacteria gave the following results. The cholera vibriones have the appearance of brightly luminescent yellowish-green small rods, puffed up slightly and curved to different degrees, in the dark field of the fluorescence microscope. The peripheral portion of the cells (see the Figure) fluoresces particularly brightly, in the form of a rim. We arbitrarily designated the intensity of fluorescence by plus signs (Table 1).

If the smears were treated with normal or antiplague fluorescent sera, fluorescence of the cholera vibriones was not observed and they could be seen only in the phase-contrast microscope.

The specificity of the fluorescent anticholera sera was tested by means of treating smears of 24-hour cultures of the cholera vibrio, V. paracholerae and other vibriones, pathogens of dysentery, typhoid, salmonellas, Proteus vulgaris, the colon bacillus and B. faecalis alcaligenes, which we arbitrarily designated "other" bacteria, with the sera. The results of these

Table 1

Intensity of Fluorescence of Bacteria in Smears Treated with Fluorescent Cholera "O" Serum of Series No 11

Вид бактерий (1)	Число штаммов (2)	Число штаммов с интенсивностью свечения (3)				Число штаммов, не давших свечения (4)
		++++	+++	++	+	
V. cholerae	10	6	4	—	—	—
V. El-Tor	1	1	—	—	—	—
V. Celebes	1	1	—	—	—	—
V. paracholerae	3	—	—	3	—	—
V. phosphorescens	1	—	—	1	—	—
V. metschnikovi	1	—	1	—	—	—
B. typhi abdominalis	2	—	—	—	1	1
B. paratyphi	1	—	—	—	1	—
B. dysenteriae	3	—	—	—	1	2
B. typhi murium	1	—	—	—	—	1
B. enteritidis	1	—	—	—	—	1
B. proteus vulgaris	1	—	—	—	—	1
B. faecalis alcaligenes	1	—	—	—	—	1
E. coli	1		—	—	1	—

Arbitrary Designations: + Very slight fluorescence, cell morphology poorly seen; ++ Slight fluorescence, morphology of microbes comes out quite distinctly; +++ Bright fluorescence, cell morphology well seen; ++++ Very striking fluorescence in the form of a fluorescent rim along the periphery of the cells.

Key: 1. Species of bacterium; 2. Number of strains; 3. Number of strains with a fluorescence intensity of; 4. Number of strains which did not show fluorescence.

experiments with one of the sera tested (they were similar with sera Nos 16 and 22) indicate that bright fluorescence was shown only by the true cholera vibriones and the El-Tor and Celebes vibriones, indistinguishable from them in an antigenic respect. Of the "other" bacteria only some showed slight fluorescence (+).

The cholera-like vibriones and V. metschnikovi showed fluorescence of different degrees of intensity under the influence of anticholera serum, which is undoubtedly connected with the similarity of their antigenic structure to that of the cholera pathogen. However, this fluorescence is not so bright, as a rule, as that of the cholera vibrio and does not appear in the form of a rim along the periphery but the entire surface of the cell fluoresces.

The intensity of fluorescence of different strains of the cholera vibrio under the influence of fluorescent serum varies somewhat and, in

addition, it changes in the same strain in accordance with the age of the culture. The most striking fluorescence of the cells was found in smears of four-five-six-10-hour cultures; cells from 24-48-hour cultures fluoresce somewhat less intensely; even less bright fluorescence is observed in the majority of cells in smears of three-five-day cultures.

In smears of 10-12-day cultures markedly altered and degenerated cells are found which fluoresce poorly in the form of a pale green background on which various puffed-up cells, which are round or oval in shape, are distinguished by their bright fluorescence. Approximately the same picture was observed in smears of a dried (by the sublimation method) microbial mass of vibriones kept under refrigerator conditions for two, three, five and eight years. In old cultures of the cholera vibrio, apparently, only various individuals preserve the power of adsorbing specific antibodies, whereas in young cultures this capacity is particularly distinctly expressed and is inherent in all cells of the population.

Therefore, the investigations made showed that anticholera fluorescent serum, being a unique immunochemical indicator, makes it possible quickly (in one and a half-two hours) to identify various cholera vibrio cells directly in microscopic preparations. However, in reacting with the antigen in accordance with the antigen-antibody reaction principle, fluorescent antibodies against the cholera vibrio can react with bacteria which are similar in an antigenic respect, particularly with certain strains of V. paracholerae. Although the fluorescence of the latter is less striking and not characteristic, which makes it possible to differentiate them from the true cholera vibrio, still further work is needed for the elimination of group reactions which can be given by fluorescent anticholera serum.

Results of Testing Antiplague Fluorescent Serum

We studied antiplague sera and the conjugates prepared from them with cultures of the plague pathogen (22 strains), the pathogen of rodent pseudotuberculosis (nine strains) and with cultures of "other" bacteria from the list mentioned above.

The original sera readily agglutinated the pathogen of rodent pseudotuberculosis (to half of its titer and some strains to full titer), in addition to the homologous cultures, and in dilutions of 1:25-1:100-1:200 it agglutinated the majority of the "other" cultures tested. By comparison with horse sera, rabbit sera were somewhat more specific, because they reacted with a smaller number of "other" bacteria but readily agglutinated the pseudotuberculosis microbe.

The fluorescent antibodies prepared from these sera showed an agglutinin test titer with cultures of the plague microbe which was two-four or even 10 times less than the original sera. At the same time, they either did not agglutinate cultures of the "other" bacteria at all or agglutinated them to a slight degree (+ in dilutions of 1:25-1:50).

Only the pseudotuberculosis bacteria, which, as is well known, are similar in their antigenic structure to the plague pathogen, were quite well agglutinated by fluorescent sera, although this did not apply to all of the strains tested.

In smears treated with antiplague fluorescent serum and examined

under blue-violet rays the pathogen of plague is seen in the form of polymorphous ovoid rods which fluoresce brightly in a greenish color. In the plague microbe, like the cholera vibrio, there is also particularly striking luminescence of the periphery of the cells in the form of a rim or areola. After treatment of the smears with normal or anticholera fluorescent sera no fluorescence of the plague microbe cells was observed.

Testing the specificity of antiplague sera by means of treating the smears of plague pathogen cultures, cultures of rodent pseudotuberculosis and "other" bacteria (Table 2) showed that the capacity of fluorescent antibodies for producing fluorescence in bacteria coincides, by and large, with the results of experiments of the full-scale agglutinin test: the sera gave striking fluorescence of the homologous bacteria and did not cause fluorescence of the "other" bacteria, with the exception of various cultures which showed a very slight fluorescence.

Table 2

Intensity of Fluorescence of Bacteria in Smears Treated with Antiplague Fluorescent Horse Serum

Вид микроба (1)	Число штаммов (2)	Число штаммов с интенсивностью свечения (3)				не давали свечения (4)
		+----	+++	++	-	
B. pestis	22	17	3	2	0	0
B. pseudotuberculosis	9	1	4	3	1	0
B. proteus vulgaris	1	—	—	—	—	1
B. paratyphi A	1	—	—	—	—	1
B. paratyphi B	1	—	—	—	—	1
B. typhi abdominalis	5	—	—	—	1	4
B. dysenteriae Sonne	1	—	—	1	—	—

The arbitrary designations are the same as for Table 1.

Key: 1. Species of microbe; 2. Number of strains; 3. With the following intensity of fluorescence; 4. Did not show fluorescence.

Cells of the pseudotuberculosis microbe were demonstrated in smears treated with antiplague fluorescent serum, whereby in the intensity of their fluorescence some strains could not be differentiated from the plague bacteria while others, just as in the experiments with the agglutinin test, did not fluoresce so brightly. Experiments with absorption of the fluorescent serum by the Castellani method also showed the similarity between the antigenic structure of the plague and pseudotuberculosis microbes, making differentiation of them difficult: after absorption of it with a

suspension of a culture of the [illegible]strain of plague microbe the fluorescent serum did not cause fluorescence of the cells of either the plague pathogen or the pathogen of rodent pseudotuberculosis. The majority of the 22 strains tested showed bright fluorescence under the influence of antiplague fluorescent serum and only two strains fluoresced to a lesser degree. In the same strain the intensity of fluorescence varied in accordance with the age of the culture. It was found that the brightest fluorescence occurred in cells of young cultures, four, five and 24 hours old, from semiliquid agar and from 24-hour and two-day cultures from ordinary agar. Bacteria from four- and six-day cultures fluoresced less brightly, and cells with altered morphology from agar cultures kept for two months in a refrigerator showed slight fluorescence. In smears prepared from three series of living 1-17 dry vaccine which had been kept for different periods (from six months to two years) the plague microbes fluoresced just as brightly as those from 24-hour cultures. It was found further that it is possible to preserve fixed smears of 24-hour cultures for quite a long time (the observation period was one and a half months) and that this has no effect on the intensity of fluorescence of cells when they are subsequently treated with labeled serum. The practical significance of the latter is obvious, because when it is impossible to make an examination in a poorly equipped laboratory, prepared and fixed smears can be sent to a laboratory where there is a fluorescence microscope and fluorescent serum.

We treated not only smears of pure bacterial cultures but also smears of artificial mixtures of the plague microbe with suspensions of the colon bacillus, S. enteritidis and microbes of the air flora with fluorescent antiplague sera. In smears of such mixtures only the plague bacteria fluoresced, which was confirmed by control phase-contrast microscopy.

Therefore, tests of the antiplague fluorescent sera which we prepared showed their suitability for purposes of rapid identification of various plague microbe cells directly in microscopic preparations. The capacity of these sera for demonstrating the pathogen of rodent pseudotuberculosis, in our opinion, cannot serve as an obstacle to their practical utilization, because the pseudotuberculosis microbe is not frequently encountered under natural conditions. However, the aim of further work should be to obtain species-specific antiplague serum which does not react with the pathogen of rodent pseudotuberculosis.

Conclusions

1. Corresponding fluorescent sera were obtained from diagnostic antiplague and anticholera agglutinating sera by means of combining their globulin fractions with fluorescein isocyanate by the Coons and Kaplan method.

2. The fluorescent sera prepared against pathogens of plague and cholera cause specific fluorescence of the homologous bacteria detectable by means of a fluorescence microscope when they are used to treat microscopic preparations.

3. By the presence of fluorescence of the bacteria, that is, by the power of the cholera and the plague pathogens for specifically adsorbing homologous fluorescing antibody, various cells of these microbes can be identified quickly directly on the microscope preparations, and after one-

two hours a tentative positive conclusion can be rendered. This affords the basis for testing the suitability of these sera in the practical work of diagnostic laboratories.

4. Further work is needed for improving the quality of the fluorescent sera tested, particularly eliminating their group reactions, which they can give with bacteria antigenically similar to the pathogens of cholera and plague.

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Rapid Identification of the Plague Microbe in Smears of Animal Organs by Means of Fluorescent Antiplague Serum

Ye. V. Chibrikova, N. I. Kuznetsova, P. K. Tabakov and Ye. I. Vel'ner (Saratov)

In 1958, 1959 a group of authors composed of Ye. V. Chibrikova, L. P. Basunova, P. K. Tabakov, I. I. Shurkina and Ye. I. Vel'ner made studies (the results have been published in the present collection) which showed that fluorescent antiplague serum can be used as an immunochemical indicator, making it possible to identify various plague microbe cells in a short time directly on microscopic preparations. The given serum did not cause fluorescence of bacterial cells which were considerably different from the plague microbe in their antigenic composition (representatives of the air flora, of the group of colon bacteria and others), and there was no difficulty in differentiating them from the latter. Only the pathogen of rodent pseudotuberculosis, very similar to the plague pathogen in its antigenic

structure, could not in many cases be distinguished from the plague microbe by the brightness of fluorescence in smears treated with antiplague fluorescent serum, just as it could not be so distinguished in a full-scale agglutinin test. However, the latter could not be an obstacle to testing the possibilities of practical application of this serum, because under natural conditions the pathogen of rodent pseudotuberculosis is rarely encountered. In addition, it should be kept in mind that the utilization of one method or another of rapid detection of pathogenic microbes does not exclude a bacteriological examination according to the classical plan with the isolation of a pure culture of the pathogen and identification of it on the basis of its total properties and characteristics.

In the present work antiplague fluorescent serum was tested for rapid identification of the plague pathogen in smear-impressions from organs of infected laboratory animals. Work along this line is interesting because the method of infecting laboratory animals is widely used in practice for detecting the plague pathogen and other pathogenic microbes, isolation of which in pure culture with subsequent identification usually takes several days, in one material or another. In addition, the possibility of specific identification of plague bacteria directly in smears from animal organs opens up new prospects also for the rapid laboratory diagnosis of plague in man, the importance of timely diagnosis of which is hard to overestimate.

As experimental animals, guinea pigs and white mice were used. They were infected with museum cultures of various pieces of bacteria grown out for 24 hours on the corresponding solid nutrient media (cultures of the tularemia pathogen were grown out for two days). The animals were infected with high doses of a mass of microbes for purposes of obtaining intense septicemia in them.

As preliminary experiments indicated, bacteria from different organs of the animals showed no differences either in the cell morphology or in the nature of fluorescence; therefore, in the main experiments on the study of animals which died and were sacrificed, only the spleen was taken, because abundant seeding of it was always observed with the infecting microbes. Smear-impressions from the spleen and cultures on agar plates were made according to generally accepted methods; the smears were fixed with ethyl alcohol for 30 minutes and then dried in air. Fixation of the smears with acetone, which was used at the beginning of the experiments with the aim of obtaining a brighter fluorescence, did not show any advantages in this respect over fixation with ethyl alcohol. The method of treating the smears with fluorescent serum has been presented in detail in the work of Ye. V. Chibrikova and coauthors; the apparatus which we used for fluorescence microscopy is also shown there.

In the first part of this work we determined the capacity of antiplague fluorescent serum for producing specific fluorescence of the plague pathogen which was in smear-impressions from the organs of the animal. With this aim in view, first of all, the same antigen (smears containing the plague microbe) was treated with different species-specific sera and, secondly, various antigens (smears with bacteria of different species) were treated with the same antiplague fluorescent serum. Simultaneously, for a comparison, smears of pure bacterial cultures were studied (Table 1).

On examination of the preparations a certain difference in the

Table 1

Fluorescence of the Plague Microbe in Smear-Impressions from the Spleens of Infected Animals

Флуоресцирующая сыворотка (1)	Степень свечения бактерий (2)		
	в отпечатках селезенки (3)		в мазках из чистой культуры чумного микроба (6)
	морских свинок (4)	белых мышей (5)	
Противочумная	++++*	++++*	++++
Нормальная	−	−	+
Противосапная	−	−	+
Противопсевдосапная	+	+	−
Противосибиреязвенная	+	+	+

Arbitrary Designations: (+) Very slight fluorescence, cell morphology poorly distinguishable; (++) Slight fluorescence, bacterial morphology demonstrated quite distinctly; (+++) Bright fluorescence, morphology of the microbes well seen; (++++) Very bright fluorescence in the form of a shiny rim along the periphery of the cells. The asterisk denotes cases in which the shiny rim was broader in the cells.

Key: 1. Fluorescent serum; 2. Degree of fluorescence of the bacteria; 3. In impressions of the spleen; 4. Of guinea pigs; 5. Of white mice; 6. In smears of a pure culture of the plague microbe; 7. Antiplague; 8. Normal; 9. Antiglanders; 10. Antipseudoglanders [melioidosis]; 11. Antianthrax.

fluorescence of plague bacteria in smears from the pure culture and impressions from the organs of infected animals, demonstrated under the influence of homologous serum, attracted attention. In both cases the bacteria fluoresced very brightly (++++); however, in smears from the organs the shiny rim in the cells was broader, and because of this their fluorescence appeared to be even brighter. The reason for this should be sought, in our opinion, in the capacity of the plague microbe for forming a capsule under conditions of the macroorganism. We ascertained this by comparing the intensity of fluorescence of plague microbe cells in smears of pure cultures, grown out under different conditions. It was found that the broadest and shiniest rim was shown by cells in smears from cultures grown out on media containing blood at 28° or on ordinary agar at 37°, that is, under conditions contributing to capsule-formation in vitro.

The data presented also show that antiplague fluorescent serum, which we used, reacted most intensely with the surface antigens of the plague microbe, which is in agreement with the data of other investigators. Thus, in the opinion of Thomason, Cherry and Moody (1937), the surface antigens play the main part, as they do in the agglutinin test, for the reaction of fluorescent antibodies in typhoid bacteria.

It should be noted that the smear-impressions [this term refers to

touching a glass slide to the cut section of an organ] from the organs should be made thin and their margins should be examined, where the most demonstrative picture is demonstrated (see the Figure).

Against the dark or slightly fluorescent background of the preparation, consisting of formed elements and tissue fluid, in the field of the fluorescence microscope various typical ovoid emerald-green plague microbe cells are seen, thereby, it is not the entire cell as a whole which fluoresces brightly but only its peripheral portion in the form of a rim or areola, which, in the opinion of all authors working with fluorescent antibodies, is a specific fluorescence. In thicker sections of the preparation, where there are accumulations of cells of the organ and brightly fluorescent bacteria, the morphology of the latter cannot be readily seen, because all become confluent into an amorphous luminous mass.

Only antiplague fluorescent serum caused a bright fluorescence of the plague microbe (++++, as on photomicrography). The other sera either did not show up the plague microbe cells or caused a very slight fluorescence of them (+, where the bacteria is seen in the form of hazy shadows without a characteristic rim along the periphery).

In subsequent experiments smear-impressions from the spleens of animals infected with various species of bacteria were treated with the antiplague fluorescent sera. Species of gram-negative bacteria were chiefly used; under natural conditions these can cause septicemia in animals and they are not readily differentiated by their cell morphology from the plague pathogen (Table 2).

The characteristic and bright fluorescence in smear-impressions from animal organs was shown only by cells of the plague pathogen. The other nine species of bacteria were not found on fluorescence microscopy, although in the infected animals there was intense septicemia. The presence of the latter was evidenced by the results of detection of the infecting bacteria both in smears stained by the Gram method and in plates made on the appropriate nutrient media.

Data obtained with respect to the pathogen of rodent pseudotuberculosis are of special interest. Cells of this microbe in smears of pure cultures fluoresced almost as brightly as the plague bacteria, and in smear-impressions from the spleens of infected animals they did not fluoresce. Although in the smear-impressions stained by the Gram method very few pseudotuberculosis bacteria were encountered, as a rule, there were still a sufficient number of them for detection, if they fluoresced. Apparently, under conditions of the macroorganism the cells of the pseudotuberculosis microbe have antigens on their surfaces which do not react with antiplague fluorescent serum, that is, are different from the antigens of the plague microbe. This does not contradict the well-known data that the plague pathogen has a somatic antigen and a specific capsular antigen in common with the pseudotuberculosis microbe (Schütze, 1932; N. N. Zhukov-Verezhnikov, 1940) and at the same time permits us to suppose that the somatic antigen of the pseudotuberculosis microbe is not located on the surface of the cells under conditions of the macroorganism.

As early as after the completion of our experiments, American investigators Winter and Moody (1959) published a paper in which they report obtaining a strictly specific antiplague fluorescent serum which does not

Table 2

Results of Testing of the Specificity o.. Antiplague Fluorescent Serum after Treatment of Smear-Impressions from the Organs of Animals Infected with Different Microbes and from Pure Cultures of These Microbes

Вид микроба (1)	Степень свечения бактерий		Число исследованных животных (5)	Число животных с обнаруженными инфицирующими бактериями (6)	
	в мазках из чистых культур (3)	в мазках-отпечатках из селезенки животных (4)		в мазках-отпечатках по Граму (7)	путем высева (8)
B. pestis	++++	++++	13	13	13
B. pseudotuberculosis rod.	+++	— или ± (9)	14	14*	14
Salm. enteritidis	—	—	3	3	3
E. coli	—	—	3	3	3
B. ozaenae	—	—	4	4	4
B. bipolaris septica	±	—	8	7	8
Listerella monocitogenes	—	—	2	2	2
B. proteus vulgaris	—	—	3	3	3
Pneumococcus	—	—	1	1	1
B. tularense	—	—	1	1 (10) (окраска по Романовскому)	1

Note. The arbitrary designations are the same as for Table 1. The asterisk denotes cases where the bacteria were found in small numbers (single cells and not in every field).

Key: 1. Species of microbe; 2. Degree of fluorescence of the bacteria; 3. In smears from pure cultures; 4. In smear-impressions from the spleens of animals; 5. Number of animals investigated; 6. Number of animals in which the infecting bacteria were found; 7. In smear-impressions stained by the Gram method; 8. By means of plating out; 9. Or; 10. Staining by the Romanovsky method.

cause fluorescence of the pseudotuberculosis microbe in smears from pure cultures and does not agglutinate it. The authors obtained this serum by means of immunization of rabbits with plague microbe cells containing the capsular antigen or fraction 1 in large quantity, to which growing out the culture on a special medium at 37° contributed.

Therefore, the results of our experiments permit us to conclude that by means of antiplague fluorescent serum it is possible quickly and specifically to identify various plague microbe cells in smears from organs of infected animals under conditions of an intense septicemia, when a large number of plague bacteria is found in the smears. After ascertaining this, we, in the second series of experiments, attempted to determine the advantage

of using fluorescent serum in the everyday work practice of plague specialists, that is, for the detection of plague microbe cells in the dead bodies of experimental animals which had undergone decay to different degrees.

With this aim in view, we studied the spleens of three guinea pigs and 30 white mice which had died of experimental plague for five days. The bodies of the animals were kept at room temperature (25°-30°) in the summertime; as early as the second day they showed signs of putrefaction.

The bodies of five white mice and pieces of the spleens of three guinea pigs were examined daily. For the purpose of detecting the plague microbe in them the following methods were used: 1) smear-impressions of the animals' spleens were treated with antiplague fluorescent serum and then examined under the fluorescence microscope; 2) a culture was made on agar containing gentian violet and sodium sulfite by the usual method, and observations of the cultures were made for three days; 3) an examination was made by the Tumanskiy accelerated method (by means of plague bacteriophage introduced into the material at the time of streaking it) with reading of the results after four hours. The last method was chosen as the simplest and most convenient of the group of accelerated methods for detection of the plague microbe proposed. The results of these experiments are presented in Table 3.

By the method of fluorescent antibodies the plague bacteria were found in all animals during the entire observation period. We obtained a positive result, as a rule, one and a half-two hours after the beginning of the examination (preparation and fixation of the smears, treatment of them with serum, microscopy). Thereby, in the smears, both in the first few hours after the death of the animals and in the next five days of the investigation of the bodies, brightly fluorescent cells of the plague microbe were equally numerous. However, even in the smears prepared 24 hours after death of the animals, swollen spheroidal cells appeared along with the plague microbe cells with typical morphology. After two-three days the number of these altered bacteria increased, and in smears prepared after the bodies had stood for four-five days, they constituted the main mass.

Plague bacteria were isolated by the bacteriological method from all animals only in the first few days after death, and from the majority, in two and three days. After four-five days growth of the plague microbe colonies could be obtained but not from all investigated bodies of animals. Thereby, plating out the material on the first day after the animals' deaths gave an abundant growth of the plague pathogen, and in the next two or three days the number of plague microbe colonies which grew out decreased sharply. In plates made from the spleens of the bodies of animals which had undergone putrefaction for three-five days, the abundant growth of putrefactive bacteria was noted on the plates, among which occasional, small "stifled" colonies of the plague microbe could be found only under the microscope. The colonies were found chiefly after 48 hours of incubation at 28°.

The presence of the plague microbe could be detected by the accelerated method, by the presence of lysis of the growing culture in the early periods of the observation, only in the first few hours and first day after death of the animals, when a culture of the plague pathogen usually grew out abundantly on the plates. During the two days it was not possible in all animals to detect the plague microbe and in none of them was it isolated

Table 3

Comparative Detection of Plague Microbe in Bodies of Infected Animals by Means of Fluorescent Serum and by the Bacteriological Method

Время, истекшее после гибели животного, сутки (1)	Число исследованных животных (2)	Число животных, у которых в селезенке обнаружены чумные микробы (3)		
		методом флуоресцирующих антител через 1½–2 часа после приготовления мазков (4)	высевом на агаровые чашки через 24–48 часов после посева (5)	ускоренным методом (по Туманскому) по истечении 4 часов после посева (6)
		(7) Морские свинки		
0	3	3	3	3
1		3	3	3
2		3*	3	1
3		3	2	0
4		3*	2	0
5		3*	0	0
		(8) Белые мыши		
0	5	5	5	2
1	5	5	5	1
2	5	5*	3**	0
3	5	5*	4**	0
4	5	5*	5**	0
5	5	5*	3**	0

*Cases where there were many large spheroidal swollen cells in the smears.

**Abundant growth of putrefactive flora; few colonies of the plague microbe; they were small, "stifled," seen only under the microscope.

Key: 1. Time elapsing after death of the animal, days; 2. Number of animals examined; 3. Number of animals in which plague microbes were found in the spleen; 4. By the method of fluorescent antibodies one and a half-two hours after preparation of the smear; 5. By plating out on agar plates 24-48 hours after streaking; 6. By the accelerated method (after Tumanskiy) four hours after streaking; 7. Guinea pigs; 8. White mice.

after three days.

It should be emphasized that we considered only the presence of plaques found four days after streaking of the material a positive result. We examined the plates again after one and two days. It was found that growth of the plague microbe colonies occurs in approximately the same way as after ordinary streaking (see above), but we did not consider these results positive, because this was no longer early diagnosis by the presence of lysis of the original growth of the plague microbe culture.

As a control for the last experiments a study was made of 10 bodies

of uninfected white mice, which were also kept at room temperature, by the same methods.

During the entire observation period (five days) no fluorescent bacteria were found in the smears of the spleens of the animals, although in smear-impressions of bodies which had undergone considerable decay (fourth-fifth day after the animals were killed) there was a mass of bacteria seen only under the phase-contrast microscope and in smears stained by the Gram method. In these cases the abundant putrefactive flora was readily detected by the bacteriological method also.

Therefore, the studies made showed that in the rapidity of obtaining positive results (one and a half-two hours after beginning of the examination) the method of fluorescent antibodies has an indubitable advantage over the ordinary bacteriological method and the accelerated method (with the introduction of plague bacteriophage into the culture material at the time it is seeded). This advantage is particularly appreciable if we consider that in our experiments, during the bacteriological examination we counted only the time when the growth of plague microbe colonies appeared, without consideration of the time required for at least a tentative identification of them. In addition, the fluorescent antibody method makes it possible specifically to identify the plague pathogen in smears from the organs of the infected animal and gives absolutely reliable results if there is intense septicemia in the animal. By this method it is possible to detect plague bacteria also in decaying material from dead bodies, when plating out on nutrient media does not give positive results. However, in this case, it should be taken into consideration that plague microbe cells in decaying bodies show a markedly altered morphology, even though they have the bright characteristic fluorescence.

Conclusions

1. The use of antiplague fluorescent serum and fluorescence microscopy makes it possible quickly to identify plague bacteria directly in smear-impressions from organs of animals infected with the plague pathogen if they have intense septicemia.

2. This method can be used for the study of fresh and decaying bodies of animals.

The results obtained afford the basis for the belief that the fluorescent antibody method can be used also for the rapid diagnosis of plague in man.

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Early Detection of the Plague Microbe by the Method of Infecting Laboratory Animals

I. I. Kurayev
(Saratov)

In a previous report (1960) mention was made of the increase in sensitivity and acceleration of the course of plague with a fatal outcome in guinea pigs by means of blocking the protective systems of the body by means of hen's egg yolk and trypan blue.

Recently, a number of factors have been studied which effectively increase the sensitivity of experimental animals to a number of infectious diseases. Among these factors are certain chemical agents as well as preparations of the suprarenal glands and hypophysis.

Many investigators point to a reduction of the resistance of animals to experimental infections under the influence of large doses of cortisone and desoxycorticosterone acetate (Z. A. Popenkova, 1956; M. P. Pokrovskaya, 1959; S. L. Blaykher, 1958; Ye. I. Shmeleva, 1959) and the adrenocorticotropic hormone (Hawashida, 1957; Selye, 1951). Thereby, not only pathogenic and conditionally pathogenic microbes but, in a number of cases, also saprophytes were found capable of multiplying actively in the body of the animal and causing a serious disease septic in nature with a fatal outcome.

Ye. M. Shamayeva and S. S. Pan'kova (1957) indicate the important part of chloroethylamines in the development of the infectious process and the inhibition of immunity in the animals. Specifically, novembichin, administered to the experimental animals in sublethal doses, leads to a delay in antibody production and a marked reduction of their level in the body.

Based on these premises, it was interesting to test a number of preparations which reduced the resistance of laboratory animals to infectious diseases.

For this purpose a comparative study was made of the effects of cortisone, ACTH, desoxycorticosterone acetate, cortin, hen's egg yolk, histamine, novembichin and trypan blue.

The experiment was performed on 500 white mice weighing 16-18 grams. The preparations were given to the animals four hours before infection, simultaneously with the plague microbe culture. Thus, four hours before infection with a virulent culture of the plague microbe one group of animals was injected intramuscularly with cortisone in a quantity of 3.75 or 5 milligrams. Another group of animals was given the preparation simultaneously with a culture of the plague pathogen subcutaneously: hen's egg yolk, 0.5-1 cc; novembichin, 0.[illegible] 5 and 0.75 milligram; histamine, 25, 50 and 75

milligrams; cortisone, 3.75 and 5 milligrams. All the experimental and control animals were infected by means of subcutaneous injection of a suspension of the virulent culture of plague microbe, strain 708, in a quantity of 25 or 10 microbes. The infecting dose of the strain used was less than 1 CLD with the aim of creating the most favorable conditions for the groups of control animals with respect to the possibility of survival and prolongation of the lifespan, which, in turn, would make it possible to find a more active preparation for increasing the sensitivity of the animals used for the biological test (Tables 1 and 2).

Table 1

Effect of Preparations Tested on the Course of Plague in White Mice Infected with a Plague Microbe Culture (Strain 708)

1 Наименование препарата и схема введения его в организм	2 Число животных	3 Число павших животных	4 Средняя продолжительность жизни павших от чумы животных, дни
5 Доза 25 микробных тел			
6 Желток куриного яйца 0,5 мл (вместе с культурой)	10	10	3,4
7 Новоэмбихин 0,75 мг (вместе с культурой)	10	10	2,8
8 Контроль	10	9	9,0
9 Доза 10 микробных тел			
10 Желток куриного яйца 0,5 мл (вместе с культурой)	20	20	3,4
11 То же 1 мл	10	10	3,2
12 Новоэмбихин 0,3 мг (вместе с культурой)	10	10	3,1
13 То же 0,5 мг	30	28	3,8
" " 0,75 "	20	20*	2,3
14 Контроль	30	20	8,0

Note. *, The culture of plague microbe was not isolated from the organs or tissues of nine mice.

Key: 1. Name of preparation and means of administration of it; 2. Number of animals; 3. Number of animals which died; 4. Average lifespans of animals which died of plague, days; 5. Dose of 25 microbes; 6. Egg yolk, 0.5 cc (together with culture); 7. Novembichin, 0.75 milligram (together with culture); 8. Control; 9. Dose of 10 microbes; 10. Egg yolk, 0.5 cc (together with culture); 11. The same, 1 cc; 12. Novembichin, 0.3 milligram (along with the culture); 13. The same, 0.5 milligram; 14. Control.

Table 2

Effect of Preparations Tested on the Course of Plague in White Mice Infected with a Plague Microbe Culture (Strain 708, 10 Microbes)

(1) Наименование препарата и схема введения его в организм	(2) Число животных	(3) Число павших животных	(4) Средняя продолжительность жизни павших от чумы животных, дни
(5) Кортизон 5 мг (за 4 часа до заражения)	10	4	6.3
(6) То же 3,75 мг	10	9*	6,1
(7) Кортизон 5 мг (вместе с культурой)	10	10**	6,6
(8) То же 3,75 мг	10	8	4,5
(9) Гистамин 25 мг (вместе с культурой)	10	8	5,0
(10) То же 50 мг	30	26***	5,0
" " 75 "	10	10****	6,0
Контроль	30	20	8,0

Note. The culture of plague microbe was not isolated from the organs and tissues as follows: * in one mouse; ** in two mice; *** in six mice; **** in eight mice.

Key: 1. Name of preparation and means of administering it; 2. Number of animals; 3. Number of animals which died; 4. Average lifespan of animals dying of plague, days; 5. Cortisone, 5 milligrams (four hours before infection); 6. The same, 3.75 milligrams; 7. Cortisone, 5 milligrams (together with the culture); 8. Histamine, 25 milligrams (together with the culture); 9. The same, 50 milligrams; 10. Control.

An analysis of these experiments (see Tables 1 and 2) showed that the activity of various preparations in increasing the sensitivity of white mice to the plague pathogen differed: the defense systems of the animals against the plague microbe were suppressed to the greatest degree after the administration of egg yolk, novembichin, histamine, and cortisone and desoxycorticosterone acetate, ACTH and cortin exerted no essential effect on the course of plague in the experimental animals. Equally unsatisfactory results were obtained in experiments in which trypan blue was injected.

From Table 1 it is seen that in the control group, 66.6-90 percent of the mice died. The average lifespans of the animals which died amounted to eight-nine days. At the same time, all mice which received hen's egg yolk died of plague. Their average lifespan was 3.2-3.4 days. White mice which were injected with novembichin died in 93.3-100 percent of the cases. The average lifespan of the animals which died of plague was equal to 2.3-3.8 days. Thereby, it should be emphasized that of the 20 white mice which received 0.75 milligram of novembichin along with 10 microbes of the plague pathogen, 11 died of plague with isolation of the plague microbe culture; nine mice died 18-22 hours after infection and no culture of the plague pathogen was isolated. In these cases, growth of colonies of the paratyphoid

bacillus, staphylococci and streptococci and Proteus vulgaris was found. Apparently, it should be considered that a dose of 0.75 milligram of novembichin is at the limit of tolerance, in connection with which the death of mice which are more reactive followed in periods too short for the reproduction of the plague microbe; therefore, in these mice a dissemination of the autoinfection was noted.

The data presented in Table 2 permit us to note that white mice, which received 3.75 milligrams of cortisone simultaneously with the plague microbe culture, died most quickly. In this case, eight out of 10 mice died with an average lifespan of 4.5 days. As far as the injection of 3.75 milligrams of cortisone four hours before infection with plague is concerned, here eight out of the 10 animals also died of plague but with an average lifespan of 6.1 days and, which should be emphasized particularly, one mouse died of paratyphoid infection. Approximately the same results were obtained after the injection of 5 milligrams of cortisone along with the plague microbe culture. Therefore, cortisone, like novembichin, when administered to particularly reactive animals, can cause autoinfection ending in death in some of these. At the same time, it should be noted that the administration of cortisone to the animals simultaneously with the plague microbe culture gives somewhat better results than in experiments where cortisone was injected four hours before the infection. This is convenient for the practical application of this preparation.

Reduction of the lifespans of plague-infected mice was observed also after the administration of histamine. Thus, after administration of 25 or 50 milligrams of histamine together with a culture of the pathogen, 80 percent of the infected mice died with an average lifespan of five days, whereas in the control group only 66 percent died and later than in the experimental group. At the same time, it should not be overlooked that the number of mice which died after the injection of histamine in which no plague microbe culture was isolated was very great by comparison with the number which died in the groups given cortisone or even novembichin.

Everything presented permits us to emphasize once again that egg yolk causes a considerable reduction in the natural resistance of white mice and increases their sensitivity to the plague microbe without provoking the development of autoinfection at the same time. With respect to the degree of its activity, novembichin was almost equal to egg yolk (histamine and cortisone reduced the resistance of the animal to a somewhat lesser extent). However, when novembichin is used autoinfection is observed in some of the animals, and they die of it; however, the plague microbe could not be isolated. Therefore, the utilization of animals treated with hen's egg yolk for a biological test makes it possible to make the bacteriological diagnosis of plague in a shorter period, two-four days after the beginning of the examination. When animals are used which were not treated with the preparation indicated above, the time needed for making the bacteriological diagnosis of plague still came to eight-nine days.

Reduction in the period needed for isolation of the plague microbe culture when animals treated with egg yolk were used as a biological test was observed by L. S. Malafeyeva in an epizootological examination of the territory of the Aral Sea Plague-Control Station in the autumn of 1960. White mice infected with a suspension of organs of investigated rodents in

a mixture with egg yolk died of plague two days sooner than white mice infected with the same suspension of rodent organs without the egg yolk. No less interesting data were obtained by L. S. Malafeyeva in the examination of material from a person who died of anthrax. White mice infected with the material being investigated from the cadaver in a mixture with egg yolk died of anthrax two days sooner than mice infected with the same material but without the yolk.

All this permits us to recommend the biological test on animals exposed to the effect of hen's egg yolk in the examination of material for the presence of the plague pathogen.

The unsatisfactory results obtained in experiments in which the mice were injected with trypan blue do not reduce the value of the results obtained in previous experiments on guinea pigs treated with trypan blue, when a reduction of resistance of the experimental animals to the infecting dose of the plague microbe and a reduction of the lifespans of the infected animals were observed.

The quantities of trypan blue used, evidently, could not cause a complete acute block of the reticulo-endothelial system in the white mice, as the result of which it exerted no essential effect on the course of plague in them. In subsequent experiments it will be necessary to determine the dose of trypan blue which causes acute block of the reticulo-endothelial system in white mice.

Conclusions

1. Hen's egg yolk depresses the natural resistance of laboratory animals, increases their susceptibility and sensitivity to small quantities of the plague microbe to the greatest extent, without provoking the development of autoinfection observed after the administration of histamine, novembichin and cortisone.

2. Mice which have been given cortisone simultaneously with a plague microbe culture die sooner from plague than white mice treated with cortisone four hours before the infection.

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Method of Study of Decaying Material Containing the Plague Pathogen with the Aid of Bacteriophage

V. M. Tumanskiy, N. V. Uryupina, V. A. Knyazeva and Z. Ye. Malinina
(Saratov)

Of all the methods of examination of putrefying material for plague which have been proposed to date, cultures on selective media, specifically on modified Mayer's and Batchelder's medium and on agar to which 0.1 percent hemolyzed blood and gentian violet 1:100,000 have been added, should be considered the best. On these media the plague microbe grows well because of the presence of stimulants (sodium sulfite or hemolyzed blood), and the growth of extraneous flora is inhibited by gentian violet. However, when there is a high content of Proteus vulgaris in the culture material, the diagnosis of plague becomes difficult. In 1957, we determined the fact that in cultures on agar plates of the investigated material containing a large number of plague microbes along with bacteriophage, the plaques of the latter are demonstrated after two and a half-three hours against the background of the original growth of the plague microbe, that is, much sooner than colonies of other species of bacteria manage to grow out. Our experiments showed that if Proteus vulgaris (less than 2,000,000 cells per cc), the colon bacillus, the pathogen of dysentery are present in small quantities in the material being investigated and gram-positive cocci are present in large numbers, they do not interfere with the diagnosis of plague.

Based on what has been presented above, we worked out in detail the method of examining dirty and decaying material infected with the plague pathogen.

For the cultures agar was used (pH, 7.2) with 0.2 percent hemolyzed sheep blood as well as blood agar containing an alcoholic-aqueous solution of gentian violet in a final concentration of 1:100,000. A suspension of a two-day agar culture of the plague microbe (EV strain) with a concentration

of 2,000,000,000 microbes per cc was poured out into seven test tubes in a quantity of 1 cc each. A suspension of a one-day agar culture of Proteus vulgaris, containing serially 20,000, 200,000, 2,000,000, 20,000,000, 200,000,000 and 2,000,000,000 microbes per cc, was added to the test tubes in quantities of 1 cc. After careful mixing of both microbe suspensions, 0.5 cc was plated out from each test tube onto blood agar and onto agar containing blood and gentian violet. Therefore, the plague microbe was plated out in a dose of 50,000,000 microbes, and Proteus vulgaris was plated out in quantities of 500, 5000, 50,000, 5,000,000 and 50,000,000 microbes (the first test tube served as a control) onto the agar plates mentioned above from each test tube.

The mixture of microbes on each medium was cultivated by two methods. A loop (2 millimeters in diameter) of plague bacteriophage was introduced into a drop of the mixture of cultures on a glass slide, mixed, and distributed with a spatula over the entire surface of the agar plate. On another agar plate, 0.05 cc of the material applied was first distributed over the surface of the plate with a glass spatula and then a drop of plague bacteriophage, which was allowed to seep over the diameter of the plate, was applied to the area so streaked with a fine pipet. The results were read after three, four, five and 24 hours. The results of the repeated experiments are shown in the Table.

From the Table it is seen that on blood agar, after three and four hours of incubation, the presence of bacteriophage plaques is noted on plating out all mixtures of the plague microbe with Proteus vulgaris. After five hours, the plaques on the agar plate with a culture made from the mixture containing 50,000,000 plague microbes and 50,000,000 Proteus vulgaris microbes almost disappeared. After six hours, the plaques almost disappeared, and on the agar plates with the cultures from other mixtures of plague microbe and Proteus vulgaris and in the culture made from the last test tube they disappeared completely. This is explained by the fact that during the first five hours of incubation Proteus vulgaris, particularly when cultured from test tubes containing it in comparatively low concentrations, does not mask the initial growth of the plague microbe or the production of plaques by bacteriophage. In the culture from the last test tube, where there is a large number of cells of Proteus vulgaris, it is impossible to detect the presence of bacteriophage plaques even after five hours.

In experiments in which bacteriophage is applied in the form of a tract, an almost similar picture is noted, that is, during the first few hours of incubation, on all the seeded sections a sterile tract was noted; after six hours of incubation, Proteus vulgaris began to overgrow some of the streaks on the tract, and by the 22nd hour Proteus vulgaris had covered the entire surface of all the agar plates. Therefore, our experiments show that by means of plague bacteriophage it is possible to determine the presence of the plague microbe in material being examined which has been markedly contaminated by Proteus vulgaris on blood agar, if the cultures are observed three-five hours after the seeding.

Cultures from the mixture of plague microbe with Proteus vulgaris on agar containing blood and gentian violet did not show any growth of Proteus vulgaris not only during the first few hours of incubation but even after 22 hours. On agar plates, with cultivation of a mixture of microbes to

Detection of the Plague Microbe by the Presence of Bacteriophage after Plating it from a Mixture of Microbes onto Agar with 0.2 Percent Blood and Agar with Blood and Gentian Violet

Доза чумного микроба в млн. микробных тел	Доза вульгарного протея (в микробных телах)	Наличие реакции бактериофагии при нанесении фага на агар									
		часы					часы				
		3	4	5	6	22	3	4	5	6	22
		петлей					дорожкой				
Посев на агар с 0,2% крови											
50	0	—	—	+	+	+	+	+	—	+	+
50	500	—	+	+	±	±	+	+	+	±	—
50	5 000	—	—	+	±	±	+	+	+	+	—
50	50 000	—	—	+	±	±	+	+	—	+	—
50	500 000	+	+	+	±	±	+	—	+	+	—
50	5 млн.	+	+	+	±	±	—	+	+	+	—
50	50 млн.	+	+	±	—	±	+	+	±	—	—
Посев на агар с кровью и генцианвиолетом											
50	0	+	+	+	+	+	—	+	+	+	+
50	500	+	+	+	+	+	—	+	+	+	+
50	5 000	+	+	+	+	+	±	+	+	+	+
50	50 000	+	+	+	+	+	—	+	+	+	+
50	500 000	+	+	+	+	+	+	+	+	+	+
50	50 млн.	+	+	+	+	+	—	—	—	+	+

Arbitrary Designations: + Presence of Plaques or Tracts; - Absence of Plaques or Tracts; ± Doubtful Result.

Key: 1. Dose of the plague microbe in millions of microbes; 2. Dose of Proteus vulgaris (in microbes); 3. Presence of bacteriophage reaction from application of the phage to the agar; 4. Hours; 5. As a loop; 6. As a tract; 7. Culture on agar containing 0.2 percent blood; 8. Culture on agar containing blood and gentian violet; 9. Million.

which one loop of plague bacteriophage has been added and distribution of the culture over the surface of the plate, after three hours of incubation the small plaques were found which became more noticeable after four-six hours, and after 22 hours the plague bacteriophage completely lysed the plague microbe culture.

On agar plates containing a culture of a mixture of microbes to which the bacteriophage was applied in the form of a tract, after four hours sterile patches were found which became more pronounced after five-six hours, and by 22 hours a broad marked tract with no growth was noted on all plates. We consider the absence of signs of bacteriophagia after three hours, when the experiment is performed by the second method, the effect of excess moisture on the agar.

In a similar way cultures were made from mixtures of the plague microbe with the colon bacillus as well as mixtures of the plague microbe with the dysentery pathogen and gram-positive cocci prepared in physiological

saline solution and Volga water.

On the basis of the experiments performed it was determined that the presence of the colon bacillus, Flexner, Grigor'yev-Shiga [Shiga] and Sonne dysentery bacteria, both separately and in a mixture with the colon bacillus, does not interfere with the phenomenon of bacteriophagia. The plaques and sterile tracts appear and become clearly distinguishable even before the foreign microflora develops (first three-four hours). Later, on agar without gentian violet the abundant growth of mixed microflora occurs which prevents the detection of the plaques. The presence of gentian violet is responsible for a clear-cut picture of bacteriophagia during this period.

Then, we performed a number of experiments for the detection of the plague microbe in the dead bodies of guinea pigs and white mice which had died of plague and had undergone decay.

On the examination of fresh bodies of guinea pigs and white mice which had died of acute plague, in all cases after two and a half-four hours we succeeded in determining the presence of the plague microbe when the organs of the dead bodies were cultivated on blood agar or on agar containing blood and gentian violet together with bacteriophage. After two and a half and four hours, plaques and a tract with no growth were found over the initial growth of the plague microbe on the agar plates. The presence of plague microbe can be detected by means of bacteriophage even when decay is marked. For example, in a culture on agar containing blood and gentian violet taken from the liver of a white mouse which had undergone considerable decay after lying about for five days at a temperature of 25°, plaques and a tract with no growth were found after three-four hours. After 24 hours, on the plates containing blood and gentian violet, large sterile patches and a broad tract were found, and the plates without gentian violet were overgrown with Proteus vulgaris.

We gained the impression that by means of bacteriophage introduced into the material being examined at the time of its culture it is impossible to detect the presence of plague microbe in decaying bodies only in those cases when the plague microbe has already died under the influence of Proteus vulgaris, that is, when it is not found even after ordinary culture.

Conclusions

1. The method of studying decaying material which is very much contaminated with extraneous flora for plague by means of bacteriophage introduced into this material at the time it is streaked is valuable, because it makes it possible to detect the presence of the plague microbe three-four hours after the seeding, that is, sooner than the colonies of other species of bacteria can develop.

2. The method of examination for the presence of the plague pathogen in material which is decaying and very much contaminated by extraneous flora with the aid of specific bacteriophage is simple and is possible not only in hospitals but also in field laboratories.

3. We recommend using this method for the study of bodies of people, rodents, and contaminated objects for plague.

Detection of Bacteriophage in the Bodies of Animals with the Aid of Chloroform

N. M. Sokolova and G. P. Nikitina
(Saratov)

The department of particularly dangerous infectious diseases of the Ministry of Health USSR has appealed to the "Mikrob" Institute to check the effectiveness of the method of detecting bacteriophage in the bodies of animals by means of chloroform, proposed by M. F. Bondarenko (Aral Sea Plague-Control Station), and to evaluate it. The method consists of the following.

A 15-20-hour plague microbe culture of the EV strain is inoculated in 5 cc of a suspension of organs in bouillon. After 24 hours of incubation of the suspension at 37° or after keeping it for two-three days at room temperature, chloroform is added to it according to the calculation of 16.7 percent (1:5 by volume). During the day the mixture is shaken vigorously two-three times for three-five minutes. Two-three hours after the last shaking the upper layer of fluid is examined for the presence of bacteriophage. For this, one drop of it is applied to a freshly seeded sensitive culture on a plate and incubated at 37°.

After one-two days, if bacteriophage suspension is present on the plate, a "sterile" tract appears in the direction of leakage of the drop, and along its margins there is a pronounced Twort phenomenon. If the activity of the bacteriophage is low, it is added a second time, for which purpose 0.5-1 cc of the upper layer of fluid being examined is introduced into 4.5 cc of bouillon, into which the young bouillon culture of the plague microbe had been inoculated. In some cases bacteriophage is added three or four times.

M. F. Bondarenko points out that bacteriophage can be detected in the material examined several weeks and even months (up to 10) after the addition of chloroform to it. The addition of chloroform to the material being studied is for sterilization, with consideration of the fact that it exerts no destructive effect on the bacteriophage.

It should be noted that N. M. Somova and N. A. Sergeyeva, in 1955-1956, quite often isolated pseudotuberculosis bacteriophages under natural conditions from wild rodents which they investigated after the addition of 5 percent chloroform to the suspension of the animal's organs.

As pointed out by M. F. Bondarenko, in a number of cases, at the site of application of the material being investigated on the plates, despite the addition of chloroform, the growth of extraneous microflora is noted. Taking this into consideration, we decided to solve certain problems, in preliminary test-tube experiments, dealing with the effect of chloroform on various species of microbes as well as on bacteriophage.

With this aim in view we checked the bactericidal properties of chloroform in a concentration of 16.7 percent, recommended by M. F. Bondarenko for the detection of bacteriophage in the material being investigated, as well as undiluted chloroform with respect to the following types of cultures: plague microbe, colon bacillus, Proteus vulgaris, Staphylococcus

albus and Staphylococcus aureus, Sarcina lutea, B. subtilis, B. anthracoides and B. anthracis, B. megatherium, B. mesentericus and B. mycoides.

A check of the bactericidal properties of chloroform was made by the generally accepted method, using the suspension without protection and with protection of the culture with 10 percent protein (normal horse serum). Determination of the bactericidal power of chloroform in a concentration of 16.7 percent was made with two-day cultures; of the undiluted chloroform, with 20-hour and five-day cultures.

As the experiment showed, chloroform in a concentration of 16.7 percent exerted a bactericidal effect on all nonspore-forming species of microbes without protection and with protection of them by protein during the first 24 hours and did not exert such an effect on five spore-bearing types of cultures of the six used in the experiment. With reduction of the chloroform concentration (to 5-10 percent) its bactericidal properties also were decreased. Undiluted chloroform exerted a bactericidal effect in 10-30 minutes on all nonspore-bearing and spore-bearing types of cultures with the exception of two: B. mesentericus (20-hour and five-day cultures) and B. anthracoides (five-day culture). Chloroform did not exert a bactericidal effect on these cultures even after three days. We explain this by the fact that the culture of B. mesentericus was different from the other experimental cultures in its very slimy growth, and the copious slime apparently prevented the penetration of the chloroform into the cells, because of which death of them did not occur. The culture of B. anthracoides contained a large number of spores (80-85 percent), of which there were no more than 20-30 percent in the other cultures. In this case some of the spores apparently were resistant to the effect of chloroform and were not subject to its bactericidal effect.

Experiments for the clarification of the chloroform effect on the bacteriophage titer were performed with four monovalent bacteriophages (d'Herelle, Pokrovskaya, Ivanov and Romashova) and two polyvalent bacteriophages prepared at the Stavropol' and Irkutsk Scientific Research Plague-Control institutes. The titer of the phages used in the experiment was checked prior to the addition of chloroform to them and 1.3 and five months after the addition of chloroform to them by the Appelman method. During the entire experiment the bacteriophages were kept at room temperature (20°-24°).

In our experiments the long-term effect (as long as five months) of chloroform on monovalent and polyvalent bacteriophages led to a slight reduction in their titer (by one-three dilutions), just as in the control bacteriophages kept under the same conditions but without chloroform.

After the clarification of the bactericidal properties of chloroform and its effect on the bacteriophage, we made a check of the M. F. Bondarenko method on animals using a parallel control with direct and concentration methods.

In connection with the fact that in our preliminary experiments chloroform did not exert a bactericidal effect on all species of microbes, for the purpose of demonstrating the bacteriophage in rodent organs the culture of the organs was made, aside from simple agar, on agar containing gentian violet (in a concentration of 1:100,000 in the medium). As a control a study was made of the organs of fresh rodents.

The experiments showed that plague bacteriophage in the bodies of

laboratory animals is detected by means of chloroform and by the concentration method for the same periods (as long as 15 days in the bodies of white mice; as long as 10 days in guinea pigs) after their introduction into the body. By the direct method bacteriophage is found for a shorter period (in white mice as long as five days; in guinea pigs, as long as 10 days) after introduction of it into the animal and in a smaller number of cases. Study of the organs of animals which were not given the bacteriophage (control) gave a negative result in all cases.

Therefore, methods of detecting bacteriophage in the bodies of animals by means of chloroform and concentration give the same results; poorer results are given by the direct method.

It should be noted that in various cases the detection of bacteriophage in organs of animals by means of chloroform on ordinary agar was complicated by the growth of extraneous microflora (spore-bearing bacilli) at the site of application of the drop of test material. With simultaneous testing of the same material on agar containing gentian violet, no growth of extraneous microbes was found in any case. When bacteriophage was present in the organs on medium containing gentian violet a "sterile" tract could always be noted on the seeded areas in the direction of leakage of the drop of test material. Therefore, it is better to detect bacteriophage in the test material with chloroform on agar containing gentian violet than on ordinary agar.

It should also be kept in mind that the chance contact of chloroform with the plate seeded with the plague microbe culture can simulate the bacteriophagia phenomenon. "Sterile" tracts obtained on seeded plates under the influence of chloroform were macroscopically the same as tracts obtained under the influence of bacteriophage. Only microscopic examination (absence of the Twort phenomenon) and further observation of the tract (absence of the "spreading" phenomenon) permit determining the origin of the "sterile" tract.

Conclusions

1. When bacteriophage is detected in the organs of animals by means of chloroform and simultaneously by concentration and direct methods, better results were obtained from the use of the first two methods.
2. The method of detection of bacteriophage by means of chloroform considerably facilitates the work of detecting the bacteriophage in the organs of animals by comparison with the concentration method, because in the former case the factor of filtration has been ruled out.
3. In connection with the fact that chloroform does not exert a bactericidal effect on certain spore-bearing cultures, in cases where suspensions from the organs have been contaminated with them, the test for bacteriophage should be made on seeded plates of agar containing gentian violet.
4. The long-term effect of chloroform on bacteriophage (as long as five months) under room temperature conditions (20°-24°) exerts practically no effect on its titer.
5. The results which we obtained permit us to consider that the method of detecting bacteriophage in the bodies of animals by means of chloroform proposed by M. F. Bondarenko can be recommended for practical work.

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Study of the Properties of the Plague Microbe Culture Kept on Dry Nutrient Medium

V. M. Tumanskiy, Ye. E. Bakhrakh, N. M. Sokolova, Z. A. Yurgina, G. P. Nikitina and Z. A. Obukhova
(Saratov)

At the "Mikrob" Institute a dry medium suitable for the diagnosis of the plague microbe (V. M. Tumanskiy and others, 1958) was obtained by the somewhat modified method of N. Ploskirev and others (1941) from an enzymatic meat hydrolysate.

In the present article the results of the study of the morphological, cultural, biochemical and other characteristics as well as the immunogenicity and virulence of the plague microbe kept for a long time on this medium are being given.

The work was conducted with two strains of the plague microbe: virulent 708 and vaccinal EV strains. These strains behaved in a manner typical of the plague microbe after preliminary checking of the morphological, biochemical and other characteristics. Both strains were inoculated into test tubes containing agar prepared from dry medium and, for the purpose of comparison, in test tubes containing Hottinger's agar.

After a two-day incubation at 28° some of the test tubes with cultures which had grown out on agar made of dry medium and on Hottinger's agar were kept at room temperature (20°-25°); the others were kept in a refrigerator at 4°. Under these conditions the cultures remained for 10 months without subculture, after which they were plated out on corresponding agar and bouillon. Thereby, it was noted that subcultures of both strains grow out well on solid and liquid nutrient media. The colony morphology and nature of growth on bouillon was typical of the plague microbe in all cases.

The cell morphology in smears made of agar and bouillon cultures was also typical of the plague microbe.

The biochemical activity of the strains did not change by comparison with their original activity when the cultures were observed for 35 days.

Subcultures of both strains fermented glucose, maltose, mannitol and did not split lactose, sucrose or rhamnose. The subcultures of the virulent 708 strain broke down glycerin, those of the EV vaccine strain did not ferment it. Subcultures of the EV strain gave a positive test for nitrites and nitrates; subcultures of the 708 strain, only a denitrification reaction.

Subcultures of the 708 and EV strains did not form indole or hydrogen sulfide, were readily agglutinated in the presence of antiplague agglutinating serum and were lysed by plague and pseudotuberculosis bacteriophages. The virulence was checked by means of subcutaneous injection of a test subculture of the 708 strain into guinea pigs (weighing 300-400 grams), using 10, 100, 1000 and 10,000 microbes.

As the experiment showed, in guinea pigs infected with a subculture of the 708 strain kept on agar made of dry medium at room temperature, the average lifespan was 7.9 days, whereas in guinea pigs infected with a subculture kept on Hottinger's agar under the same temperature conditions, it was 13 days. When kept on the same medium but in a refrigerator at 4° the average lifespan in guinea pigs infected with a subculture from Hottinger's agar was less than in guinea pigs infected with a subculture from agar made of dry medium.

From the data presented it follows that virulence was maintained somewhat better in subcultures on agar made of dry medium at room temperature and in the subculture from Hottinger's agar under refrigerator conditions. In the study of the immunogenicity of the subcultures of the EV vaccine strain the following method was adopted.

Guinea pigs (weighing 300-450 grams) numbering 130 were divided into 13 groups of 10 guinea pigs each. Of these, 12 groups were experimental and one, control. The experimental groups of guinea pigs were vaccinated with cultures from the agar made of dry medium and Hottinger's agar after they had been kept for 10 months under different temperature conditions.

The schema of immunization was the following.

A suspension of a two-day agar culture was injected into guinea pigs subcutaneously in doses of 1000, 1,000,000 and 1,000,000,000 microbes (10 guinea pigs were vaccinated with each dose). After 20 days, all the guinea pigs vaccinated and all the controls (nonimmunized) were infected subcutaneously with a highly virulent strain of the plague microbe (200 CLD). The results of the experiment are given in the Table.

As is evident from the Table, all control guinea pigs died of plague after five days, on the average. Animals vaccinated with subcultures of the EV strain kept on Hottinger's agar at room temperature showed a low survival rate. A greater survival was noted among guinea pigs vaccinated with a subculture kept on dry agar at room temperature. A somewhat higher survival rate was noted after vaccination with subcultures kept in the refrigerator. Thus, of 30 animals vaccinated with the culture kept on Hottinger's agar, 11 survived, whereas of those vaccinated with the culture grown out on dry agar, 10 survived.

All the vaccinated guinea pigs which did not die as the result of infection with the virulent strain were killed after 35-40 days. Study of them showed that they were not infected with plague.

As the result of the studies made it was determined that the cultural, biochemical and immunogenic properties of the plague microbe as well as its virulence when kept on agar made of dry medium are maintained just as well as on Hottinger's agar, usually utilized. Therefore, the given dry medium can be used not only for the diagnosis of the plague microbe but also for the production of bacterials as well as for the long-term storage of plague microbe cultures.

Immunogenicity of Cultures of the EV Strain of the Plague Microbe

Среда (1)	Температура хранения (2)	Иммунизирующая доза (3)	Число испытанных животных (4)	Число выживших животных (5)
(6) Сухой агар	(10) Комнатная	1 000	8	1
		1 млн. (11)	8	2
		1 млрд. (12)	10	4
(7) То же	4°	1 000	10	0
		1 млн.	10	2
		1 млрд.	10	8
(8) Агар Хоттингера	(10) Комнатная	1 000	10	0
		1 млн.	10	2
		1 млрд.	10	3
(7) То же	4	1 000	9	0
		1 млн.	10	4
		1 млрд.	10	7
(9) Контроль		0	10	0

1. Medium; 2. Temperature at which kept; 3. Immunizing dose; 4. Number of animals tested; 5. Number of surviving animals; 6. Dry agar; 7. The same; 8. Hottinger's agar; 9. Control; 10. Room; 11. Million; 12. Billion.

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The Use of Polycarbohydrate Medium for the Study of Material for Presence of the Cholera Vibrio

A. K. Adamov, O. N. Mosolova, L. M. Gol'dfarb and N. I. Kuznetsova
(Saratov)

Polycarbohydrate media of the Russell type are extensively used for the investigation of various objects for the presence of typhoid-paratyphoid and dysentery microbes (Ye. A. Avdeyeva, 1941; Ye. D. Ravich-Birger and V. N. Meshalova, 1955; M. I. Lur'ye, 1955; V. I. Buyevich, 1957; T. G. Terent'yeva, 1957; A. B. Chernomordik, 1957; I. S. Ol'kenitskiy, 1958). The use of polycarbohydrate media makes it possible to accelerate the selection of the most characteristic cultures and to reduce considerably the time for making the analysis.

In the analysis of material infected with cholera vibriones it is particularly important to select quickly the most typical cultures which must be further studied. Russell's medium is poorly suited to these purposes, because biochemical changes produced in it by the cholera vibrio

are no different from the changes caused by the majority of other species of pathogenic microbes of the colon group.

With the aim of seeking a convenient medium for the rapid selection of cultures suspected of the cholera vibrio, we made a study of a number of variants of polycarbohydrate media containing lactose, sucrose and arabinose in various combinations. The choice of these carbohydrates specifically was determined by the biochemical characteristics of the cholera vibriones and other species of intestinal microbes. The cholera vibriones actively split sucrose without gas formation, split lactose slowly and do not split arabinose. Of the other representatives of the microbes of the colon group, only some species are capable of splitting sucrose, arabinose and lactose without forming gas. We considered it advisable not to include glucose in the polycarbohydrate media being tested, because this carbohydrate is split by the majority of biochemically active microbes.

In preliminary experiments a study was made of the effect of the concentration of nitrogenous substances on changes in the pH of the medium caused by microbes in media containing lactose, sucrose and arabinose. Acid-formation was manifested most distinctly on media containing 0.5 percent peptone in which the pH of the medium was 7.4. Therefore, in the experiments this concentration of peptone was used.

Polycarbohydrate media were prepared according to the following recipe: peptone, 0.5 gram, salt, 0.5 gram, agar, 1 gram, carbohydrates depending on the experimental conditions; indicator, bromthymol blue solution, 2 cc, or Andrade's reagent, 4 cc, depending on the experimental conditions, distilled water, 100 cc; the pH of the medium was in accordance with the experimental conditions (Andrade's reagent was prepared by the generally accepted method. The solution of bromthymol blue was prepared by the following method: 0.8 cc of 5 percent sodium hydroxide solution and 24.1 cc of distilled water were added to 0.1 gram of bromthymol blue).

The medium was poured out and the culture was made on it according to the generally accepted method for culture on Russell's medium. The cultures were kept in an incubator at a temperature of 37° for 48 hours. The results were read after 24 and 48 hours.

Of the polycarbohydrate media tested which contained arabinose, lactose and sucrose in various combinations the best results were obtained on media containing 0.1 percent sucrose, 1 percent lactose (at a pH of 7.4 using Andrade's indicator). Changes caused by the vibriones on this medium were very much different from the changes caused by other species of microbes of the colon group. Cholera vibriones caused the formation of acid only in the column. Typhoid-paratyphoid and dysentery microbes as well as B. faecalis alcaligenes failed to change the color of the medium. The colon bacillus split sucrose and lactose with the formation of acid and gas.

The suitability of the medium containing sucrose and lactose for tentative determination of the culture suspected of growth of the cholera vibrio was checked on a large number of strains of different species of microbes. The results of the experiments are shown in the Table.

The Table data show that the majority of vibriones, with the exception of one strain of V. metschnikovi, split carbohydrates of the sucrose-lactose medium with the formation of acid in the column. With the growth of V. metschnikovi in this medium no acid formation was noted. The growth of

Growth of Intestinal Microbes on Sucrose-Lactose Medium

Виды микробов	Количество штаммов	Части среды	Изменения среды
(5) Холерные вибрионы	19	К / С	— / К
(6) Вибрионы El-Tor	3	К / С	— / К
(7) Парахолерные вибрионы	5	К / С	— / К
(8) Вибрион Мечникова	1	К / С	— / —
(9) Водный вибрион	1	К / С	— / К
(10) Фосфоресцирующий вибрион	1	К / С	— / К
(11) Тифо-паратифозные бактерии	2	К / С	— / —
(12) Дизентерийные палочки	3	К / С	— / —
(13) Кишечные палочки	3	К / С	К / КГ
Паракишечные палочки (14)	2	К / С	— / КГ или — (or)
(15) Щелочеобразователь	1	К / С	— / —

Note. In the column "Parts of the Medium" the letter "K" designates the slant portion of the medium; the letter "C," the column. In the column "Changes in the Medium" the letter "K" designates "acid"; the letter "Г" designates gas; the minus sign (-) denotes the absence of changes in the medium.

Key: 1. Species of microbe; 2. Number of strains; 3. Parts of the medium;

[Table continued next page]

[Table continued from previous page]. 4. Changes in the medium; 5. Cholera vibriones; 6. El-Tor vibriones; 7. V. paracholerae; 8. V. metschnikovi; 9. V. aquatilis; 10. V. phosphorescens; 11. Typhoid-paratyphoid bacteria; 12. Dysentery bacilli; 13. Colon bacilli; 14. Paracolitic bacilli; 15. 15. Alkali former.

typhoid-paratyphoid, dysentery microbes and B. faecalis alcaligenes on sucrose-lactose medium was also unaccompanied by the formation of acid products, and the color of the medium did not change. This fact is of particular significance for the differentiation of the cholera vibrio from B. faecalis alcaligenes, which in its morphological and cultural characteristics shows a certain similarity to the pathogen of cholera. The colon bacillus split carbohydrates of the sucrose-lactose medium with the formation of acid and gas.

The results of the experiments presented attest to the expediency of utilizing sucrose-lactose medium for the study of material suspected of infection with cholera vibriones. This medium makes it possible, on mass examinations, rapidly to select cultures suspected of being cholera vibriones.

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The Problem of an Accelerated Agglutinin Test by the Centrifugation Method in Brucellosis

M. G. Lokhov
(Saratov)

For the laboratory diagnosis of brucellosis and determination of the immunological condition of the body, at the present time the Wright agglutinin test, the accelerated Huddleson slide test, and the allergic test with

brucellin by the Burnet method, the complement-fixation test and the opsonocytophagic test are used. However, all these tests have a number of essential shortcomings.

The complement-fixation test and the opsonocytophagic test are rarely used in laboratories because of the difficulty in performing them.

The results of the allergic test with brucellin by the Burnet method are usually read after 24 hours. Therefore, the final diagnosis in the patient can be made only after 24 hours. In addition, the Burnet test is often negative during the first few days of a disease in man.

After the work of A. A. Uvarov and Ye. P. Konoval'tseva (1941), Ye. I. Kaytmazova (1945), M. L. Feder and N. M. Shemeneva (1947), M. V. Gorbacheva (1949) on checking the specificity and determining the diagnostic value of accelerated and simplified Huddleson tests, this test, together with the Burnet test, was widely used for diagnostic purposes in medical districts and at feldsher-midwife stations, which is explained by the simplicity of it, the rapidity with which the results are obtained and specificity. However, the value of the Huddleson test has recently been doubted by some authors (Ye. S. Dem'yanov, 1947; K. P. Studentsov, 1949; G. A. Balandin, 1955). Apparently, in connection with this, in instructions on serological diagnosis approved by the Ministry of Health USSR dated 23 March 1954, the utilization of the Huddleson test for the diagnosis of brucellosis is limited at the present time (edited by V. M. Zhdanov and Z. A. Plankina. Brutsellez. Organizatsionno-Metodicheskiye Materialy (Brucellosis. Material on Organization and Methods), 1955, p. 85). The main defect of the Wright test is: 1) the need for laboratory conditions in performing it; 2) the obtaining of the final result of the examination only after 18-24 hours.

These defects are eliminated by performing the accelerated agglutinin test by the centrifugation method.

We performed the agglutinin test by the centrifugation method in the following way. Blood was taken from people by vein. For the purpose of obtaining serum the blood was slanted in test tubes. The agglutinin test was performed with serum dilutions from 1:50 to 1:800. As an antigen killed cultures were used which we obtained from the Rostov and Odessa institutes, the Omsk and Pyatigorsk brucellosis-control stations, which contained 10,000,000,000 microbes per cc. In performing the test the plan for performing the Wright test given in the instructions mentioned above was adhered to strictly. The tests in the test tubes were centrifuged for 30 minutes immediately after performing the test and then the result was read.

In the case of a positive agglutinin test complete clarification of the fluid is obtained with the formation of a characteristic "umbrella" on the bottom of the tube, which on shaking gives clearly expressed clumps or granules, readily visible on ordinary examination. In the case of negative results, either a uniform turbidity remains or the fluid is slightly clarified. Thereby, the precipitate formed usually rises after shaking in the form of a "pigtail" and readily breaks up into a uniform turbidity.

The entire test by the centrifugation method takes up no more than an hour from the beginning of the work and no more than two hours from the time the blood is taken.

Centrifugation was performed in a hand-operated milk centrifuge, into which 120 test tubes were placed, that is, simultaneously from 20 to

30 analyses were made. This permitted us to utilize the hand-operated milk centrifuge for mass studies under field conditions.

The agglutinin test by the centrifugation method was studied during the examination of the population for the effectiveness of prophylactic inoculations with dry living vaccine, in checking for the effectiveness of prophylactic inoculations among cattle, in examining the population in foci of brucellosis for detection of patients and retrospective diagnosis, and for examination of flocks of sheep for epidemiological indications. Simultaneously, the classic agglutinin test, Huddleson test and Burnet test were performed. In a rural locality and under field conditions the examination was limited to the three methods: the agglutination test by centrifugation, the Huddleson test and the Burnet test. Not uncommonly, because of the working conditions we had to limit ourselves to two tests: the agglutinin test by the centrifugation method and the Huddleson test. In the examination of animals in some cases the complement-fixation test was performed.

The summarized results of the studies are shown in the Table.

The agglutinin test by the centrifugation method in all cases gave more positive results than did the agglutination test by the classical method. Thus, in the examination of cattle on farms unfavorable for brucellosis, 8.6 percent more positive results were obtained in the first case than in the second; in the examination of cattle in the isolation ward, 8 percent more.

In the examination of the population on farms unfavorable for brucellosis, 10.7 percent more positive results were obtained by the agglutinin test with centrifugation in one case; in another, 12.6 percent more; on checking the effectiveness of inoculations among the population, in one case, 14.1 percent more positive results were obtained; in another, 17.8 percent more; on checking sheep's milk, 14 percent more; on examining cow's milk, 17 percent more; on the examination of sheep according to epidemiological indications, 21.2 percent more; in acute foci of brucellosis in short-horned cattle, 26.5 percent more.

In our studies the agglutinin titers of the classic agglutinin test were considerably lower than the agglutination test by the centrifugation method.

On examination of the population for brucellosis in unfavorable places as well as on the examination of short- and long-horned cattle and in checking on the effectiveness of prophylactic inoculations among people, we obtained 398 positive results. The agglutinin titers for the agglutinin test by the centrifugation method in serum dilutions of 1:50 were less than the agglutinin titers of the classic method by 28.3 percent; in dilutions of 1:100, 7 percent more; in dilutions of 1:200, 19.9 percent more; in dilutions of 1:400, 1.8 percent less; in dilutions of 1:800, 4.3 percent more.

On the investigation of long-horned cattle for the effectiveness of prophylactic inoculations, 330 positive results were obtained. For agglutinin titers of 1:100 no positive results were obtained by the agglutinin test by the centrifugation method. In titers of 1:200 positive results, obtained by the centrifugation method, were 8.1 percent fewer than by the classical method; in titers of 1:400, 13.7 percent fewer; in a titer of 1:800, 13.7 percent more; 1:1600, 8.1 percent more; 1:3200, 2.2 percent more.

Comparative Study of the Immunological State of the Body in Brucellosis by Means of the Agglutinin Test with Centrifugation and Other Immunological Tests

Объекты и цель исследований	Число исследований	Число положительных результатов: число	Число положительных результатов: %	Процент положительных результатов от общего числа исследований при реакции: агглютинации центрифугированием	агглютинации классической	Хеддльсона	Бюрне	связывания комплемента
11 Проверка эффективности профилактических прививок	866	456	52,7	46,0	31,9	52,7	42,6	—
12 То же	843	399	47,3	40,5	22,7	47,3	—	—
. .	415	219	52,8	42,6	—	47,4	20,0	—
. .	259	137	52,9	47,1	—	51,3	—	—
13 Проверка эффективности прививок у телят через 20—30 дней после прививок	341	330	96,8	97,1	97,1	97,1	.	.
14 Обследование населения в неблагополучных по бруцеллезу пунктах	197	69	35,0	27,0	16,3	34,3	16,2	—
12 То же	84	53	63,1	58,9	46,3	60,7	—	—
. .	210	108	45,0	32,5	—	39,1	33,5	—
. .	208	30	14,4	12,0	—	12,0	—	—
15 Проверка овец по эпидемиологическим показаниям	91	63	69,2	36,6	15,4	55,0	—	45,0
16 То же в очагах бруцеллеза	53	43	81,1	68,0	41,5	75,4	—	—
12 То же	28	19	68,0	35,6	35,6	.	—	60,6
17 Обследование крупного рогатого скота с клиникой бруцеллеза	10	10	100,0	100,0	100,0	100,0	—	—
18 То же в изоляторе	50	43	86,0	80,0	72,0	—	—	50,0
19 То же в хозяйствах, неблагополучных по бруцеллезу	140	90	64,3	17,9	9,3	63,6	—	9,3
20 Исследование молока овец в неблагополучных хозяйствах	105	26	24,8	22,0	8,0	18,0	—	—
21 То же молока коров	127	54	42,5	40,9	23,0	33,8	—	—
22 Всего . . .	4056	2149	53,0					

1. Objects and purpose of the examinations; 2. Number of examinations; 3. Number of positive results; 4. Percentage of positive results in the total number of examinations obtained from the following test:; 5. Number; 6. Agglutination with centrifugation; 7. Classic agglutination; 8. Huddleson; 9. Burnet; 10. Complement-fixation; 11. Check on the effectiveness of prophylactic inoculations; 12. The same; 13. Check on the effectiveness of inoculation in calves 20-30 days after the inoculation; 14. Examination of the population in places unfavorable for brucellosis; 15. Check on sheep according to epidemiological indications; 16. The same in foci of brucellosis; 17. Examination of cattle with the clinical picture of brucellosis; 18. The same in an isolation ward; 19. The same in farms unfavorable for brucellosis; 20. Examination of sheep's milk on unfavorable farms; 21. The same for cow's milk; 22. Total.

In those cases where only the agglutinin test by the centrifugation method gave positive results or where simultaneously positive results were given by the Huddleson test, the agglutinin titers were low. Thus, in 27 cases where the agglutinin test was positive and the other tests for brucellosis were negative, the agglutinin titer was only 1:50. In 424 cases where positive results were obtained by the Huddleson test, the agglutinin titers were: 1:50 in 67.7 percent of the cases; 1:100, in 21.9 percent; 1:200, in 8.5 percent; and 1:400, in 1.9 percent of the cases. Low titers for the agglutination test by the centrifugation method, obtained by this method alone [the word "or" has probably been left out here] with simultaneous confirmation of the results by the Huddleson test, from our point of view, speak for its greater sensitivity than that of the classic agglutinin test. The agglutinin test with centrifugation shows more positive reactions than the Burnet test. Thus, in examining the population for the effectiveness of prophylactic inoculations, more positive results were demonstrated by the agglutination method with centrifugation than by the Burnet tests to the extent of 22.6 percent; in the examination of the population in inhabited places unfavorable for brucellosis, 9.5 percent more.

In comparing results obtained in the agglutinin test by the centrifugation method and in the complement-fixation test, controversial data were found.

In farms with an acute course of brucellosis among short-horn cattle the positive results obtained were more by the complement-fixation test than by the agglutinin test with centrifugation: in one case, by 7.4 percent; in another, 25 percent.

In farms in which brucellosis had a chronic course among the long-horn cattle, more positive results were demonstrated by the agglutination test by the centrifugation method than by the complement-fixation test: in one case, 8.6 percent more; in another, 30 percent more.

Of the five methods by which we made the examinations for brucellosis, the largest number of positive results was obtained by the Huddleson test. This test gave 1.8-7.3 percent more positive results than the agglutinin test by the centrifugation method. This is apparently explained by the fact that nonspecific reactions were obtained in some cases.

Thus, in one farm which was favorable with respect to brucellosis among long-horned cattle, 35 out of 72 head were found to react positively to brucellosis by the Huddleson test, that is, more than 50 percent. Of these, the reactions to serum doses were as follows: 0.08 percent serum, four; 0.4 percent, six; 0.02 percent, 13; 0.01 percent, 12. In all cases the agglutinin test by the method of centrifugation and the complement-fixation tests were negative.

Observation of this farm, conducted for a year, showed its complete welfare with respect to brucellosis. In this farm the finding of animals reacting positively for brucellosis can be explained only by nonspecific Huddleson tests.

For the purpose of diagnosing brucellosis the agglutinin test by the centrifugation method is specific.

We checked the following agglutinating sera with a killed culture of brucellas: typhoid, paratyphoid A and B, typhoid, paratyphoid A and B, Gärtner, Breslau [varieties of S. enteritidis], S. suipestifer, Morgan's

bacillus, the Grigor'yev-Shiga [Shiga] bacillus, Hiss-Flexner bacillus, Stutzer, and Kruze-Sonne bacilli, which showed high titers. In all cases negative results were obtained. The agglutinin test by the centrifugation method with antigens prepared from cultures of typhoid, paratyphoid A and B, Gärtner's bacillus, Breslau bacillus, S. suipestifer, Morgan's bacillus, Shiga and Hiss-Flexner bacilli, Stutzer, and Kruze-Sonne bacilli gave negative results with agglutinating brucellosis serum. Negative results were also obtained when the agglutinin test was performed by the centrifugation method in all persons who had not been sick with brucellosis and in animals on farms which showed no sign of this infectious disease.

Conclusions

1. According to our observations, the agglutinin test with the use of centrifugation for the examination of various objects gives positive results 8-26.5 percent more often than the classic Wright test.
2. A positive agglutinin test, performed with centrifugation, is found 9.5-22.6 percent more often than a positive Burnet test.
3. More positive results are found by the Huddleson test than by the agglutinin test by the centrifugation method to the extent of 1.8-7.3 percent. However, in the Huddleson test, nonspecific results can occur.
4. The agglutinin test by the centrifugation method has an advantage over the accelerated agglutinin test by the Huddleson method in the fact that it shows the quantitative characteristics of the test. The agglutinin test by the centrifugation method is specific and is not positive in other infectious diseases. The results of this test are read 40-60 minutes after the beginning of performance of it and two hours after the time of obtaining blood for examination.
5. The agglutinin test by centrifugation, technically easy to accomplish, can be performed in a rural locality in all medical and veterinary districts even for mass examinations.

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Accelerated Method for the Detection and Identification of Anthrax Bacilli in the Soil

L. S. Petrova and V. S. Larina
(Saratov)

A considerable number of papers has been given over to the problem of detection and differential diagnosis of anthrax bacilli in the literature of recent years. A description has appeared of accelerated method for the identification of the anthrax pathogen. Some of them are used for differentiation of microbes which have already been isolated (Jensen, Kleemeyer, 1953; Brown and Cherry, 1955; M. N. Meysel' and others, 1957; E. N. Shlyakhov, 1960; A. N. Dolgov, 1960); others for the detection of the bacilli directly in the objects being examined (P. I. Pritulin and M. A. Kuz'min, 1959; V. Ya. Mikhaylov and others, 1960). As a rule, both are based on some single characteristic of the anthrax microbe.

Ivanovic and Felders (1958) suggested identifying anthrax bacilli by their power of forming phosphatase, by specific phage lysis and by sensitivity to penicillin. V. Ya. Antonets and others (1959) recommend using the action of anthrax bacilli on lecithin, red blood cells, methylene blue in Borisov's medium with consideration of the nature of growth on bouillon for this purpose. However, despite the application of these methods, the detection of anthrax bacilli in the soil, which is one of the sources of anthrax infection, is still difficult.

Thus, for example, A. V. Koronnyy (1958) isolated only 18 cultures of anthrax bacilli from 1000 samples of soil and water investigated, taken from anthrax foci. Such a very small number of findings is possibly explained by the fact that the soil is the most complicated object for bacteriological examination, because it contains various microorganisms, many of which are antagonists to the anthrax bacilli.

Therefore, on the basis of data presented in the literature it may be considered that for the purpose of detecting anthrax bacilli in the soil there are no sensitive and, at the same time, available reliable accelerated methods to date. Therefore, further search along this line is of very great current importance. The present work has been given over to this purpose.

Method of Investigation

For the examination a sample of soil from a garden-orchard plot was taken, 1 gram of which contained about 16,000,000 different microorganisms with the predominance of spore-forming saprophytes. Viable spores of anthrax

bacilli were added to the soil samples in quantities of $8 \cdot 10^4$, $8 \cdot 10^3$, $2 \cdot 10^3$, $1 \cdot 10^3$, suspended in 1 cc of physiological saline solution. The soil was infected with standard spore material prepared from four different strains of anthrax bacilli. Then, each sample of soil was suspended in 3 cc of distilled water, carefully shaken up and allowed to settle for 10 minutes. The supernatant fluid was poured off and treated by the Dold method at 37°-40° on a water bath for 10 minutes, after which it was centrifuged at 1000 revolutions a minute for 30 minutes. The bulk of the fluid was removed, and the lower portion of the supernatant fluid in a volume of 0.4-0.5 cc was taken for examination. The examination was made by the bacteriological method and with the aid of biological tests on animals. For this purpose, 0.1 cc of the supernatant fluid was applied to Hottinger's agar plates (pH, 7.2) without blood and with 5 percent sheep blood, and three plates with the corresponding medium were seeded serially with a spatula. In parallel, white mice were infected with the soil centrifugate. As is well known, the usual method of isolating the anthrax pathogen from different objects by means of biological tests on animals requires from three to 10 days and does not always give a positive result. According to the data of a number of authors, the sensitivity of animals to pathogenic microbes can be increased by means of administering the yolk of a hen's egg (M. L. Kapusto and V. I. Kuzin, 1937; I. I. Kurayev, 1960) and corticosteroid hormones (de Lamater and others, 1953) to them. On the basis of these data, we infected the animals with the soil centrifugate together with yolk or in combination with the following hormones: cortisone, desoxycorticosterone or insulin. For this purpose the centrifugate being investigated was mixed in a volume of 0.1 cc with 0.5 cc of egg yolk diluted with physiological saline solution (1:2), and the mixture was injected into mice subcutaneously in the area of the right groin. The hormones were injected into the thigh muscle of the left hind extremity directly before infection (cortisone and insulin were injected in a dose of 2.5 and 0.02 unit; desoxycorticosterone, 0.5 milligram).

Impressions were taken from the organs of mice which had been killed or which had died, on which the biological test was being performed, with subsequent seeding on Hottinger's agar (pH, 7.2) containing 5 percent blood and without it, using loops for this purpose. Smear-impressions were made from the organs on a glass slide for detection of the capsule (staining by the Romanowsky method). The spleen and tissue from the infection site were tested for the presence of anthrax antigen by the method of diffusion in gel, using standard agar plates (V. V. Akimovich and others). Cultures of the organs were examined after eight, 10, 18 and 20 hours of growth at 37°.

Colonies suspicious because of their characteristic morphology and the absence of hemolysis were inoculated into Hottinger's bouillon (pH, 7.2) for the performance of the "pearl necklace" test, determination of the nature of growth, motility in a hanging drop; these colonies were studied by the method of diffusion in gel precipitation, using standard agar plates. The results of the test were read after five, six, eight and 12 hours. The growth characteristics on bouillon were noted after six hours.

Results of the Studies Made

On direct bacteriological examination anthrax bacilli could be found in the soil only when there was a relatively high content of the viable spores (from $8 \cdot 10^3$ to $8 \cdot 10^4$ per gram of soil). The isolation of anthrax microbes from the soil is difficult, chiefly because of the abundant growth of soil microbes, which frequently form colonies which in many characteristics resemble colonies of anthrax bacilli (in morphology and sometimes even in the absence of hemolysis); in addition, in a number of cases the colonies of the true anthrax bacilli caused the occurrence of more or less distinct zones of hemolysis toward the end of the first day of cultivation; therein our data are in agreement with those of a number of other investigators (Buza, 1940; A. D. Melikhov, 1958). Frequently, even in the first few hours of growth the soil spore-forming aerobes formed broad zones of hemolysis; as the result it was very difficult to differentiate the colonies of the true anthrax bacilli growing in the immediate vicinity of these colonies.

Therefore, direct bacteriological examination of the soil for the presence of anthrax bacilli is not a very sensitive method.

More encouraging results were given by the method of biological tests on animals. For the purpose of solving the problem of which of the stimulants of the infectious process in animals is most effective for the biological tests, an experiment was performed on 63 white mice which were divided into five groups of 12-15 each. The animals were studied after their deaths. Mice of the first group were injected only with a soil centrifugate; mice of the second group, with a centrifugate containing egg yolk; mice of the third group, with a centrifugate containing egg yolk, cortisone and desoxycorticosterone; mice of the fourth group, with a centrifugate containing insulin; mice of the fifth group, cortisone and desoxycorticosterone before infection (Table 1).

As is seen from Table 1, in the first, third and fourth groups of animals infected with the soil centrifugate, using, respectively, yolk, insulin, cortisone and desoxycorticosterone, approximately the same results were obtained. The animals of these groups died 50 hours after infection. In all the mice which died the anthrax antigen was found in the spleen and in the tissues taken from the infection site. In each group eight pure and three-four mixed cultures were isolated from the animals. In the first group of mice, cultures isolated from 12 mice were identified as anthrax by all the tests used, with the exception of two cultures which did not give the "pearl necklace" phenomenon because of the presence of extraneous flora. In the third group of 12 mice the anthrax pathogen was isolated in 10 cases; in the fourth, from 11 out of 12 animals. In mice of the second group the anthrax pathogen was isolated from only three out of 12 animals; true enough, death occurred much sooner than in the animals of the first, third and fourth groups. Death of the control animals occurred after 60 hours, that is, 10 hours later than in the experimental animals (first, third and fourth groups). Diffusion in gel showed precipitation with the organs of animals in 13 cases. However, a pure culture could be isolated from only seven of the animals which died. A mixed culture was isolated from the other eight mice, which

Table 1

Detection of Anthrax Bacilli in Mice Which Died Depending on the Preparation with Which They Were Treated

(1) Номер группы	(2) Вводимый материал	Число мышей	(4) Срок гибели мышей после заражения (часы)	(5) Число мышей, у которых обнаружена культура: (8) чистая	(9) смешанная	(6) Число мышей, органы которых дали преципитацию в агаре	(7) Число мышей, органы которых дали положительные: (10) преципитацию в агаре	(11) тест «жемчужного ожерелья»
1	(12) Желток	12	50	8	4	12	12	10
2	Желток, кортизон и дезоксикортикостерон (13)	12	34	3	9	10	4	3
3	Инсулин (14)	12	50	8	3	12	10	10
4	Кортизон и дезоксикортикостерон (15)	12	50	8	4	12	11	11
5	Контроль (без обработки) (16)	15	60	7	8	13	7	7

Note. 1000 spores of anthrax bacilli were introduced into 1 gram of soil.

1. Number of groups; 2. Material administered; 3. Number of mice; 4. Period of survival of the mice after infection (hours); 5. Number of mice in which the culture was found; 6. Number of mice whose organs showed precipitation in the diffusion in gel test; 7. Number of mice whose organs were positive; 8. Pure; 9. Mixed; 10. In the diffusion in gel test; 11. In the "pearl necklace" test; 12. Yolk; 13. Yolk, cortisone and desoxycorticosterone; 14. Insulin; 15. Cortisone and desoxycorticosterone; 16. Control (without the use of a stimulant).

could not be identified because of the abundance of extraneous microorganisms.

Therefore, with the separate administration of yolk, insulin or corticosteroid hormones there was only a very slight shortening in the survival period of the biological test animals.

With the aim of accelerating the detection of anthrax bacilli in the soil, in the second series of experiments we sacrificed the mice six, 12, 18 and 24 hours after infection. The experiment was performed on 195 mice according to the same plan as for the first (Table 2).

Bacteriological examination of the organs of mice sacrificed after six hours gave a negative result in all cases. The best results were obtained in the first group of mice, infected with the soil centrifugate together with yolk. From mice sacrificed after 12 and 18 hours, a culture of the anthrax pathogen was isolated and identified in 11 to 12 mice out of 15. Somewhat poorer results were obtained from the examination of mice of the second group, infected with hormones along with the yolk and corticosteroids; even poorer results, in the group of animals infected with the soil centrifugate together with insulin. Anthrax bacilli could be isolated from animals infected with the centrifugate together with cortisone and

Table 2

Detection of Anthrax Bacilli in Killed Mice Depending on the Preparation with Which They Were Injected

(1) Номер группы	(2) Вводимый материал	(3) Число мышей	(4) Срок гибели мышей после заражения (часы)	(5) Число мышей, у которых обнаружена культура		(8) Число мышей, органы которых дали преципитацию в агаре	(9) Число мышей, органы которых были положительные	
				(6) чистая	(7) смешанная		(10) преципитация в агаре	тест „жемчужного ожерелья" (11)
1	Желток (12)	15	12	9	2	0	11	11
		15	18	11	1	12	12	12
		12	24	7	0	7	7	7
2	Желток, кортизон и дезоксикортикостерон (13)	15	12	4	3	1	7	5
		12	18	5	7	10	11	9
		12	24	7	4	11	10	7
3	Инсулин (14)	12	12	2	0	1	2	2
		12	18	7	0	6	7	7
		15	24	7	1	10	8	7
4	Кортизон и дезоксикортикостерон (15)	12	12	1	0	0	1	1
		12	18	4	2	6	6	6
		11	24	9	2	11	11	10
5	Контроль (без обработки) (16)	21	12	0	0	0	0	0
		15	18	5	0	5	5	5
		15	24	8	1	8	8	8

Note. The same as for Table 1.

Key: 1. Number of groups; 2. Material administered; 3. Number of mice; 4. Period of survival of the mice after infection (hours); 5. Number of mice in which a culture was found; 6. Pure; 7. Mixed; 8. Number of mice whose organs showed precipitation in the diffusion in gel test; 9. Number of mice whose organs were positive; 10. In the diffusion in gel test; 11. In the "pearl necklace" test; 12. Egg yolk; 13. Yolk, cortisone and desoxycorticosterone; 14. Insulin; 15. Cortisone and desoxycorticosterone; 16. Control (without stimulant).

desoxycorticosterone and sacrificed after 12 and 18 hours in only a small percentage of cases. From 11 animals sacrificed after 24 hours it was possible to isolate and identify 10 anthrax cultures by all tests. From mice of the control group, sacrificed after 12 hours, no culture of anthrax could be isolated even after infection with a soil centrifugate containing from 8000 to 80,000 viable spores per gram. From 15 mice sacrificed after 18 hours an anthrax culture was isolated and identified in five; after 24 hours, in eight mice.

Conclusions

1. With the use of the ordinary bacteriological method of examining the soil we were able to find anthrax bacilli when there were no less than 8000 viable spores per gram of soil.

2. When animals were infected with a soil centrifugate (1000 spores per gram) together with an emulsion of hen's egg yolk, anthrax bacilli were isolated from all animals used in the experiment.

3. In the group of animals infected with a soil centrifugate together with a hen's egg-yolk emulsion, the anthrax bacilli were isolated in almost all animals (in 11-12 out of 15) sacrificed after 12-18 hours.

4. Anthrax bacilli could be identified in mixed cultures with the greatest frequency by the method of specific diffusion in standard agar plates. The latter method can be applied successfully to the detection of the antigen of anthrax bacilli in the tissues of animals which have died and which have been killed (after 18 hours).

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Specific Diffusion in Gel Test as a Method of Identification of Anthrax Bacilli and Detection of Anthrax Antigens in the Organs of Laboratory Animals

V. V. Akimovich, N. S. Goncharova and I. N. Zemtsova
(Saratov)

The bacteriological diagnosis of anthrax is based on the totality of many characteristics inherent in the pathogen of this infectious disease. This not only complicates but also prolongs the time needed for identification of the microbes isolated. Naturally, under such conditions, there has been no discontinuance of the search for new methods which might independently or in combination with other methods be reliable and, which is particularly important, which might assure the detection and identification of anthrax bacilli in the shortest possible time.

In our previous study it was made clear that group antigens are present in B. anthracoides and B. pseudotuberculosis in somewhat smaller quantities, as a rule, than in the anthrax pathogen. This fact constituted the basis for utilization of the diffusion in gel test by the Ouchterlony method (1948) for differential diagnosis. (O. Ouchterlony. Acta Path. et Microbiol. Scandinav., 1948, 25, 1-2, 186-191.) In connection with this, the need arose for modifying somewhat the technique for performing the diffusion in gel test so as to assure, first of all, standard conditions for the test and, secondly, the early appearance of the precipitate; and, thirdly, the development of a positive reaction with a minimum number of microbes investigated. Considering that the antigen and serum diffuse equally in different directions, it was necessary to limit the mass of the agar in order to reduce maximally the unproductive absorption of the reactants and, by the same token, increase their concentration in the zone in which precipitation develops. By means of reducing to a certain limit the distance between the wells containing the serum and antigen, it is possible to bring about a considerable acceleration in the appearance of zones of the precipitate, although thereby the number of them may be reduced. It was possible to achieve this by means of utilizing agar plates of minimum size and by means of maximum shortening of the distance between the wells containing the

reactants.

For this purpose we constructed a stamp which made it possible to prepare a standard agar plate 23x10x3 millimeters, with a distance between the wells of 6 millimeters and with well diameters of 5 millimeters.

The experiment was performed with clarified 1 percent agar in physiological saline solution at a pH of 7.0, containing merthiolate (1:10,000) as a preservative. Fused agar in a quantity of 20 cc was poured out into Petri dishes with strictly horizontal bases; under such conditions the thickness of the agar layer amounts to 3 millimeters. A small drop of fused agar was applied to the bottoms of the wells so as to prevent leakage of the fluids under the agar plate.

In the experiment an examination was made of 55 strains of different species of bacilli; of these, 10 were strains of anthrax bacilli; 30, of B. anthracoides; three, of B. pseudoanthracis; five, of B. mesentericus; two each of B. megatherium, B. subtilis, B. mycoides; and one strain of B. cereus. The bacilli were grown out at 37° on the agar plates containing blood (5 percent) from 10 to 20 hours in different experiments. Then, with a loop, part of the colonies were removed and the bacterial mass was suspended in a drop of physiological saline solution (0.05 cc), which had first been introduced into the well for the antigen; simultaneously, 0.05 cc of whole anthrax precipitating serum was poured into the other well. The agar plates were put into a dryer and incubated at 37°. The results of the test were recorded four, eight, 16 and 20 hours after the test was performed (Table 1).

Table 1

Results of Diffusion in Gel Test in Standard Agar Plates

Вид микробов (1)	Число штаммов (2)	Число штаммов, давших положительную реакцию преципитации с появлением линий (1, 2) преципитата в течение (3)							
		4 часов (4)		8 часов		16 часов		20 часов	
		1	2	1	2	1	2	1	2
B. anthracis	10	0	0	10	0	10	7	10	9
B. antracoides	30	0	0	2	0	2	4	2	7
B. pseudoanthracis	3	0	0	1	0	1	0	1	2
B. mesentericus	5	0	0	0	0	0	0	0	0
B. megatherium	2	0	0	0	0	0	0	0	0
B. subtilis	2	0	0	0	0	0	0	0	0
B. myco des	2	0	0	0	0	0	0	0	0
B. cereus	1	0	0	0	0	0	0	0	0

1. Species of microbe; 2. Number of strains; 3. Number of strains which gave a positive diffusion in gel test with the appearance of a precipitation band (one, two) in:; 4. Hours.

It was noted that in the remote period it was possible to find two and, very rarely, three precipitation bands between the wells: The first band was broad, compact, located near the well containing serum; the second and third bands were fine, clearly outlined, located the same distance between the wells or somewhat closer to that containing the antigen. The first precipitation band appears in the earliest period; then comes the second, and, finally, comparatively rarely, the third.

As is seen from Table 1, after four hours the diffusion in gel test was negative in all cases; after eight hours, 10 strains of the anthrax bacilli produced a distinct first line. This zone of precipitate was recorded in two strains of B. anthracoides and one strain of B. pseudoanthracis. After 16 hours, in some strains of the microbes a second precipitation band appeared; after 20 hours it was observed in almost all of the strains of anthrax bacilli tested, in seven strains of B. anthracoides and two strains of B. pseudoanthracis. All the other spore-forming saprophytes failed to give a positive diffusion in gel test with immune anthrax serum.

From the data presented it may be concluded that the observation period for final recording of the results of the diffusion in gel test can be limited to eight hours. The diffusion in gel test performed along this line cannot constitute a reliable method for the identification of anthrax bacilli, since B. anthracoides, although in relatively rare cases, can also produce a first precipitation band.

In this connection, in the next experiment we made an attempt to utilize diluted anthrax precipitating serum in a proportion of 1:2 and 1:4.

It was found that two strains of anthrax bacilli with serum in a dilution of 1:4 gave no precipitation reaction. Serum in a dilution of 1:2 caused a positive reaction with all anthrax strains, but at the same time the precipitation phenomenon was absent with two strains (5, 8) of B. anthracoides. The results of this experiment could have been seen beforehand, because in these strains of B. anthracoides the quantity of antigen giving rise to the development of the first precipitation band was approximately the same as in the true anthrax microbes.

After failure with the utilization of diluted immune sera, we made an attempt to use the sera after their adsorption by different antigens. It should be stated that we did not place great hopes on the possibility of obtaining strictly specific anthrax sera, because some strains of B. anthracoides and B. pseudoanthracis not only share the specificity of the antigens of anthrax bacilli but the content of these antigens in these species of microbes can also be approximately the same.

As adsorbents the following were utilized: 1) B. anthracoides, strain 5, having a capsular haptene in almost the same quantity as in anthrax bacilli; 2) B. anthracoides, strain 18, characterized by the presence of antigen which gives the second precipitation band similar to that given by anthrax microbes; 3) B. anthracis, of Tsenkovskiy's first vaccine; 4) the anthrax capsular substance. The sera were absorbed twice: after the addition of antigen the mixture was placed in a refrigerator for 16 hours, and then centrifuged at 6000 revolutions per minute for 20 minutes; to the clear serum the same quantity of the same antigen was again added, and the entire procedure was repeated. For the absorption the microbe suspension was added so that, in the final analysis, there be 15,000,000,000 microbes

per cc of serum. After absorption of the serum with the capsular substance the latter was added in a quantity of 1 cc to 4 cc of serum. In all, four series were utilized (137, 146, 221, 175) of the equine anthrax precipitating sera.

With the use of absorbed sera, we were unable in all cases to differentiate anthrax bacilli distinctly from microbes similar to them. As the result of absorption, the sera lost almost all their antibodies or preserved them only to the capsular haptene, or, finally, the sera produced only the second precipitation band. The behavior of anthrax bacilli and certain of the B. anthracoides to the anthrax absorbed sera was the same and, therefore, any possibility of differentiating between them was ruled out.

Of the 33 strains of B. anthracoides and B. pseudoanthracis, three strains were serologically identical with the anthrax bacilli. Therefore, the diffusion in gel test could not constitute an independent method of identification of the anthrax cultures isolated.

It was natural to expect that the diffusion in gel test would give more encouraging results in the study of organs of animals infected with material suspected of anthrax.

The experiments were performed on mice. The mice were infected with spores of different strains (7) of anthrax bacilli subcutaneously in the area of the groin. The organs of the animals which died were studied several hours and two-four days after they died. Tissues from the infection site and the spleens of the mice which died were put into a separate Petri dish, cut up with scissors to a pulp, and a small portion of the tissues which had been cut up was transferred to the wells in the agar plate containing the drop of physiological saline; the other wells were filled with whole anthrax precipitating serum. Agar plates were put into a dryer and incubated at 37°. The residual, greater portion of the cut-up material was put into an agglutination tube, poured over with 1 cc of physiological saline solution and heated on a water bath at 100° for five minutes. After boiling, the fluid was carefully poured off or, if it was turbid, was filtered off through a filter. Part of the tissue extract was used for the diffusion in gel test; the other part, for the Ascoli test. Thermal extracts of the organs of animals which had undergone considerable decay maintained a marked opalescence or even a distinct turbidity even after repeated filtration through an asbestos filter, which complicated the reading of the Ascoli test and, in a number of cases, apparently was the cause of a negative test. An investigation was made of 179 white mice which had died after infection with the spores of anthrax bacilli.

The results are presented in Table 2.

It should be noted that with increase in the period between the time of death of the animal and the beginning of the examination a gradual increase is observed in the content of extraneous microflora and, when this interval reached four days, in many cases it was impossible to detect anthrax bacilli in the cultures, particularly in the spleen. The leading extraneous microbes were the colon bacillus and Proteus vulgaris. In this experiment the test was read after 18-20 hours.

A positive diffusion in gel test was observed with the greatest regularity with an antigen from the spleens of mice examined several hours after death (95.7 percent). A positive test was observed in only 67.4 percent of

Table 2

Results of Diffusion in Gel Test with Tissues of Mice Dying of Anthrax Depending on the Period Elapsing after Death

Период с момента гибели мышей до исследования	Число мышей	Из них реакция преципитации при исследовании				Число мышей, у которых реакция Асколи с органами		Число мышей, от которых выделена культура при бактериологическом исследовании					
		селезенки		ткани с места заражения				селезенки			места введения		
		положительная	отрицательная	положительная	отрицательная	положительная	отрицательная	чистая	смешанная	посторонняя	чистая	смешанная	посторонняя
В пределах первых часов	46	44	2	31	15	33	13	41	5	0	45	1	0
2 дня	73	69	4	62	11	48	25	19	51	3	23	42	8
4 дня	60	55	5	52	8	34	26	16	18	26	24	18	18
Всего . .	179	168	11	145	34	115	64	76	74	29	92	61	26

1. Period from the time of death of the mice to the examination; 2. Number of mice; 3. Of these, the results of the diffusion in gel examination; 4. Of the spleen; 5. Of tissues from the infection site; 6. Number of mice in which the Ascoli test with organs was:; 7. Number of mice from which a culture was isolated on bacteriological examination; 8. Positive; 9. Negative; 10. Pure; 11. Mixed; 12. Extraneous; 13. In the first few hours; 14. Days; 15. Total.

all cases with tissues taken from the infection site. In the examination of mice two days after death the number of positive results of the diffusion in gel test with the spleen decreased somewhat (94.5 percent); however, the test with the tissues from the infection site was positive in 85 percent of the cases; four days after death the number of positive results with the spleen decreased to 91.7 percent and the number with tissues from the infection site increased to 80.0 percent. However, the greatest percentage of positive tests, regardless of the time of the examination of the tissues after death of the mice, was found in cases where the spleen was used as the object examined. However, this does not mean that in performing the diffusion in gel test the utilization of tissues taken from the infection site should be stopped, because in a number of cases they give a positive diffusion in gel test where the spleen gives a negative test. If we take this fact into consideration, the total percentage of positive reactions on examination of mice four days after death reaches 96.6 percent.

If we judge by the results of bacteriological examination of the spleens of mice whose bodies had shown decay, it may be noted that the number of negative diffusion in gel tests increases as the number of extraneous microorganisms in the cultures increases. The impression is created that in organs of mice which have undergone considerable decay a destruction of the antigens of the anthrax bacilli may occur.

In parallel with the main experiments, we performed, as has been described above, the thermoprecipitation test by the Ascoli method. Most often, this test was positive (71.7 percent) when the organs of mice were examined shortly after death; in more remote periods, when anthrax bacilli were isolated in the form of cultures mixed with extraneous microbes or when they could not be detected at all, the Ascoli test was positive in only 56.6 percent of the cases.

Therefore, regardless of the time of examination of the tissues of the animals which had died, a positive Ascoli test was found much less often than the diffusion in gel test using standard agar plates. It must be supposed that this phenomenon is determined by several factors. First of all, in the preparation of the extract the antigen is very much diluted and, thereby, its concentration is reduced, which cannot be immaterial for the outcome of the test. In addition, in thermal processing part of the soluble antigen is apparently extracted by the coagulated proteins of the tissues. Finally, the possibility has not been ruled out that there is partial denaturation of the antigen as the result of the effect of relatively high temperature. The reliability of these ideas has been confirmed by studies in which a thermal extract of the spleen was used as an antigen for the diffusion in gel test. In this case the test was positive in only 52 percent of the cases.

Therefore, the best results in serological examination of the organs of laboratory animals dying of anthrax are obtained from the diffusion in gel test with native spleen in standard agar plates. This test is specific: We did not once observe a positive diffusion in gel test with normal equine or rabbit serum.

Since some strains of B. anthracoides and B. pseudoanthracis produce distinct precipitation with anthrax serum, it was to be expected that the organs of animals infected with these microbes would also produce a positive

precipitation test with this serum.

For the purpose of solving this problem white mice were injected intraperitoneally with a suspension of an 18-hour agar culture. Some of the mice infected with B. anthracoides (strain 5) in a dose of 1,000,000,000 microbes died (five out of 10 examined); from them the corresponding microbes could be isolated in the form of single colonies on agar plates. Antigens similar in their serological properties to the antigens of anthrax bacilli could not be found in the organs of these animals. From three mice out of five which died after infection with B. pseudoanthracis 1312 (100,000,000 microbes) the corresponding microbes were isolated in pure culture from all objects examined. In three cases the diffusion in gel test with the spleens of the animals which died was negative.

In the next experiment 20 white mice were given an intraperitoneal injection of 100,000 microbes of an 18-hour agar culture of Tsenkovskiy's first vaccine. In this experiment all the mice died on the third-fourth day; a pure culture of anthrax bacilli was isolated from the blood and internal organs. In the diffusion in gel experiment a clear-cut positive test was obtained with the spleens of only four mice; in three cases the Ascoli test was also positive. This phenomenon is entirely to be expected, since, according to our previous data, the bacilli of Tsenkovskiy's first vaccine form a capsule very inconstantly.

Based on the data presented, it may be stated that a positive diffusion in gel test is observed with the greatest constancy with the organs of mice infected with virulent anthrax bacilli. It should be emphasized that the diffusion in gel test in the examination of the organs of animals, on which the biological tests were performed, is accomplished primarily with the capsular antigen, which is evidenced by the nature of the precipitation band; its union with the precipitation zone is conditioned by the capsular substance and, finally, the precipitation test remains negative with anthrax serum which has first been absorbed by the specific capsular antigen (see the Figure).

For the purpose of evaluating the diffusion in gel test on standard agar plates, it was interesting to determine the minimum time in which its results can be read. With this aim in view, we made observations of the development of the diffusion in gel test dynamically.

The organs of anthrax-infected mice which had died were examined on the third-fourth day, that is, during the period of pronounced putrefaction. On bacteriological examination either mixed or extraneous microbe cultures were isolated as a rule. The test was recorded six, eight, 10, 14 and 18 hours after the beginning of the experiment.

The results of the experiment are shown in Table 3.

A distinct broad and compact precipitation band obtained with the spleen was found as early as six hours in 38 out of 42 mice. After 10 hours a negative test was noted in only two mice, in which the test continued to be negative in the subsequent hours of the observation.

Therefore, it may be considered that the diffusion in gel test should be read six hours after its performance, and the final reading should be made after 10 hours. Longer observation periods are apparently superfluous.

Table 3

Results of the Diffusion in Gel Test as a Function of the Time They are Recorded

Часы регистрации реакции (1)	Число мышей (2)	(3) Из них реакция преципитации при исследовании			
		селезенки (4)		ткани с места введения микробов (5)	
		положительная (6)	отрицательная (7)	положительная (6)	отрицательная (7)
6	42	38	4	10	32
8	42	39	3	12	20
10	42	40	2	33	9
14	42	40	2	38	6
18	42	40	2	38	6

1. Hours of recording the reaction; 2. Number of mice; 3. Of these the diffusion in gel test on examination of the; 4. Spleen; 5. Tissue from the site of injection of the microbe; 6. Was positive; 7. Was negative.

Conclusions

1. The utilization of standard agar plates considerably accelerates the appearance of the precipitation area with a comparatively small quantity of reacting antigen.

2. The diffusion in gel test can constitute a supplementary method of differentiating anthrax bacilli from microbes similar to them.

3. By means of the specific diffusion in gel test on standard agar plates the anthrax antigen can be detected in the organs of dead mice which have undergone putrefaction, on which the biological test was performed; the diffusion in gel test was positive in 96.6 percent of the cases.

4. The diffusion in gel test with the organs of mice which have died of anthrax becomes distinct after six hours; the final reading of the test should be made 10 hours after its performance.

Rapid Identification of the Glanders and Melioidosis Pathogens by Means of Fluorescent Sera

S. V. Anokhina, L. P. Bazunova and P. K. Tabakov
(Saratov)

The laboratory diagnosis of glanders and melioidosis is at the present time based on the cultural, morphological, biochemical and biological characteristics of the pathogens. Serological and allergic tests have acquired great popularity. However, it has been difficult to differentiate between these absolutely different species of microorganisms, because identification of them by their motility, capsule formation, time needed for growth on synthetic nutrient media is imperfect and requires a long time.

In recent years, numerous reports have appeared in the literature on the possibility of identifying the pathogens of many infectious diseases by labeled specific, so-called fluorescent antibodies with the use of fluorescence microscopy. The method is based on the utilization of immune purified serum, which after special treatment with the dye, acquires the power of causing luminescence of the homologous antigen.

The fluorescent antibody method was first proposed by Coons and others (1942). In the Soviet Union the first to begin to work this method out were M. N. Meysel', Ye. N. Levina, Ye. A. Kabanova and M. M. Pishchurina (1957). Studies on the use of labeled fluorescent antibodies with the aim of identifying the pathogens of melioidosis were first made by Moody, Goldman and others (1956). In smears of pure cultures they succeeded in finding 220 M. pseudomallei microbes. It is particularly important that the presence of extraneous microorganisms in a quantity of 10,000,000 cells per cc did not interfere with the detection of the pathogen of melioidosis. Such differential luminescence has been obtained successfully with all species of microorganisms used in the experiment with the exception of the glanders bacillus. The latter, as the result of treatment with labeled melioidosis serum, fluoresced to the same degree as did the bacillus of melioidosis. The authors did not have antiglanders serum.

The task of our studies was that of obtaining fluorescent antiglanders and antimelioidosis sera and testing their fluorescent effect with homologous and heterologous cultures. We had six strains of B. mallei (5584, 54, 55, 56, 5 and 6) and two of M. pseudomallei (S-141 and 51-274).

The sera were obtained by immunizing rabbits with killed formol vaccine. The gamma-globulin fraction was isolated from the sera obtained by the method of precipitation with 50 percent ammonium sulfate.

The sera were labeled by the Coons and Kaplan method. Subsequently, the globulin was purified of the excess dye by means of dialysis against a 0.01 M phosphate buffer at a pH of 7.0.

For microscopy a fluorescence apparatus was used consisting of an OI-18 condenser and an opaque OI-17 illuminator with an SVD-120A quartz lamp with SZS-7, FS-1, SS-8 (ZhS-18) light filters attached to it. An MI-90 1.25 objective and a 5X ocular were used. Fluorescence microscopy was always checked by phase-contrast microscopy.

First, we made an attempt to find the most favorable conditions for

assuring the effective luminescence of the microorganisms studied. The best fixative was ethyl alcohol. The optimum time of action of the labeled serum on the smear-preparations in the wet chamber amounts to 15-20 minutes. The smears are best prepared from one-two-day cultures grown out on meat-infusion or Bottinger's agar containing 3 percent glycerin (pH of 7.0).

The intensity of fluorescence was determined by the generally accepted method. For the purpose of checking on the specificity of fluorescent immune gamma-globulins, each time gamma-globulin of normal serum treated by the same method was used. The quality of the native, purified and labeled sera was determined by the agglutinin test, with a strain used as an antigen for immunization of rabbits. We shall indicate various changes which we made in the method when we come to them. Studies with the first antiglanders gamma-globulins showed that good fluorescence was noted in the B. mallei No 5584 strain, from which a vaccine was prepared for immunization. The other strains fluoresced slightly. Antimelioidosis labeled antibodies showed up both strains of M. pseudomallei and one strain of B. mallei equally (Table 1).

Table 1

Characteristics of Glanders and Melioidosis Fluorescent Globulins Obtained in the First Series of Experiments

(1) Сыворотка	(2) Номер	(3) Агглютинационный титр с гомологичным штаммом: (4) нативной сыворотки	(5) γ-глобулиновой фракции	(6) меченой γ-глобулиновой фракции	Серия изоцианата флуоресцеина (7)	Разведения меченого γ-глобулина (8)	(9) Результат люминесцентной микроскопии со штаммами: B. mallei 55-4	B. mallei 51	B. mallei 55	B. mallei 56	M. pseudomallei C-141	M. pseudomallei 51-271
(10) Противосапная (штамм 5584)	1	1:30 000	1:70 000	1:4 000	C. 25	1:8	+++	++	+	+	++	++
	2	1:40 000	1:60 000	1:10 000	C. 25	1:8	+++	++	+	+	++	++
	3	1:2 000	1:1 000	1:700	ИРЕА	1:10	+++	++	+	+	++	++
	4	1:1 000	1:2 000	1:800	ИРЕА	1:10	+++	++	+	+	+++	++
(11) Контроль		1:100	1:100	1:80	C. 25	1:8 1:10	—	—	—	+	+	+
(12) Противомелиоидозная (штамм С-141)	5	1:600	1:1 000	1:500	C. 25	1:10	+++	+	+	+	+++	+++
	6	1:2 000	1:3 000	1:900	ИРЕА	1:10	+++	++	+	+	+++	+++
	7	1:3 000	1:5 000	1:2 000	ИРЕА	1:4	+++	+	+	+	+++	+++
(13) Контроль		1:100	1:100	1:80	C. 25	1:4 1:10	+	+	—	+	+	+

Note. ++++ and +++, Specific luminescence; ++ and +, Nonspecific luminescence.

Key: 1. Serum; 2. Number; 3. Agglutinin titer with homologous strain; 4. Of native serum; 5. Gamma-globulin fraction; 6. Of labeled gamma-globulin fraction; 7. Series of fluorescein isothiocyanate; 8. Dilution of labeled gamma-globulin; 9. Result of fluorescence microscopy with the following strains:; 10. Antiglanders (strain 5584); 11. Control; 12. Antimelioidosis (strain S-141).

Therefore, such labeled gamma-globulin could not be used for the identification of the pathogens of glanders and melioidosis. It could not be used either for the diagnosis of glanders, because different strains of this pathogen are antigenically different. This phenomenon was unexpected for us: We obtained similar results in the agglutinin test. This paradoxical phenomenon was pointed out by V. D. Krylov (1929), S. N. Vyshelesskiy and F. A. Terent'yeva (1954) and others.

Unexpected also was the disappearance of the fluorescence of the strain B. mallei 5584 and M. pseudomallei S-141 after the action of homologous labeled gamma-globulins on them. Our idea of the short lives of the labeled sera was not justified. After the passage of these pathogens through the bodies of susceptible animals (after passage of the first strain through the body of a cat and of the second through a guinea pig), they again fluoresced after treatment with the same fluorescent antibody.

The second series of experiments was performed with new antiglanders sera obtained by the same method. For the immunization, as an antigen, vaccines were used which had been prepared from strains of B. mallei 54, 55 and 56 which did not fluoresce when treated with labeled sera in the first series of experiments. The gamma-globulin fraction of these sera was obtained by water-alcohol precipitation in the cold. Subsequent treatment of the globulin was carried out by the Cohn method; this method made it possible to prepare gamma-globulins five-seven times more quickly. The gamma-globulins were labeled, aside from IREA fluorescein isothiocyanate, with new dyes, isomers of I and II isothiocyanate (dry). For the purpose of comparing their effects the volume of each serum used was divided into three parts, and each of these was labeled with one of the dyes mentioned. In contrast to the first experiment, in the labeled gamma-globulins we determined the percentage of protein (by the micro-Kjeldahl method with subsequent isothermal distillation in Conway diffusion dishes), which made it possible to calculate their effective dilution.

The quality of labeled gamma-globulin depends on the dye used, which was determined not only by the intensity of fluorescence but also by its specific effect on homologous microorganisms. The best dye was the first isomer of isothiocyanate. Gamma-globulin labeled with the second isothiocyanate isomer was almost as good as gamma-globulin labeled with the first isothiocyanate isomer.

Gamma-globulins labeled with fluorescein isothiocyanate were worse: none of the sera labeled with it were capable of differentiating glanders from melioidosis, as in the first experiment. Nor was there specific fluorescence even in those strains from which the sera had been prepared. At the same time, the same gamma-globulins but labeled with the first and second isothiocyanate isomers gave good specific fluorescence of all the B. mallei strains used in the experiment; the M. pseudomallei strains used fluoresced slightly, nonspecifically, which permits differentiating these pathogens (Table 2).

Advantage was taken of the favorable results of this experiment; we decided to repeat the treatment of the antiglanders gamma-globulin obtained from immunization of rabbits with vaccine made of the 5584 strain and the antimelioidosis gamma-globulin obtained by the same method from the S-141 strain with the use of the isothiocyanate isomer. In this experiment also

Intensity of Fluorescence of Glanders and Melioidosis Pathogens as a Function of the Nature of the Fluorochrome Used for Marking the Antiglanders Gamma-Globulins

(1) Номер кроличьей сыворотки	(2) Номер штамма, взятого для иммунизации	(3) Предельное разведение сыворотки	(4) Процент белка	(5) Результат свечения со штаммом					
				M. mallei				M. pseudomallei	
				5584	54	55	56	C-1	52-274
(6) *Изоционат флуоресцеина (ИРЕА)*									
8	54	1:8	0,15	++++	++	+++	++	++	++
9	55	1:8	0,15	+++	++	++	++	++	++
10	56	1:16	0,09	++++	++	++	++	++	++
—	54+55+56	1:16	0,08	+++	++	+++	+++	++	++
(7) *Первый изомер изотиоцианата*									
8	54	1:10	0,23	++++	++++	++++	++++	++	++
9	55	—	—	(9) Не испытывалась					
10	56	1:20	0,1	++++	++++	++++	++++	+	+
—	54+55+56	1:20	0,17	++++	++++	++++	++++	++	++
(8) *Второй изомер изотиоцианата*									
8	54	1:16	0,15	++++	++++	++++	++++	+	++
9	55			Не испытывалась					
10	56	1:10	0,15	+++	++++	+++	+++	++	++
—	54+55+56	1:10	0,14	++++	++++	++++	++++	++	++

1. Number of rabbit serum; 2. Number of strain used for immunization; 3. Maximum dilution of the serum; 4. Percentage of protein; 5. Result of fluorescence with strain; 6. Fluorescein isothiocyanate (IREA); 7. First isothiocyanate isomer; 8. Second isothiocyanate isomer; 9. Not tested.

the antiglanders labeled gamma-globulins showed up the glanders bacteria very well and showed the melioidosis bacteria much more poorly. Labeled antimelioidosis gamma-globulins gave the same fluorescence with glanders and melioidosis strains (Table 3).

These data are in agreement with the studies of Moody, Goldman and others (1958), who also failed to differentiate between glanders and melioidosis pathogens with labeled melioidosis gamma-globulin. We obtained similar results with the agglutinin test.

With the last series of labeled gamma-globulins we were able to perform experiments for determining the specificity with species of bacteria distant in an antigenic respect. With the aim of eliminating nonspecific reactions, the smears were treated with labeled gamma-globulins in the diluted form. As an effective dilution the dilution was used which gave bright fluorescence to the same degree as undiluted gamma-globulin (Table 4).

In the majority of microorganisms used in the experiment there was no

Table 3

Characteristics of Glanders and Melioidosis Fluorescent Gamma-Globulins Labeled with the First Isothiocyanate Isomer

Сыворотка (1)	Номер сыворотки (2)	Агглютинационный титр с гомологичным штаммом (3)			Разведение меченого γ-глобулина (4)	Процент белка (5)	Результат люминесцентной микроскопии со штаммами (6)							
		нативной сыворотки (7)	γ-глобулина (8)	меченого γ-глобулина (9)			B. mallei						M. pseudomallei	
							5584	54	55	80	5	6	C-141	51-274
(10) Противосапная (штами 5581)	11	1:4 000	1:9 000	1:2 000	1:20	0,1	++++	++++	++++	++++	++++	++++	++	++
(11) Противомелиоидозная (штамы С-141)	12	1:1 000	1:2 000	1:1 000	1:15	0,1	++++	+++	+++	++++	+++	++++	++++	+++
(12) Контроль		1:100	1:100	1:80	1:15	—	—	—	—	—	—	—	+	+

1. Serum; 2. Number of serum; 3. Agglutinin titer with homologous strain; 4. Dilution of labeled gamma-globulin; 5. Percentage of protein; 6. Result of fluorescence microscopy with strains:; 7. Native serum; 8. Gamma-globulin; 9. Labeled gamma-globulin; 10. Antiglanders (strain 5584); 11. Antimelioidosis (strain S-141); 12. Control.

.ble 4

Characteristics of Fluorescenc. . Various Types of Bacteria Treated with Labe..ed Antibodies

Вид бактерий (1)	Число штаммов (2)	Флуоресцирующие противосапные γ-глобулины (3)	Флуоресцирующие противомелиоидозные γ-глобулины (4)	Меченые γ-глобулины сыворотки (5)
B. mallei	6	++++	++++	—
M. pseudomallei	2	++	++++	+
B. pyocyaneum	2	—	—	—
B. coli	1	—	+	+
B. anthracoides	1	—	—	—
V. cholerae	2	—	—	—
V. paracholerae	2	—	—	—
B. pestis «EB» (6)	1	—	+	+
Proteus vulgaris	1	—	—	—
Группа Brucell (7)	3	—	—	—

1. Species of bacteria; 2. Number of strain; 3. Fluorescent antiglanders gamma-globulins; 4. Fluorescent antimelioidosis gamma-globulins; 5. Labeled serum gamma-globulins; 6. EV strain of B. pestis; 7. Brucella group.

fluorescence. The colon and plague bacilli showed very slight nonspecific fluorescence. Therefore, we can recommend labeled glanders and melioidosis antibodies for differentiation between glanders and melioidosis pathogens, on the one hand, and species of microorganisms which are antigenically distant.

The last stage of the investigations of labeled fluorescent glanders and melioidosis gamma-globulins was a determination of their sensitivity. The detection of fluorescent cells was possible in smears of B. mallei or M. pseudomallei suspensions containing 500,000 microbes per cc. The fluorescent cells could be detected but inconstantly in smears made of suspensions containing 5000 microbes per cc. On testing glanders and melioidosis bacteria in a mixture with 500,000,000 other microbes (E. coli and B. pyocyaneus) the same results were obtained as in the testing of smears made of pure culture.

In conclusion, we checked the lives of the fluorescent gamma-globulins studied. The latter preserved their activity when kept for a year at 4°. Thymol was used as a preservative.

Conclusions

1. Antiglanders and antimel..oidosis fluorescent gamma-globulins possess a high degree of specificity.

2. Antiglanders fluorescent gamma-globulins are suitable for differentiation between glanders and melioidosis bacteria.

3. Antimelioidosis fluorescent gamma-globulins cannot be used for differentiating melicidosis from glanders, because the pathogens of these infectious diseases are not different in the intensity of fluorescence.

4. The best dye for the gamma-globulins studied was found to be the first isomer of isothiocyanate.

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The Use of Artificial AKR-7 Resin for Making Conway Diffusion Vessels

P. K. Tabakov
(Saratov)

At the present time, the microdiffusion method worked out by Conway and others originally for the determination of ammonia and urea is widely used for the analysis of a number of compounds. They proposed a special diffusion apparatus, widely known by the name of Conway diffusion vessels. Afterwards, it was determined that the principle and apparatus are applicable to many microdeterminations (Conway, 1947).

S. R. Mardashev and N. N. Lestrovaya (1949) have improved the design of the Conway diffusion vessel by means of introducing two transverse partitions in the outside chamber, which makes it possible to utilize these modified instruments for a larger group of analytical determinations, particularly for the determination of enzyme activity (asparaginase, glutaminase, urease and others).

To date, the fact that glass Conway diffusion vessels modified by S. R. Mardashev and N. N. Lestrovaya cannot be purchased is a limitation to more extensive incorporation of this type of analysis into laboratory practice.

Many laboratories are attempting to get out of this difficulty by means of making paraffin cells, poured into special molds (S. R. Mardashev and N. N. Lestrovaya, 1949; A. N. Belozerskiy and N. I. Proskuryakov, 1951;

M. Ya. Fel'dman, 1957, 1958). However, the time spent in making paraffin vessels is too great, and the possibility of using them is limited to one-two analyses. In addition, the ready fusibility of paraffin in the summer-time leads to deformation of the vessels and, the main thing, does not permit conducting the microdiffusion analyses at incubator temperatures, which is necessary for the study of enzyme activity. These defects are entirely eliminated if the Conway cells are prepared of plastic rather than paraffin.

For this purpose we used artificial AKR-7 resin [acrylic resin consisting of a powder—polymethylmethacrylate with a catalyst—and fluid—methylmethacrylate—stained and mixed with inhibitor], which is being used for making dental prostheses.

For the purpose of preparing the plastic Conway vessels a simple mold, cut out of aluminum or other metal, is needed.

The mold which we are proposing consists of four parts: a mold frame,* matrix, insert, and cover; the mold and dimensions are shown in Figs 1-4.

Making the Diffusion Vessels

Thirty-six grams of AKR-7 emulsion powder, weighed out on a technical balance, is poured out into a porcelain beaker with a volume of 100 cc; 16 cc of the monomer is added and mixed with a glass rod until the powder is completely wet with the monomer.

The mass is allowed to stand at room temperature for 15-30 minutes until it can be drawn out in the form of long threads, until it has the consistency of soft dough and until it sticks to the hands.

The mass obtained is placed in the assembled mold and pressed into all of its recesses by means of a spatula or handle of a scalpel, covered with cellophane and then with the cover, and put under a press for 10-15 minutes. After this, the mold is immediately pressed in a stirrup bolt (Fig 5) and put into a water bath filled with cold water. The bath is heated to boiling for 30-40 minutes and boiled for 30 minutes.

After complete cooling, the polymerized Conway diffusion vessels are carefully removed from the molds. For this purpose, first the insert is taken out with the bolt or stirrup bolt, and then the cover and, finally, the diffusion cell are removed, separating the latter from the mold with a knife. Then, with a file the projecting edges are removed, and the sharp edges are filed down.

Before use, the diffusion cells are washed with a wire brush, dried in the inverted position and are poured over with fused paraffin. The necessary reagents are measured out into the prepared vessels and covered with a piece of glass or Petri dish heated over a burner. Thereby, the paraffin remaining on the edges of the vessel are melted, and the glass sticks firmly to the diffusion vessel.

On completion of the analysis all the diffusion vessels are dropped into a pot containing hot water and boiled for 5-10 minutes. The paraffin with which the diffusion vessels were covered floats up and is removed with the hot water, and the diffusion vessels are dried and used again. The Conway diffusion cells which we made from AKR-7 resin can be used for three years (the limit of our observations), are unbreakable, do not deform, are not attacked by acids and only to a negligible degree by strong alkalis.

[Russian: obichayka]

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REVIEW

The Effect of Certain Cultivation Conditions on the Virulence of the Plague Microbe

(Review of Abstracts)

Ye. E. Bakhrakh
(Saratov)

In 1942, Devignat and Schootter first checked the effect of elevated temperature in combination with aeration on the virulence of plague microbes and showed that after several serial subcultures of the plague microbe on bouillon under conditions of aeration at 37°, two highly virulent strains became avirulent. However, for a long time no attention was paid to this fact. Summing up the material on conditions leading to the loss of virulence of the plague microbe in 1954, Politzer, speaking about such factors as frequent subculture on synthetic nutrient media, the effect of bacteriophage, cultivation in a bile medium, in bouillon containing alcohol, long-term incubation in bouillon at 32°, the passage of virulent strains through the immune organism and others, even failed to mention aeration as a possible factor bringing about a loss of virulence of a plague microbe culture.

The effect of aeration at an elevated temperature on the virulence of the plague microbe cultures and some associated problems have recently been discussed in detail in papers published in 1957-1960 in the J. Bact. In the first work of this group (Fukui, Ogg and others, 1957) three virulent strains isolated from man were investigated: two glycerin-negative (Alexander and Saka) and one glycerin-positive (Yokohama) strains. As liquid medium, bouillon from a heart infusion to which 0.25 percent xylose and 0.06 percent $MgSO_4 \cdot 7H_2O$ and bouillon from a demineralized acid casein hydrolysate were added was used (Higuchi and Carlin, 1957). The agar media were prepared in a heart muscle infusion. Living cells were counted by plating out on blood agar plates (Difco), to which 0.. percent glucose and 0.04 percent sodium sulfite had been added. Virulence was checked on white mice, which were injected intraperitoneally with 0.2 cc of the corresponding dilution of the culture in a buffer solution.

The studies made showed that bouillon cultures incubated under aeration conditions at 26° and agar cultures grown out at 26° and 37° remained virulent, whereas aerated bouillon cultures incubated at 37° lost their virulence. Thereby, when a large inoculation dose ($4-12 \cdot 10^8$ cells) was used an appreciable progressive reduction of virulence was observed only after several serial passages; where small inoculation doses were used ($1 \cdot 10^4$ cells) an equivalent reduction of virulence occurred as early as the first generation (Table 1).

The authors suggested that the loss of virulence by the plague microbe culture is associated with the selection of avirulent mutants introduced into the culture or formed in it during the process of growth.

Avirulent mutants, in contrast to the original strains, were characterized by three features: 1) the absence of virulence; 2) the inability to

Table 1

The Effect of the Inoculation [illegible] and the Incubation Temperature on the Virulence [illegible] the Plague Microbe

Штамм (1)	Температура инкубации, ° (2)	Посевное число (живые клетки) (3)	Урожай (живые клетки) через 40 часов (4)	Число генераций (5)	LD_{50}	Vi-антиген (6)
Saka	26	168,00	9·200	5–6	7	
		17,50	15·400	10	5	
		1,71	13·000	13	8	
		0,02	840	15	9	
	37	230,00	1·810	3	9	+
		23,00	2·190	7	104	-
		2,42	3·420	10–11	> 2·800	+
		0,02	291	13–14	>15·800	—
Alexander	26	745,00	14·900	4	11	
		58,00	15·0 0	8	15	
		5,65	13·900	11		
		0,06	920	14	40	
	37	312,00	1·580	2	15	+
		1,80	780	9	> 3·500	+
		0,19	1·040	13	> 1·400	—
		0,02	198	13	16·800	—

1. Strain; 2. Incubation temperature, degrees; 3. Inoculation dose (living cells); 4. Yield (living cells) after 40 hours; 5. Number of generations; 6. Vi antigen.

produce Vi antigen (the latter was determined by diffusion in gel by the Burrows and Bacon method, 1956); 3) by the capacity of the avirulent cells for growing more quickly than the virulent cells in aerated bouillon cultures at 37°.

If, under active aeration conditions at 37°, the avirulent variants multiply more rapidly, as the authors of the work supposed, the loss of virulence must have been directly connected with the number of cell generations: in other words, with increase in the number of bacterial generations in the culture there must have been an increase in the ratio of avirulent to virulent cells and a gradual shift of the population in the direction of the predominance of avirulent individuals in it.

Actually, the number of cell generations in a culture cultivated from small inoculation doses ($1 \cdot 10^4$ cells) after 40 hours was approximately equal to the total number of generations produced from three-four serial subcultures with an inoculation dose equal to $1 \cdot 10^9$ (12–14 generations).

It was shown by specially performed experiments that a culture which developed from an inoculation dose of $2 \cdot 10^6$ cells produced three avirulent colonies out of 10; a culture grown out from an inoculation dose of $2 \cdot 10^7$, five avirulent colonies. In cultures grown from inoculation doses equal to

$1 \cdot 10^4$-$1 \cdot 10^6$ cells all 10 colonies were avirulent.

The work of Ogg, Fricman, Andrews and Surgalla, who obtained two mutants from the virulent Alexander strain, a work which was published in 1958, constitutes corroboration of this theory of selection of avirulent mutants in aerated bouillon at 37°: a virulent strain, the LD_{50} of which was less than 10 cells for mice on intraperitoneal infection, and an avirulent strain, which did not cause the death of mice even when used in a dose of $1 \cdot 10^6$ living cells. Both strains produced smooth colonies. The cultures were made with different proportions of virulent and avirulent cells. The concentration of the inoculation suspension in each sample amounted to $1 \cdot 10^8$ cells per cc of bouillon (Table 2).

Table 2

The Virulence of Plague Microbe Cultures Grown Out at 26° and 37° from a Mixture of Virulent and Avirulent Cells

Отношение вирулентных и авирулентных клеток в смеси	LD_{50} культуры		
	до посева	после выращивания в течение 40 часов при	
		26°	37°
9:1	10,0	8,8	118
5:5	16,8	8,8	555
1:9	86,0	72,0	3 000

1. Ratio of virulent to avirulent cells in the mixture; 2. LD_{50} of the culture; 3. Before cultivation; 4. After cultivation for 40 hours at.

As is seen from Table 2, in cultures incubated at 37° the avirulent cells grew out better, which was evidenced by the increasing LD_{50}. In cultures incubated at 26° the relationship between virulent and avirulent cells did not change, which is confirmed by the constant LD_{50}.

A check on the virulence of a culture grown out from a small inoculation dose ($1 \cdot 10^4$ virulent cells per cc), performed at certain intervals, showed that with each new generation the number of avirulent individuals increased. After six hours, a reduction in the number of living cells was noted; however, the LD_{50} did not change. With further increase in the number of living cells the LD_{50} also increased.

The observations of Wessman, Miller and Surgalla (1958), who noted the toxic effect of glucose on the virulence of the plague microbe in chemically defined media are interesting.

The authors mentioned above studied growth of seven virulent and five avirulent strains under aeration conditions at 37° in a chemically defined medium. As a source of energy glucose, xylose, galactose or mannose was added to the medium.

It was found that all the avirulent and two virulent strains developed normally in the presence of all these carbohydrates; for the five virulent

strains a larger [illegible] required in the presence of mannose and particularly glucose, and [illegible]y a reduction in the number of viable cells was observed. Only then [illegible] number of living cells regained and did the culture develop logarith[illegible]y. The specificity of the glucose effect is indicated by the fact [illegible] in the presence of glucose with an equal quantity of xylose the num[illegible]f living cells decreased to the same degree as when they were grown on [illegible] media containing glucose alone. In the presence of xylose alone the n[illegible]ber of viable cells did not decrease.

Reduction of the number of living cells, observed in the majority of virulent strains incubated on media with glucose, depended on the incubation temperature, which was confirmed by a series of experiments with careful control of the temperature conditions. All cultures, virulent and avirulent, showed normal growth curves at 26° but at 37° and 38° the virulent microbes produced, as always, a smaller number of viable cells. Determination of the virulence of these cells showed a reduction of it. Thus, cultures of the Alexander strain, after serial subcultures on synthetic medium containing glucose, rapidly lost virulence for mice; if the source of energy was xylose, galactose or mannose, virulence changed to a much lesser degree (Table 3).

Cultures of the plague microbe which had lost virulence on being cultivated under aeration conditions at 37° did not show an increase in it as the result of repeated passages on agar at 27° and 37° or after long-term incubation (even after 14-15 generations) in cellophane bags sewn into the abdominal cavity of the guinea pig (Ogg and others), although under these conditions usually no loss of virulence was noted. On this basis, the authors concluded that the loss of virulence by strains of the plague microb. observed when they were cultivated in aerated bouillon at 37° is the result of creation of some kind of conditions inhibiting the growth of virulent mutants in the medium and contributing to more active propagation of avirulent strains. The latter, as the authors supposed, appear as the result of mutations and selection during the growth process or as the result of selective growth of avirulent mutants which appeared for the first time in the material being seeded. (The data obtained, attesting to loss of virulence by plague microbe strains under aeration conditions, may be the result of cultures inhomogeneous with respect to virulence, which are introduced at the time of inoculation, with the subsequent development of avirulent forms or the result of adaptive variation in combination with the selection of avirulent individuals best adapted to the given cultivation conditions (author's note).)

What are the conditions inhibiting the growth of virulent cells? This question cannot be considered conclusively answered. However, even now a number of factors have been demonstrated contributing to preservation of the virulence of cultures incubated at 37° in an aerated medium.

In the studies quoted above as well as in investigations published somewhat later, the following factors were noted which, to one degree or another, prevent the loss of virulence by plague microbe strains when cultivated under aeration conditions at 37°: a) the addition of filtrates of bouillon cultures of a number of microbes, including the plague microbe, to the nutrient medium; b) setting the initial pH of the medium equal to 7.7-7.8; c) cultivation in an atmosphere of pure nitrogen or with an admixture of 1 percent carbon dioxide; d) the addition of certain substances to the

Table 3

The Effect of Serial Subcultures on Media with Different Sources of Energy on the Virulence of the Plague Microbe (Alexander Strain)

Источник энергии	Число пересевов	Минимальное число жизнеспособных клеток, 10^6	Число жизнеспособных клеток, 10^6	Примерное число генераций	Возраст культуры, часы	LD_{50} для мышей
Глюкоза	1	3,7	110	5,9	72	234(90—608)
	2	0,77	240	8,2	48	4300(1410—1310)
	3	9,8	480	5,7	32	$48 \cdot 10^5$
	4	11,4	1500	7,1	32	$4 \cdot 10^5$
Ксилоза	1	6,5	580	6,5	48	11(4,6—25)
	2	4,7	340	6,2	48	12(5—29)
	3	8,4	330	5,3	48	46(17,7—120)
	4	3,5	220	6,0	48	62(24—161)
Галактоза	1	6,5	380	6,9	48	10(4,1—23)
	2	3,7	150	5,3	48	5(2—12,5)
	3	3,7	260	6,1	48	18(6—54)
	4	3,3	170	6,7	48	35(14,4—138)
Манноза	1	6,5	110	4,1	48	9,2(3,7—23)
	2	0,26	190	9,3	48	45(17,6—175)
	3	48,5	350	6,2	32	78(29 - 211)
	4	11,1	1 200	6,8	32	485(187—1260)

1. Source of energy; 2. Number of subcultures; 3. Minimum number of viable cells, 10^6; 4. Number of viable cells, 10^6; 5. Approximate number of generations; 6. Age of the culture, hours; 7. LD_{50} for mice; 8. Glucose; 9. Xylose; 10. Galactose; 11. Mannose.

medium; sodium bicarbonate, glutamic or aspartic acid, calcium salts, etc.

Let us analyze the effect of these factors in somewhat greater detail.

Ogg, Friedman and others (1958) noted that cultivation of a virulent strain from small inoculation doses in a medium consisting of a mixture of bouillon and heart infusion with filtrates of bouillon cultures of an avirulent plague microbe strain, strains of the pseudotuberculosis microbe, colon bacillus or Sonne dysentery bacillus prevented or reduced the attenuation of virulence of the plague microbe when it was grown from small inoculation doses under aeration conditions at 37°. The filtrates of the cultures were obtained after seeding bouillon made of heart infusion with an avirulent strain of the plague microbe or with the other species of microbes indicated above and incubating them with shaking at 37° for four hours, after which the cells were precipitated by centrifugation, and the supernatant fluid was filtered through a glass filter. The filtrate was mixed with bouillon in different proportions (from 1:1 to 3:1). It was found that even a culture which grew out on a medium consisting of one part filtrate and one part bouillon made of heart infusion had a low LD_{50}, that is, it did not lose virulence.

The same authors showed that in a bouillon culture grown out under

aeration conditions [illegible] no l[illegible] virulence was observed if the initial hydrogen ion concentration of the [illegible]dium was close to 7.8.

The results of the experiments in which the reaction of the medium changed within limits of 7.0-7.8 showed that such a slight difference in the initial pH of the medium as 0.2 has an influence on the LD_{50}.

No reduction of virulence of the plague microbe occurs, according to Weesman's, Miller's and Surgalla's data (1958), if glucose is present in the synthetic medium, if the initial pH of the medium is no less than 7.7-7.8.

The inclusion of a number of substances in the medium contributed to preserving the virulence of the plague microbe when cultivated under aeration conditions at 37°: 0.25 percent glutamic or aspartic acid, 0.1 percent sodium bicarbonate (Delwiche, Fukui and others, 1959) as well as calcium salts (Higuchi and others, 1959).

The data of a number of authors permit us to believe that reduction of the oxygen tension plays a great part in maintaining the virulence of the plague microbe growing on bouillon at 37°. Thus, Ogg, Friedman and others (1958) noted that in cultures grown out from small inoculation doses (approximately $1 \cdot 10^4$ cells per cc) on a medium containing reducing substance (sodium thiogluconate), no reduction of virulence is observed in the case of growth under static conditions. Delwiche, Fukui and others (1959) showed that cultivation of the plague microbe under aeration conditions at 37° in an atmosphere of nitrogen with the complete absence of oxygen led to obtaining cultures which were approximately $1 \cdot 10^4$-$1 \cdot 10^6$ more virulent than cells of the same strain grown out in an atmosphere of air. Virulence was even better preserved when 1 percent carbon dioxide was introduced into the nitrogen atmosphere. (It is well known that under the influence of carbon dioxide there is an increase in the toxicity of the culture. This should be kept in mind in evaluating the effect of CO_2 on the virulence (author's note).) The authors believe that preservation of the virulence by the plague microbe culture under conditions of reduced oxygen tension and under the influence of carbon dioxide may have the following mechanism.

The oxidative metabolism of avirulent cells is more effective than that of virulent cells. Therefore, the avirulent mutants contained in the inoculation suspension will always grow more rapidly than the virulent cells under aerobic conditions, and the culture as a whole will lose its virulence. In the absence of oxygen (in a nitrogen atmosphere) the virulent cells can grow as quickly as the avirulent cells and, therefore, the virulence of the culture is maintained.

Carbon dioxide, by suppressing the oxidative processes, inhibits the original growth rate of both virulent and avirulent cells. However, thereby the virulent cells continue to develop, as the result of which the original proportion of virulent and avirulent cells remains constant and the virulence of the culture does not change. From this viewpoint the effect of sodium bicarbonate becomes clear; with introduction of it into the medium carbon dioxide is introduced, particularly since it has been shown by special experiments that the concentration of sodium in the medium plays no part in preserving the virulence. True, the effect of sodium bicarbonate can be attributed to an increase in the pH of the medium. However, preservation of the virulence by the culture in the presence of sodium bicarbonate cannot be ascribed to a certain increase in the pH of the medium for the following

reasons. First of all, the concentration of it used only slightly increases the reaction of the medium (from 7 to 7.2). Secondly, it has been determined that in cultures buffered at a pH of 6.8-7.0 sodium bicarbonate also contributes to maintaining the virulence of the culture. This property of bicarbonate is demonstrated particularly in an atmosphere of nitrogen and an atmosphere of nitrogen with carbon dioxide, in which the pH of the medium is appreciably reduced. These facts permit us to conclude that the preservation of virulence by the plague microbe culture in the presence of sodium bicarbonate is not associated with the change of the pH of the medium and can only be the result of introduction of carbon dioxide into it.

The authors attempt to explain the preservation of virulence by the plague microbe when filtrates of broth cultures of different species of microbes, including the plague microbe, are added to the nutrient medium also by the fact that they contain a large quantity of metabolically produced carbon dioxide.

It is difficult to say whether aspartic and glutamic acids act as sources of carbon dioxide, directly suppressing the Krebs cycle in the avirulent mutant or by some other unknown method.

Therefore, the investigations mentioned above showed that selective growth of avirulent mutants at 37° under aeration conditions is suppressed with a high pH of the medium and in the presence of carbon dioxide.

On the other hand, some factors were established favoring the growth of virulent cells at 37°. Higuchi, Kupferberg and Smith (1959) showed that the presence of calcium ions in the medium is necessary for the growth of virulent cells at 37°. Introduction of the appropriate quantities of calcium salt (0.02-0.04 mole) into the bouillon prevented the loss of virulence by the plague microbe when subcultured in a liquid aerated medium.

In contrast to virulent strains, avirulent strains did not require calcium ions in the basic medium if there was a certain quantity of magnesium ions there (up to 0.025 mole). However, reduction of the magnesium content in the medium caused these strains to require calcium. The salts of some other bivalent metals (strontium and zinc) could replace calcium in the nutrient medium.

Although the reason for the inhibition of growth of virulent cells at 37° in the absence of calcium ions is unknown so far, the study by Higuchi and others mentioned above made it possible to reach an interesting conclusion from a practical respect.

Higuchi and Smith (1961) worked out and proposed a differential agar medium suitable only for the growth of avirulent cells at 37°, because the growth of virulent cells was suppressed on it.

The principle underlying the making of this medium was that of practically completely removing the calcium salt by addition of sodium oxalate to an agar medium and introducing magnesium salts in place of them. Therefore, conditions were created favorable only for the growth of avirulent cells at 37°.

The authors showed that by using this medium it is possible to detect the number of avirulent mutants present in a virulent culture of the plague microbe and to study quantitatively the variation of the plague microbe from virulence to avirulence.

Burrows (1960) points out that on medium with a calcium deficiency

there is a selective suppression of the growth only of those microbes which contain V and W antigens. Since there are avirulent strains which produce these antigens, the growth of these on this medium is also difficult. Among these strains is the EV vaccine strain, which, according to the data of Higuchi and Smith, like the virulent strains, does not grow on medium with a calcium deficiency. Determination of the fact that cultivation of the plague microbe under aeration conditions at 37° causes a marked reduction of virulence of the plague microbe, explained by the creation of conditions unfavorable for the growth of virulent individuals in the culture, was responsible for the trend of subsequent studies toward studying the nutritional requirements of virulent and avirulent cultures of the plague microbe at an elevated temperature. A number of studies has already been made along this line. A review of these will be given later.

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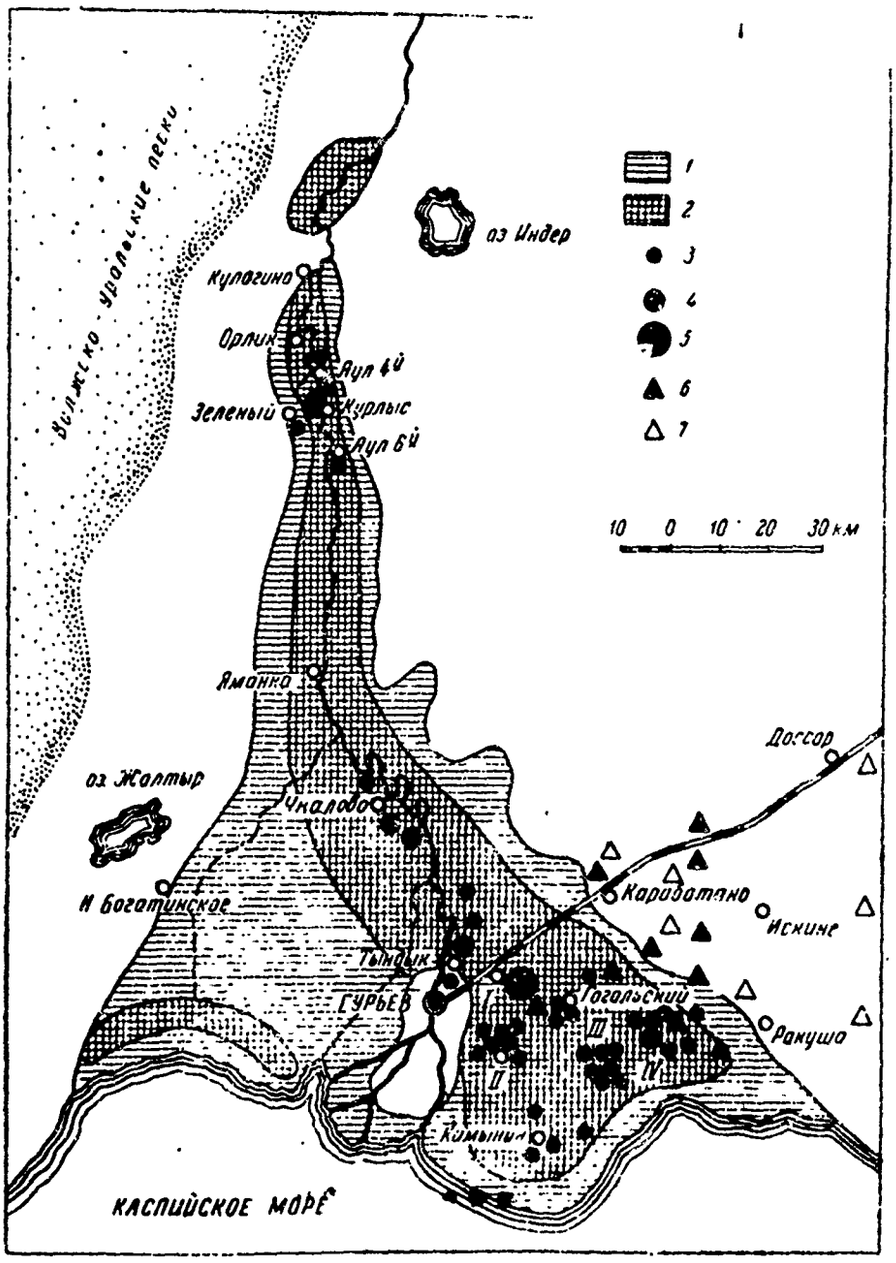


Figure 1. Diagram of Distribution of Epizootics in 1958

1. flood zone in 1957; 2. zone of increased mouse census in the summer of 1958; 3. places in which house mice or fleas were found from which from one to ten plague cultures were isolated; 4. the same, from 11 to 20 cultures; 5. the same, over 20 cultures; 6. places in which plague-infected great sand rats or their fleas were found in 1958; 7. the same, in 1957. I. "Kirpichnyy Zavod"; II. "Bol'shoy Mokryy" section; III. "Gogol'skiy" section; IV. "Bezymyannyy" section.

(To article by Fenyuk et al. pp 2-21)

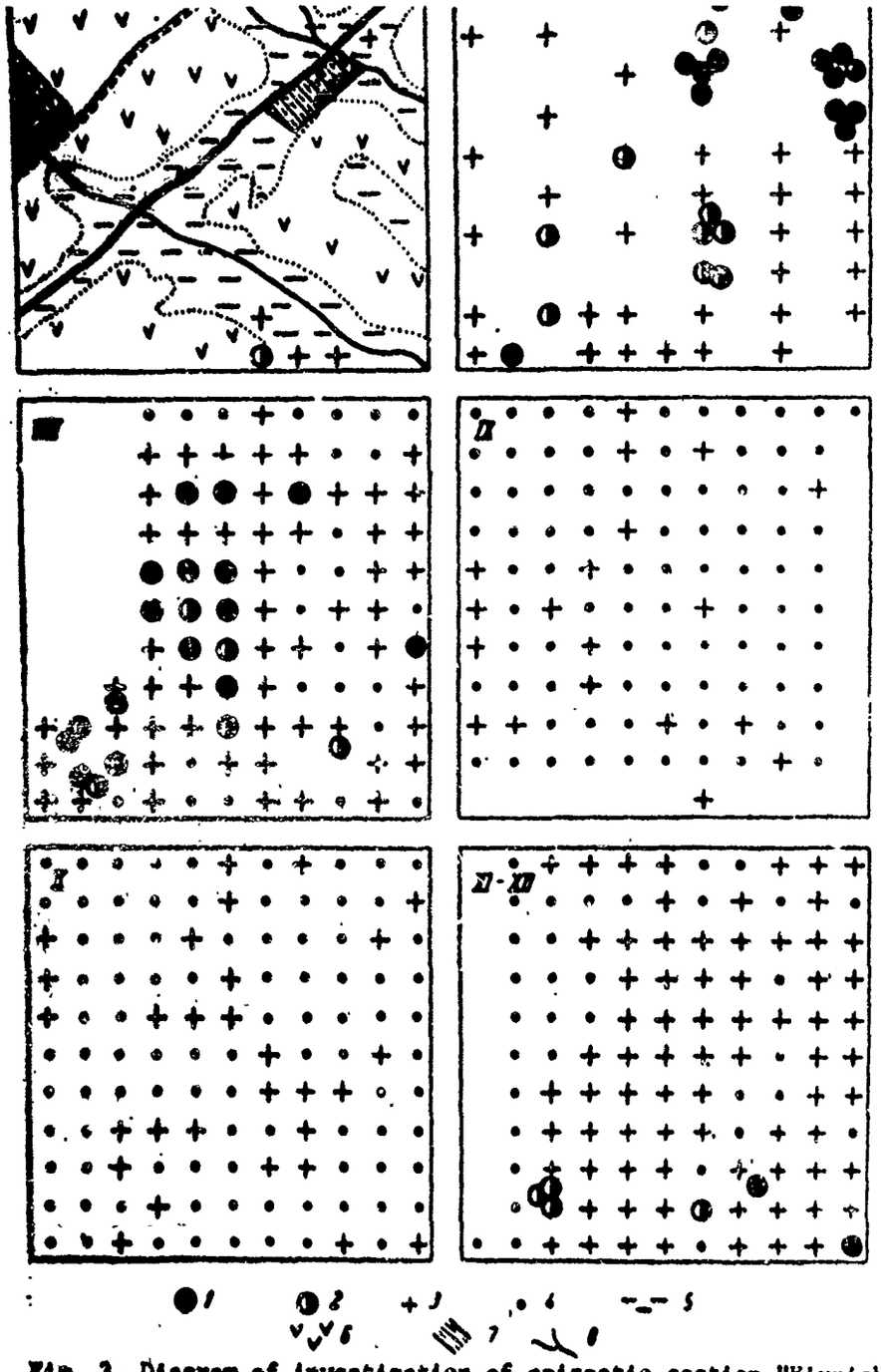


Fig. 3. Diagram of investigation of epizootic section "Kirpichuyy Zavod" (25 square kilometers) and the course of the epizootic on it in 1958. 1) plague microbe culture from house mice; 2) plague microbe culture from house mice fleas; 3) and 4) places from which the field material was obtained (3. where the rodents or fleas were caught; 4. where they were not caught); 5) depression in the terrain covered with beggar weed vegetation; 6) elevated areas; 7) orchards; 8) eriks [shallow channels at the delta of a river].

(To article by Fenyuk et al. pp 2-21)

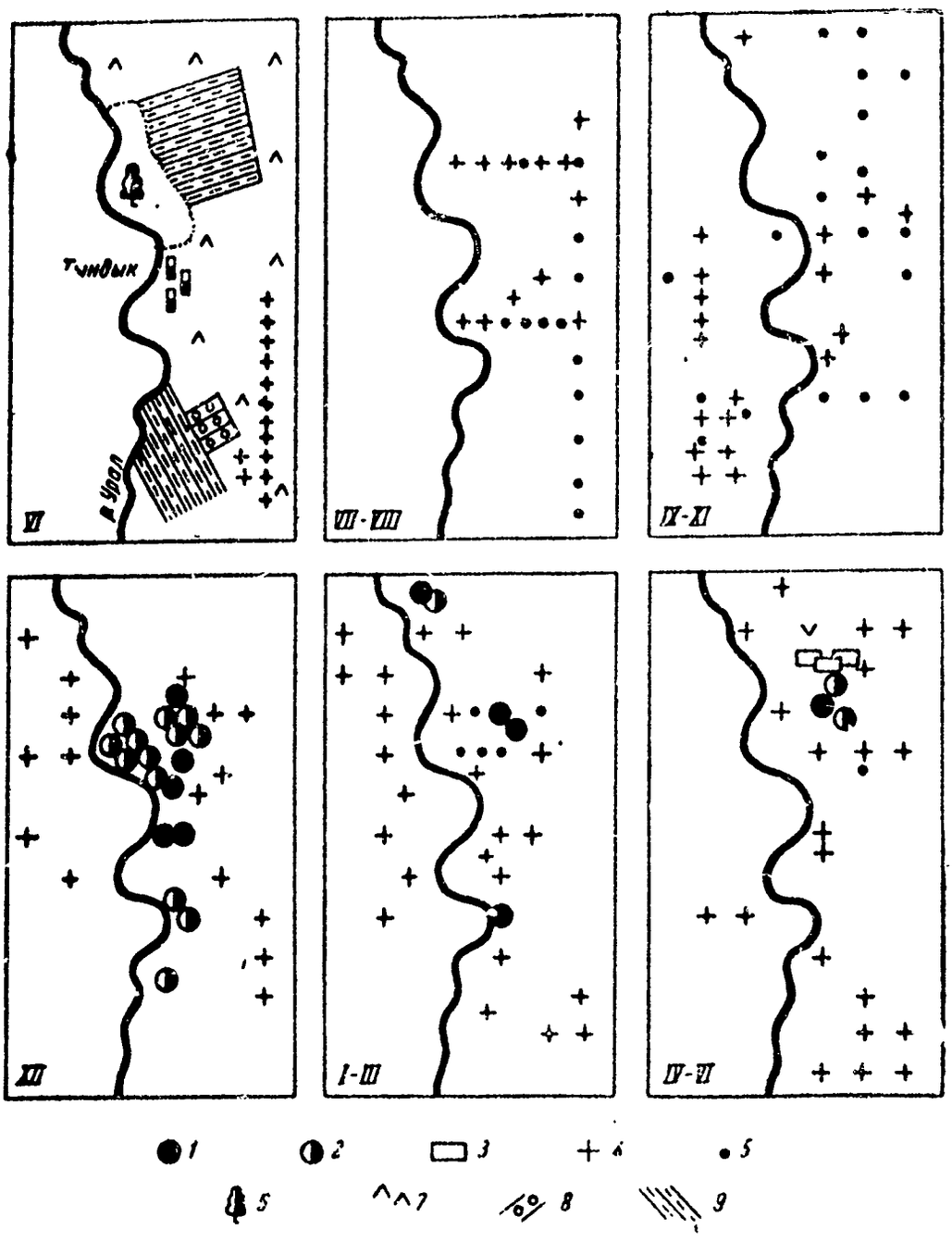


Fig. 4. Diagram of investigation of epizootic section Tyndyk (50 square kilometers) and the course of the epizootic on it in 1958 and 1959.
1) plague microbe culture from house mice; 2) plague microbe culture from house mice fleas; 3) plague microbe culture from ectoparasites of the yellow suslik; 4) and 5) places where the field material was taken (4. where the fleas and rodents were caught; 5. where they were not caught); 6) willow grove; 7) pasture; 8) fruit garden; 9) orchards.

(To article by Fenyuk et al. pp 2-21)

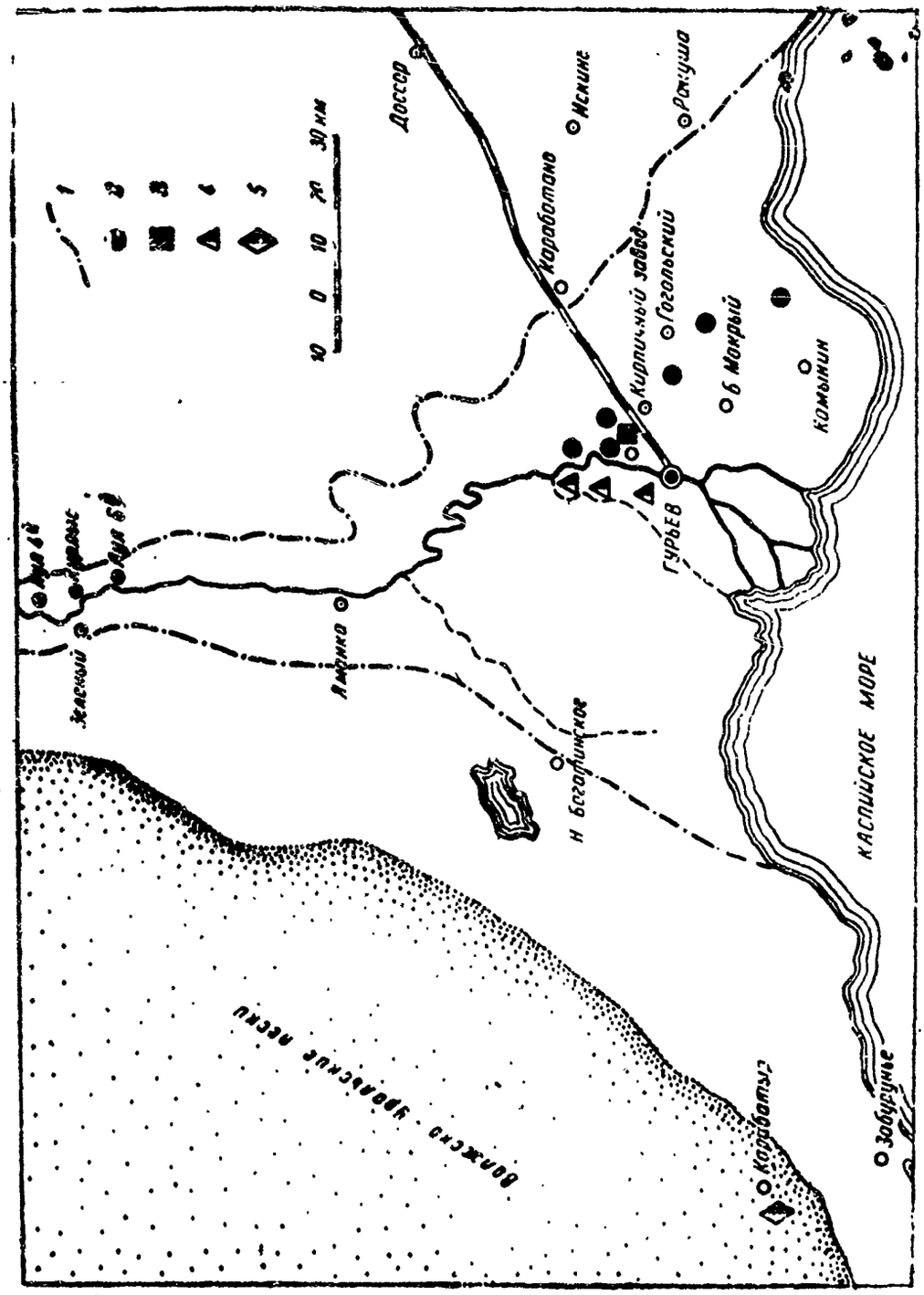


Fig. 5. Diagram of distribution of epizootic in 1959. 1. boundaries of the flood zone in 1957; 2. places in which plague-infected house mice and fleas were found; 3. places in which plague ectoparastes taken from a yellow suslik caught in its holes were found; 4. place of finding plague-infected dwarf susliks and their ectoparasites; plague microbe culture from meridional jird.

(To article by Fanyuk et al. pp 2-21)

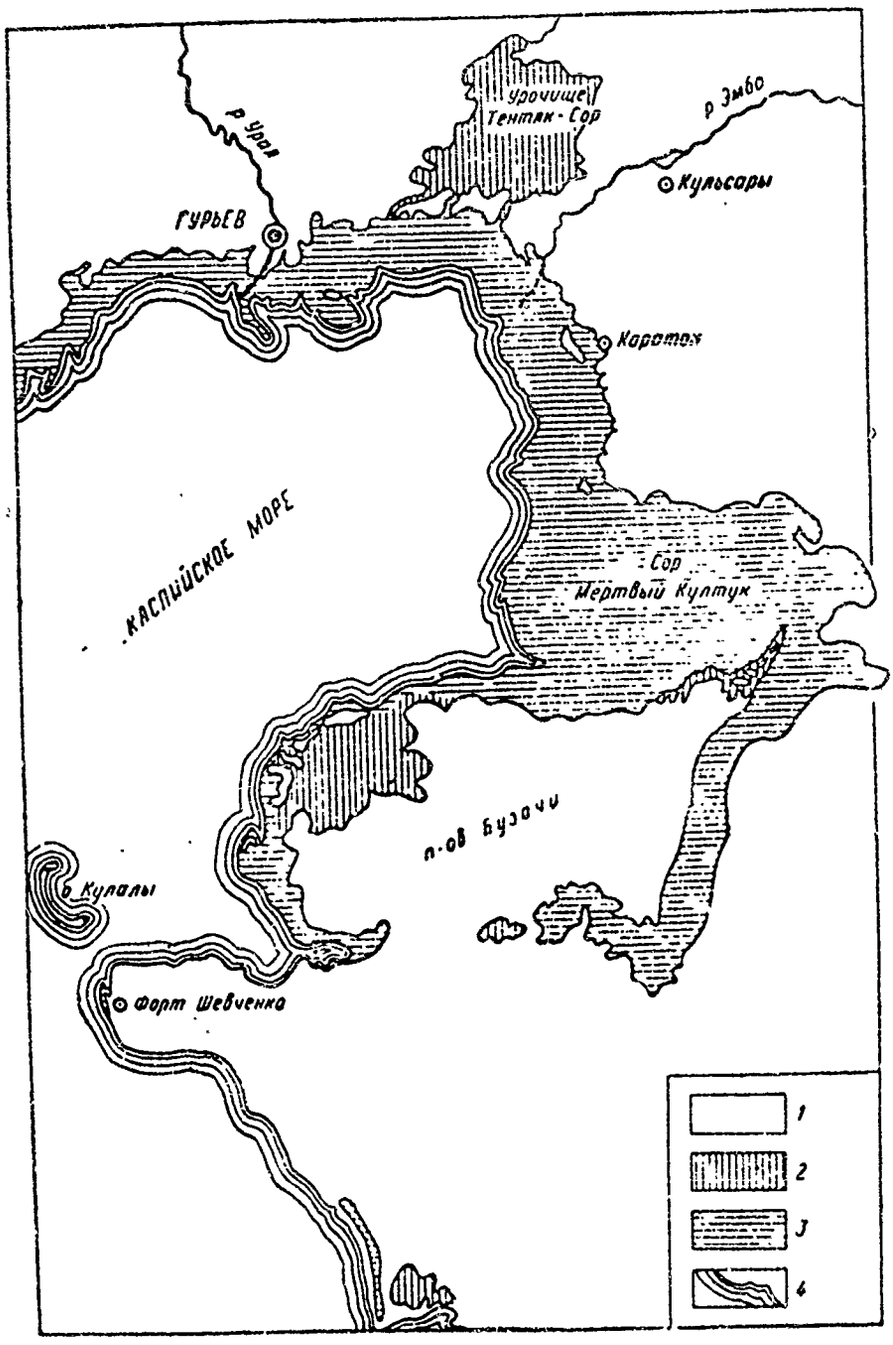


Fig. 1. Territory which emerged from under the sea because of a regression of the Caspian Sea. 1) ancient continental areas of dry land and islands; 2) territory which was under water until 1910; 3) territory which was under water until 1929; 4) present-day outlines of the sea.

(To article by Lavrovskiy, pp 40-58)

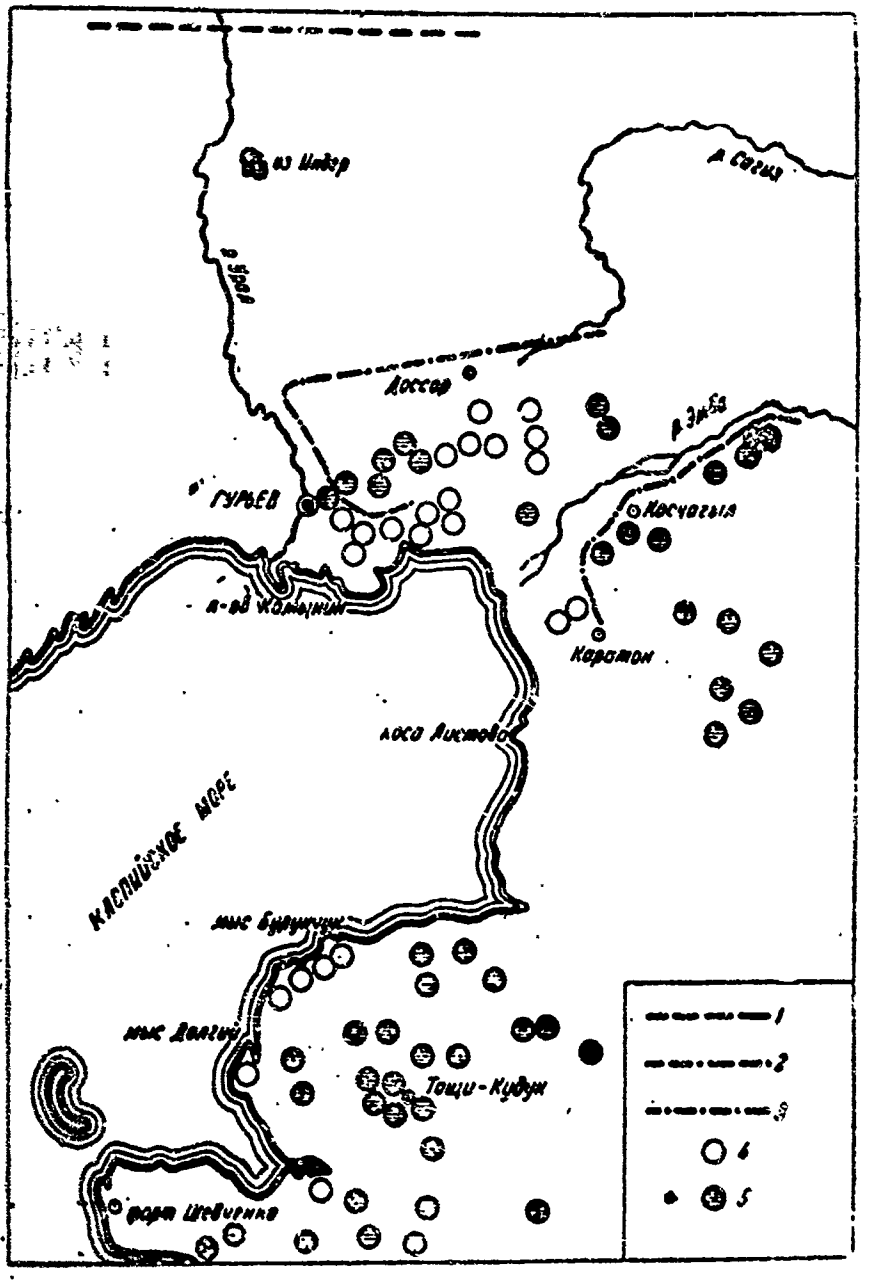


Fig. 2. Diagram of distribution of plague epizcotics and change in the boundaries of the area of distribution of the great sand rat in the northeast and east Caspian 1) boundary of area of distribution of the great sand rat in the Pleistocene (after I. M. Gromov); 2) the same in 1959; 3) the same in 1935; 4) epizootic areas on new territories; 5) the same on the main [original] shore.

(To article by Lavrovskiy, pp 40-58)

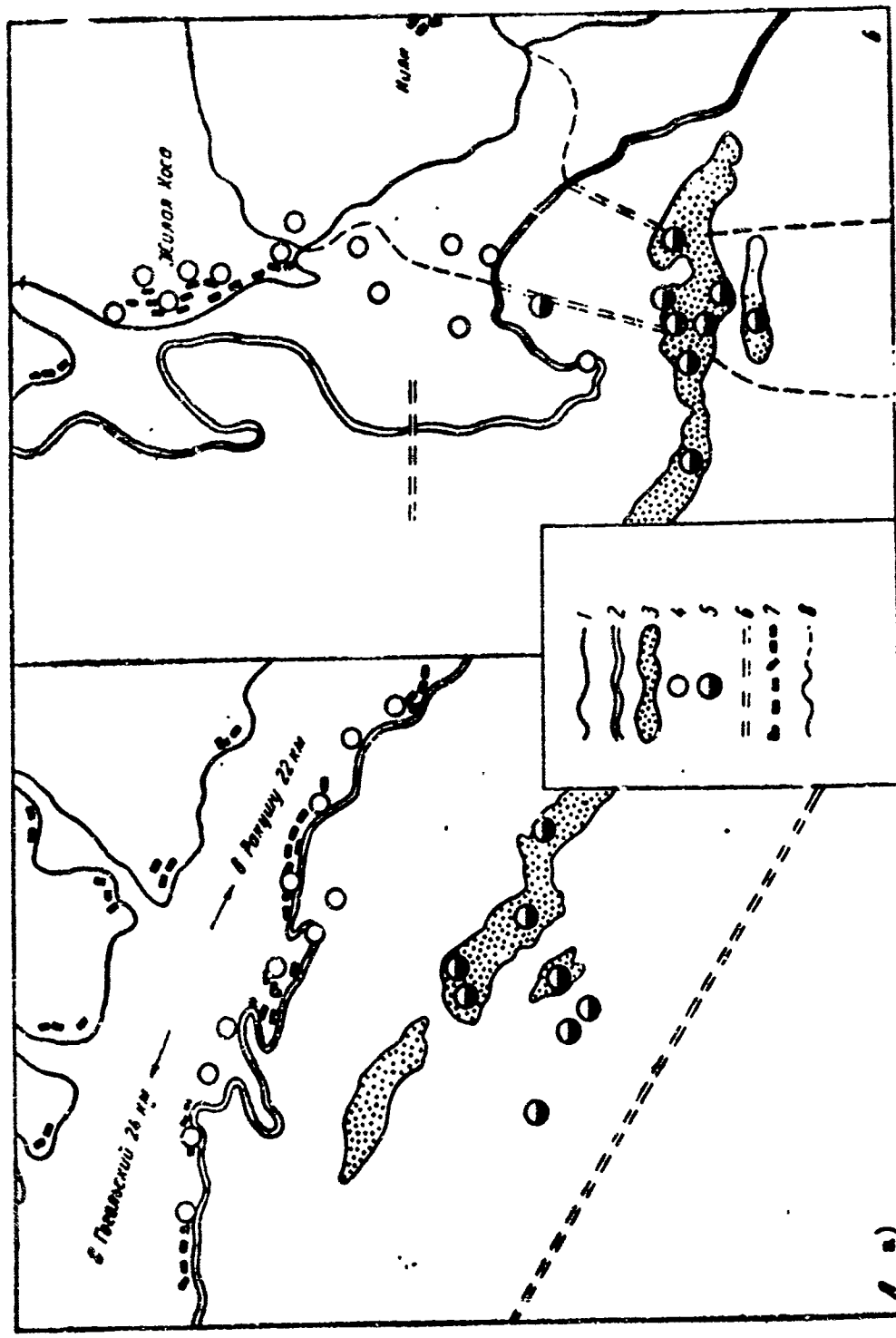


Fig. 3. Dispersal and settlement of the Great Sand Rat. A. Atrau Natural Landmark; B. Zhilaya Kosa. 1. boundary of the original shore; 2. outlines of the sea shore in 1929; 3. cumulative sand ridges on dried-up territory; 4. disposition of colonies of the great sand rat in the winter of 1956/57; 5. disposition of great sand rat colonies in the autumn of 1958; 6. canals; 7. ruins of villages; 8. dried-up arms of the Ural River.

(To article by Lavrovskiy, pp 40-58)

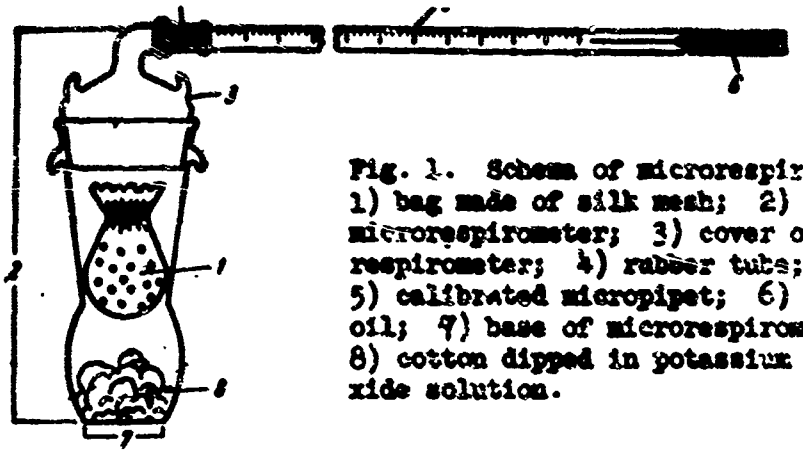


Fig. 1. Scheme of microrespirometer. 1) bag made of silk mesh; 2) glass microrespirometer; 3) cover of microrespirometer; 4) rubber tube; 5) calibrated micropipet; 6) apricot oil; 7) base of microrespirometer; 8) cotton dipped in potassium hydroxide solution.

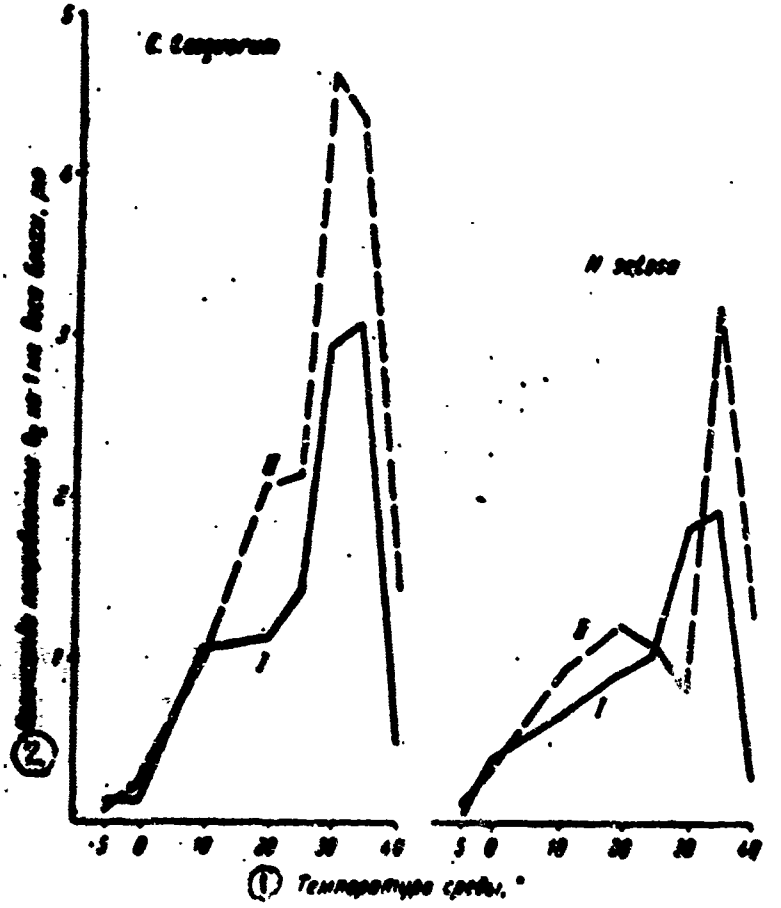


Fig. 2. Oxygen consumption by satiated fleas. I - females; Iа - males; 1) ambient temperature; 2. quantity of oxygen consumed per mg of weight of the flea, cc.

(Both figures to article by Kondrashkina, pp 71-77)

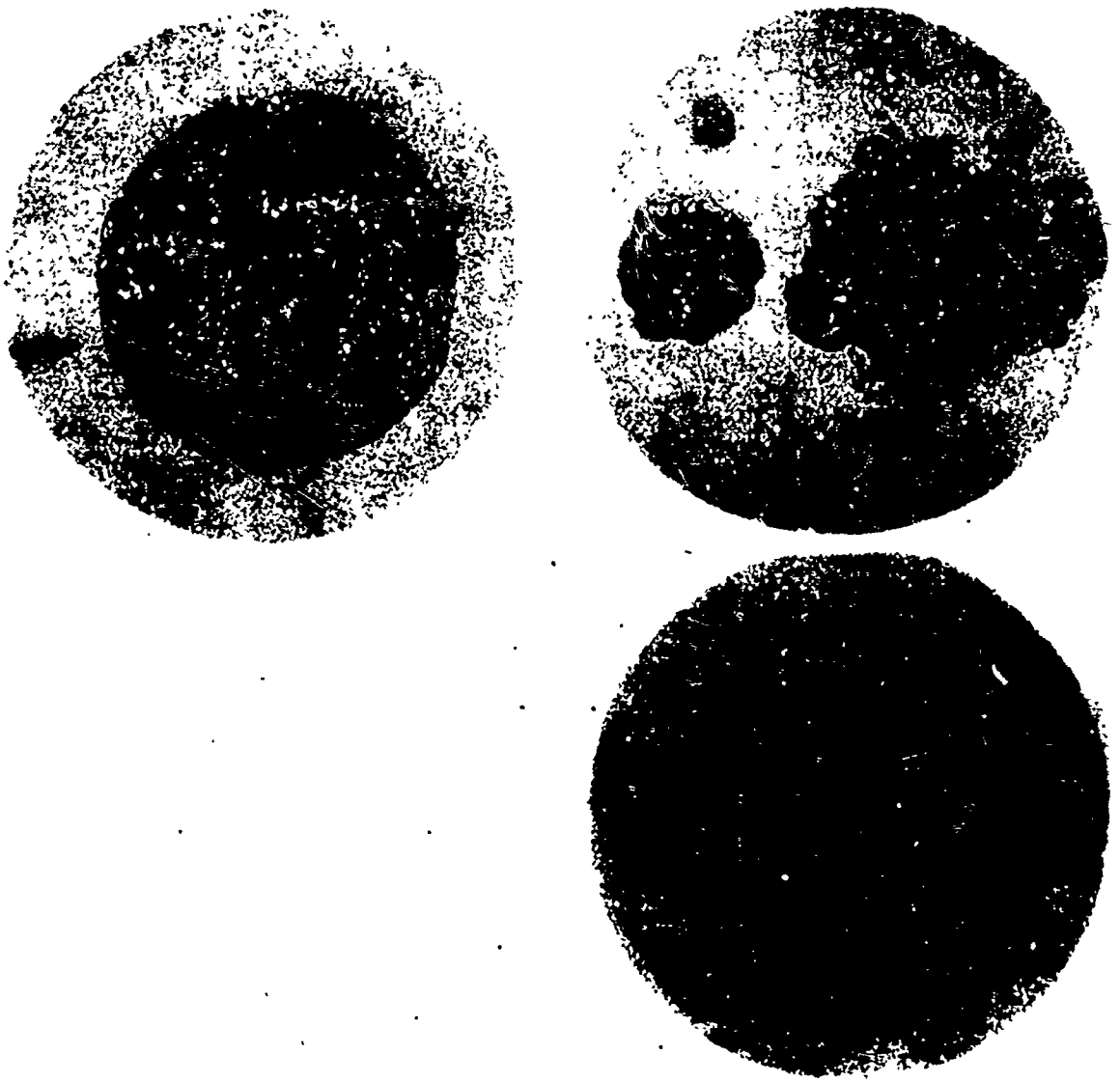


Fig. 1. Heterogenous structure of colonies of the plague microbe exposed to streptomycin effect.

(To article by Malinina pp 131-134)

Fig. 2.

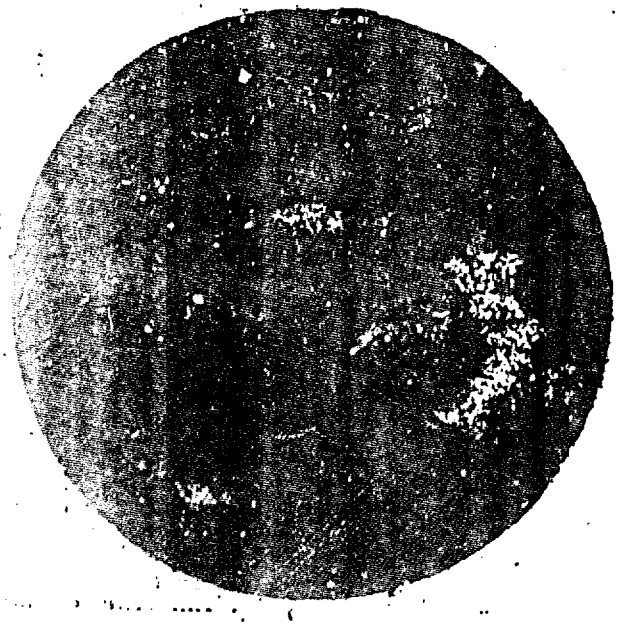


Fig. 3.

Fig. 2. Smears of plague microbe cultures altered under the influence of streptomycin.

Fig. 3. Dissociation of plague microbe culture under the influence of streptomycin into S and R variants.

(To article by Malinina, pp 131 - 134)

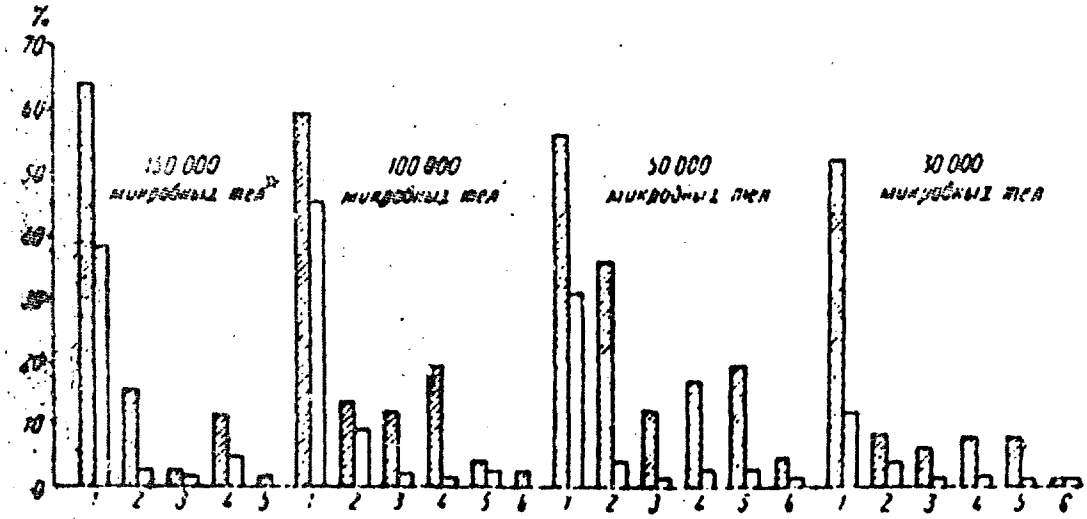


Fig. 1. Comparative data on the growth of the EV vaccine strain from cultures of organs and tissues of inoculated white mice in semiliquid agar (0.15%) with blood (0.3%), Gentian violet (1:200,000) (Hatched columns) and on agar containing blood (3%) and Gentian violet (1:100,000) (White columns).

1) from the injection site;
2) from the regional lymph node;
3) from the liver;
4) from the spleen;
5) from blood;
6) from the lung.

w) microbe bodies

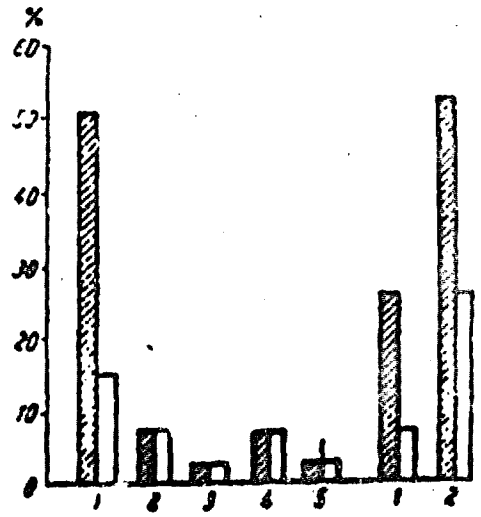


Fig. 2. Comparative data on the growth of vaccine strain of the plague microbe from the organ cultures of guinea pigs inoculated with the EV vaccine in semiliquid agar (0.15%) containing blood (0.3%) and Gentian violet (1:200,000) (Hatched columns) and on agar containing blood (3%), Gentian violet (1:100,000) (White columns). 1) from the injection site; 2) from the regional node; 3) from the liver; 4) from the spleen; 5) from the blood.

(To article by Samoylova, pp 155-160)

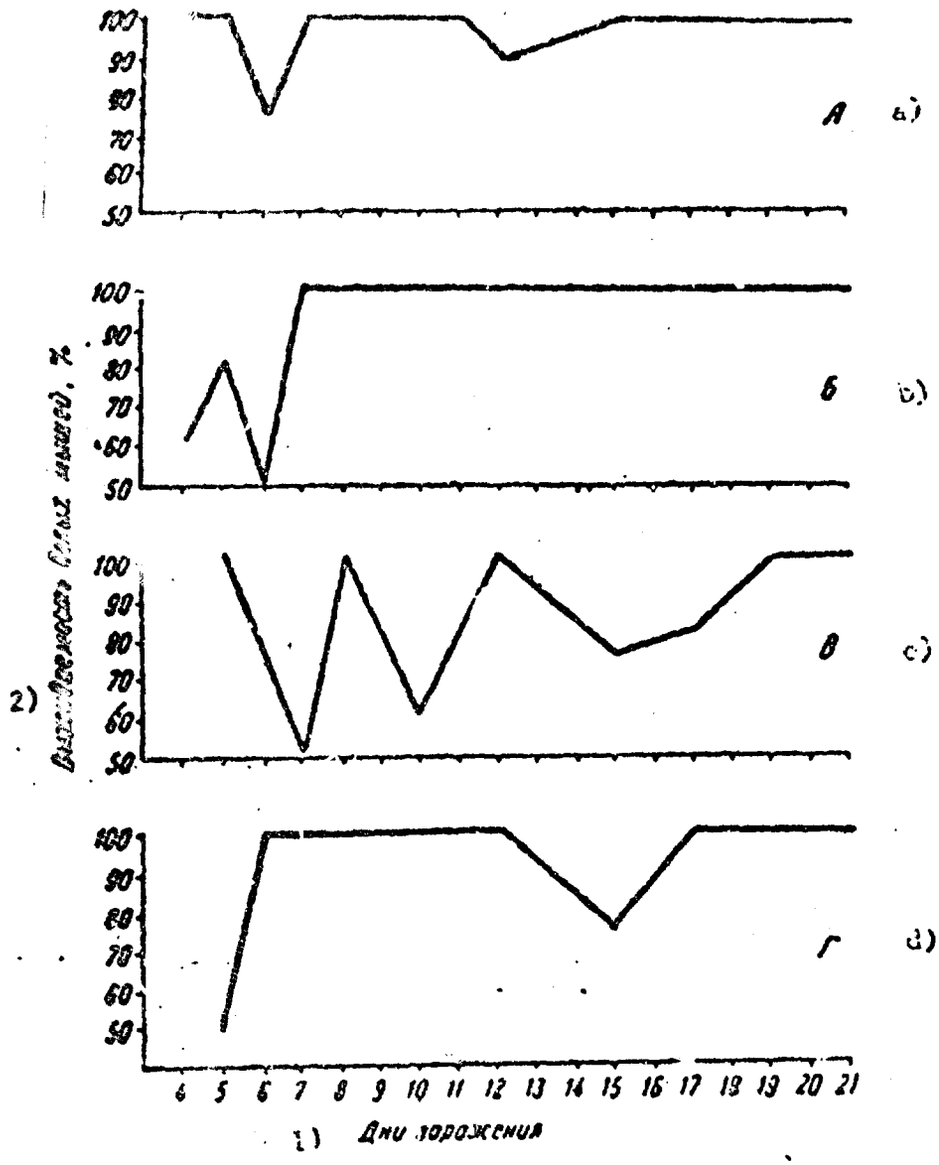


Fig. 3. Survival of white mice after infection by vaccination. a - dose of 130,000 microbes; b - dose of 100,000 microbes; c - dose of 50,000 microbes; d - dose of 30,000 microbes. 1) days of infections; 2) survival of white mice, %.

(To article by Samoylova, pp 155-160)

(Captions to these figures on following page)

Figure 2

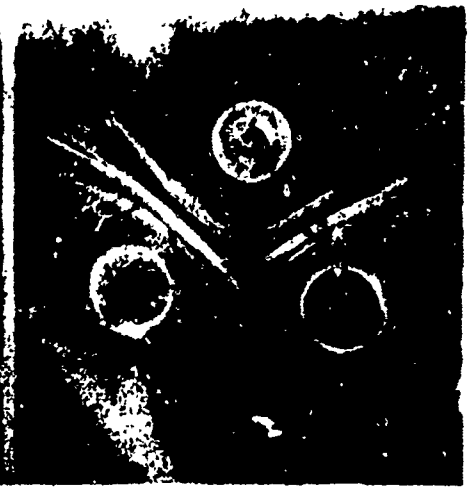


Figure 3

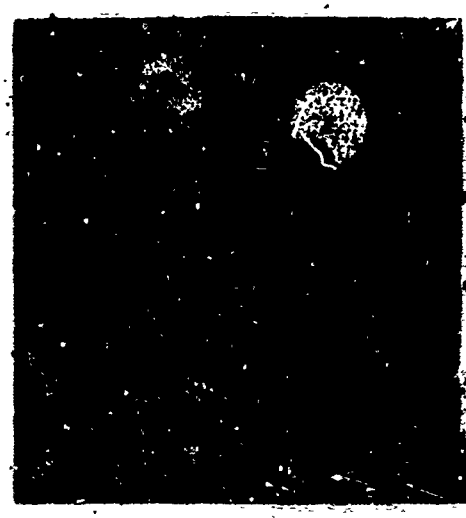


Figure 4

Figure 5

Figure 6

(To article by Pustovalov et al. pp 160-167)

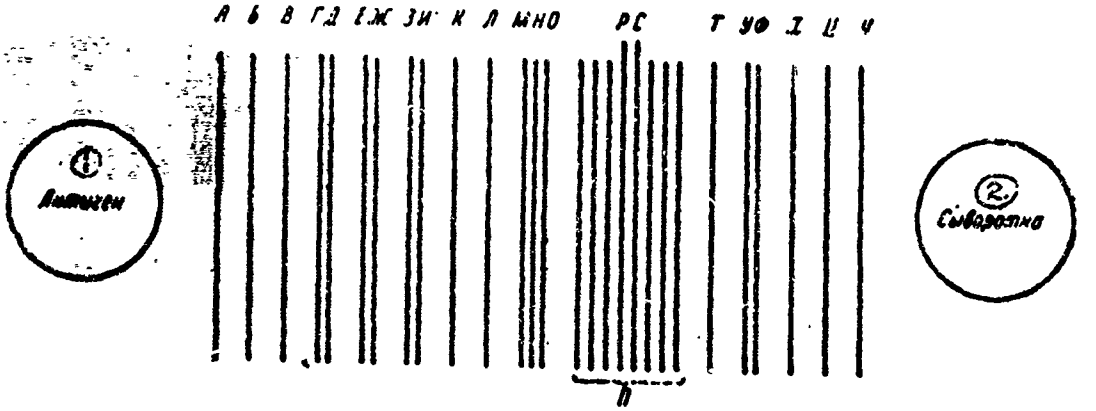


Fig. 7. Schema Showing the Arrangement and Designation of Antigens of P. Pestis EV NIIEG. 1. Antigen; 2. Serum

Captions to Figures on preceding page

Fig. 2. Precipitation Bands of Living and Acetone-Killed Plague Microorganisms (P. Pestis EV of the NIIEG). 1. Suspension of living microorganisms (75,000,000,000); 2. Suspension of acetone-killed microorganisms (75,000,000,000); 3. Normal horse serum; 4. and 5. Physiological saline solution; 6. Extract of sterile nutrient medium with physiological saline solution; 7. Plague agglutinating serum.

Fig. 3. Diffusion in Gel of Extracts of a Bacterial Mass of P. Pestis EV of the NIIEG. E_1. First extract (26.8 mg); E_2. Second extract (9.6 mg); E_3 Third extract (1.5 mg); P. Physiological saline solution; A.C. Plague agglutinating serum (explanation in the text).

Fig. 4. Diffusion in Gel of Antigens of Combined Extract of Bacterial Mass of P. Pestis EV of the NIIEG. 1. Extract (5 mg); 2. Extract (1 mg); 3. Extract (0.5 mg); 4. Extract (0.3 mg); 5. Extract (0.2 mg); 6. Extract (0.1 mg); 7. Plague agglutinating serum.

Fig. 5. Diffusion in Gel of Antigens of Combined Extract of Bacterial Mass of P. Pestis EV of the NIIEG. 1. Extract (15 mg); 2. Plague agglutinating serum; 3. Plague agglutinating serum absorbed by fraction 1A (explanation in text).

Fig. 6. Diffusion in Gel of Antigens of Combined Extract of Bacterial Mass of P. Pestis EV of the NIIEG (EXPLANATION IN TEXT). 1. Extract (15 mg); 2. Plague agglutinating serum; 3. Plague agglutinating serum absorbed by the 1A fraction.

(To article by Pustovalov et al. pp 160-167)

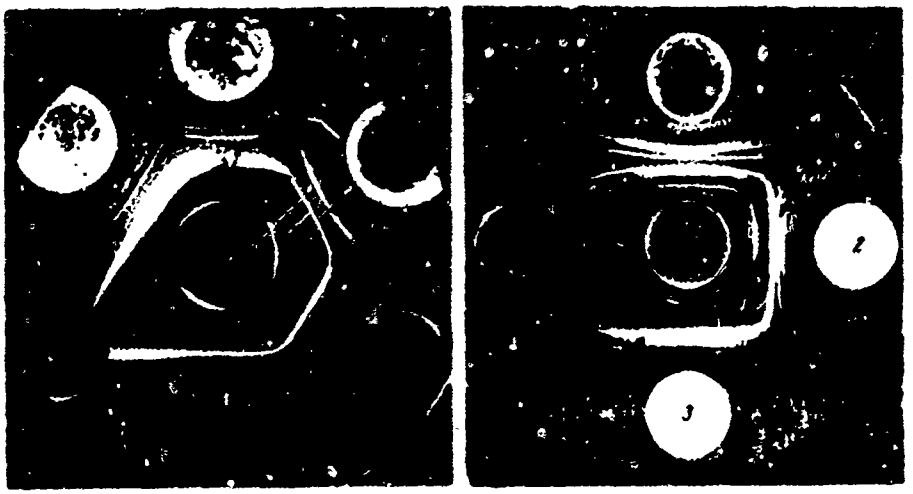


Fig. 8. Diffusion in Gel of Antigens of Extract of a Bacterial Mass of P. Pestis EV NIIEG.
1. Extract (100 mg);
2. Extract (30 mg);
3. Extract (20 mg);
4. Extract (10 mg);
5. Extract of sterile nutrient medium with physiological saline;
6. Extract of bacterial mass (200 mg);
7. Plague agglutinating serum (explanation in text).

Fig. 9. **Diffusion in Gel of Antigenic Fractions** from Extract of P. Pestis EV of the NIIEG .
1. Toxic fraction (14 mg);
2. Extract of bacterial mass (32 mg);
3. Residual fraction (24 mg);
4. Physiological saline solution;
5. Plague agglutinating serum (explanation in text)

(To article by Pustovalov et al. pp 160-167)

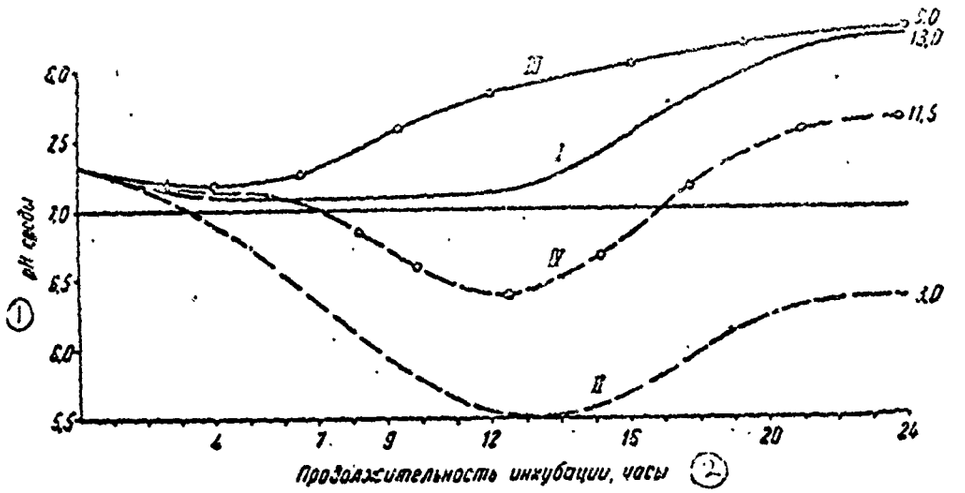


Fig. 1. Change in the Reaction of the Medium During the Course of Growth of the Plague Microbe Under Aeration Conditions on Two Series of Hottinger's Bouillon. I. bouillon of series 21 without glucose; II. bouillon of series 21 with glucose; III. bouillon of series 10 without glucose; IV. same with glucose. The figures on the curves denote the number of microbes (in billions/cc) after 24 hours of growth. 1. pH of medium; 2. duration of incubation, hours.

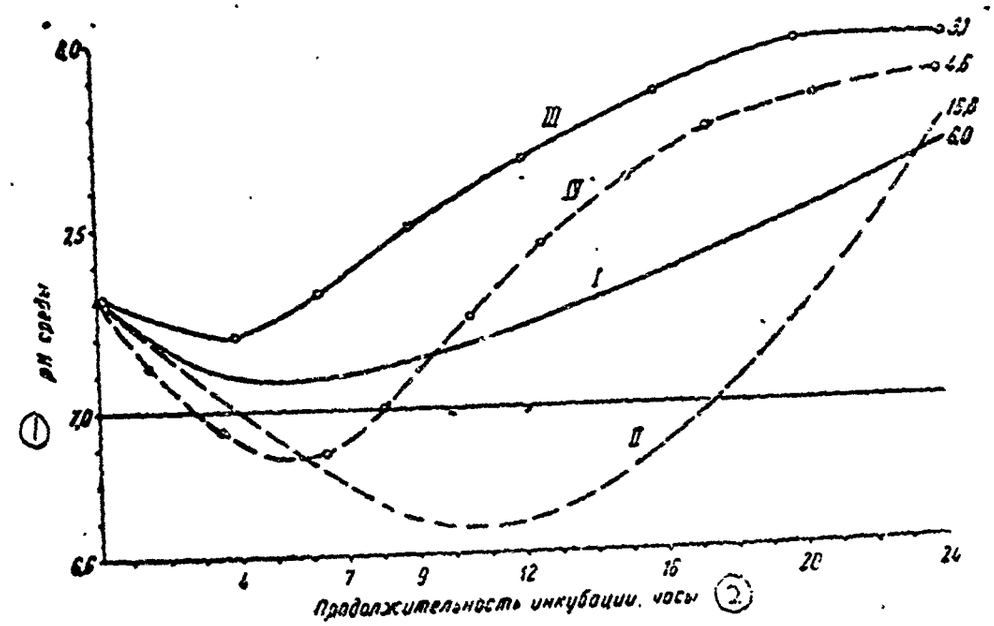


Fig. 2. Change in the Reaction of the Medium During the Course of Growth of the Plague Microbe Under Aeration Conditions on Casein Bouillon Containing Certain Inorganic Salts and Without Them. I. bouillon containing salts; II. bouillon containing salts plus glucose; III. bouillon without salts; IV. bouillon without salts plus glucose. 1. pH of medium; 2. duration of incubation, hours.

(Both figures to article by Drozdovskaya et al. pp 229-238)

Upper Figure: Specific fluorescence of cholera vibriones treated with luminescent cholera serum.

(To article by Chibrikova et al. pp 250-257)

Lower Figure: Smear impressions of mouse spleen infected with plague microbe treated with antiplague fluorescent serum

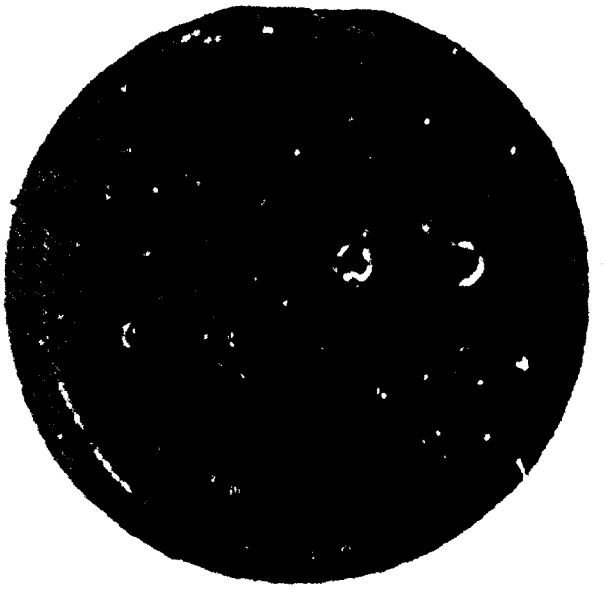
(To article by Chibrikova et al. pp 257-265)

Upper Figure: Specific fluorescence of cholera vibriones treated with luminescent cholera serum.

(To article by Chibrikova et al. pp 250-257)

Lower Figure: Smear impressions of mouse spleen infected with plague microbe treated with antiplague fluorescent serum

(To article by Chibrikova et al. pp 257-265)



Diffusion in Gel Test on Standard Agar Plates with the Organs of Mice which Died of Anthrax. Those nearer to the wells containing anthrax-precipitating serum are located along the same compact precipitation band.

(To article by Akimovich et al. pp 294-302)

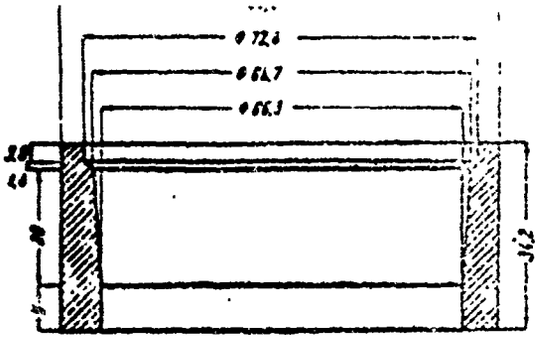


Fig. 1. Drawing of Mold Frame [obichayka]

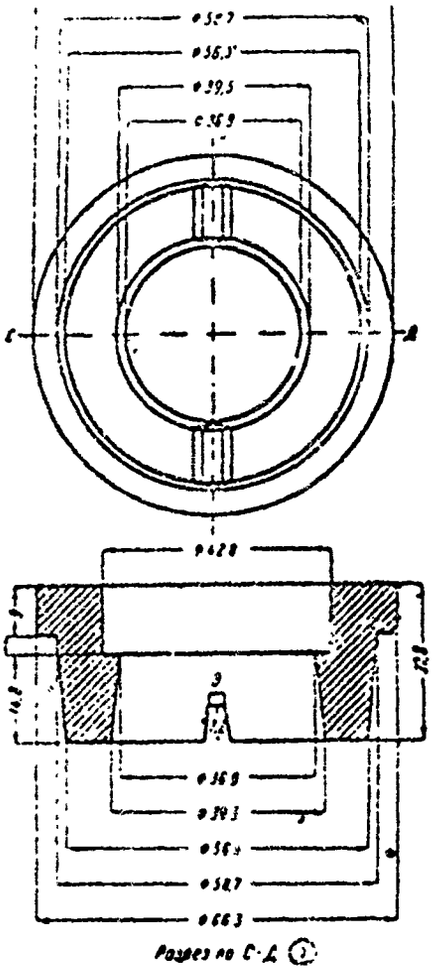


Fig. 2. Drawing of Mold Matrix
1. section through C-D

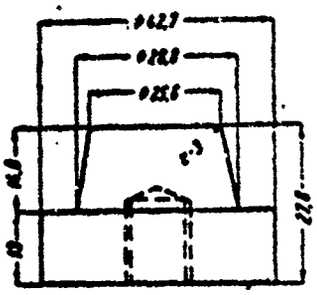


Fig. 3. Drawing of Mold Insert

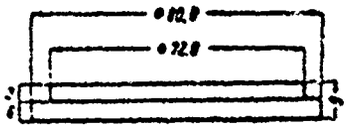


Fig. 4. Drawing of Mold Cover

(To article by Tabakov, pp 308-310)

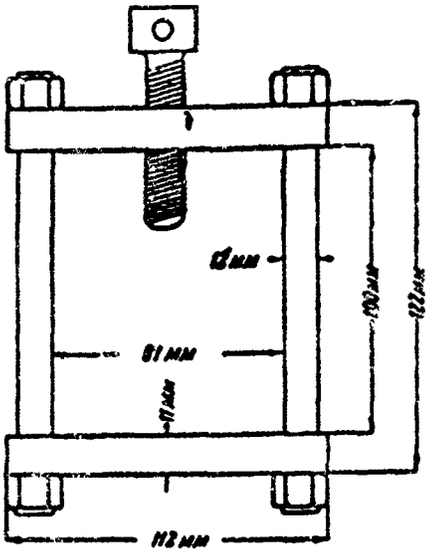


Fig. 5. Drawing of Mold Stirrup Bolt

(To article by Tabakov, pp 308-310)

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