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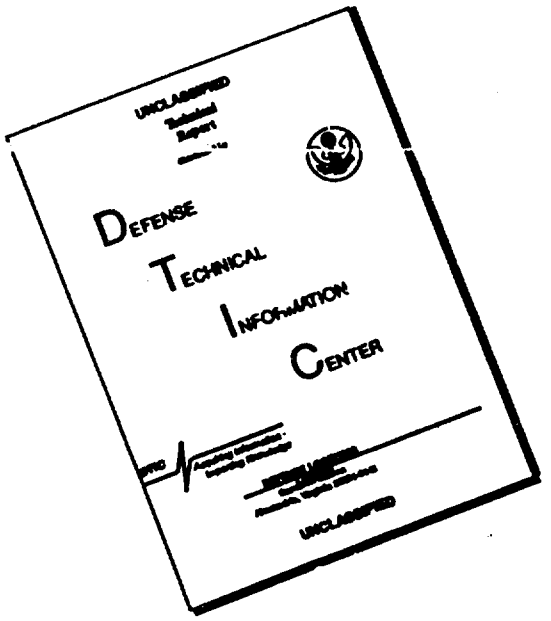

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CHANGES OF THE L-FORMS OF BACTERIA AND THEIR RESISTANCE WHEN KEPT AT VARIOUS TEMPERATURE CONDITIONS

/Following is the translation of an article by K. P. Shakhovskiy in the Russian-language journal Zhurnal Mikrobiologii, Epidemiologii i Immunobiologii (Journal of Microbiology, Epidemiology and Immunobiology), No 10, 1963, pages 105-111./

From the Second Moscow Institute imeni Pirogov

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The question of the role of the L-forms of bacteria in infectious pathology and epidemiology calls for further study, in particular, the resistance of these forms to the effect of various unfavorable factors must be clarified. Studies in this area are few in number and restricted chiefly to the Proteus group (Dienes and Weinberger, Tulasne and Minck, Bloss-Bender, Higmans and Kastelein, et. al.). Data characterizing the resistance of the L-forms of pathogenic species of bacteria in the literature available to us has not been found. Accordingly, we decided to study the resistance of the L-forms of certain pathogenic species of bacteria to the effect of various physical, chemical, and biological agents. Serving as the experimental material, from among gram-negative pathogenic species, were stable L-forms of typhus abdominales bacteria and of the gram-positive species -- the stable L-forms of α- and β-hemolytic streptococcus. For comparison we used a culture of stable L-forms of Proteus vulgaris.

As is known (savoyskiy), bacterial form of certain gram-negative bacteria of the intestinal group are preserved without reculturing up to 6-7 months under refrigerator conditions. At room temperature and at 37 degrees, when the life processes are intensified, and drying of media increases, the vital activity of the bacteria is reduced -- they are preserved for up to 1-4 months (Kantley, et. al., Vogralik, and Savoyskiy). As far as the vital activity of hemolytic

streptococci are concerned, it proves less active under corresponding conditions. For example, at the refrigerator temperature these bacilli are usually retained up to 2-½ months (Shchegolev). Longer (3-6 months) in storage without reculturing at different temperatures (4, 20, and 37 degrees) proves destructive for hemolytic streptococci (Nikkols).

Based on these data, according to which the limiting survival time of vegetative forms of bacteria under nutrient medium conditions is 1-7 months, we set ourselves the task of discovering the storage time of L-forms under nutrient medium conditions at different temperatures.

Used as material for the investigation were five strains of L-forms obtained from the Institute of Epidemiology and Microbiology imeni Gamaleya. Included was one strain of α-(No 409) and two strains of β-hemolytic streptococcus (No 190 and 196), one strain of typhus abdominales bacteria (No 152) and one strain of Proteus vulgaris.

The last two strains were obtained in 1954, the strain of β-hemolytic streptococcus -- in 1957 on media containing 125 IU of penicilin per 1 ml, and the strain of β-hemolytic streptococcus was isolated in the L-form from the blood of a rheumatism patient in 1960. The more abundant growth of the L-colonies was observed in the passage of the cultures in media containing 250-1000 IU of penicilin per ml. The strain of β-hemolytic streptococcus No 190, during the passages of which on nonpenicillin media individual colonies of streptococcus was sometimes found in addition to L-colonies, this strain No 190 was classed among the provisionally stable L-forms, while we recorded the other strains not reverting during 21-28 days as stable.

These strains were cultivated on semifluid (0.3% agar) seral (10%) penicilin (250-2000 IU per ml) media. The media for cultivation of streptococcal L-forms were prepared on agar of the tryptic digestion of beef heart. By way of osmotic stabilizers they contain saccharose (20%) and magnesium sulfate (0.1-0.2%). Before the beginning of the experiment the original cultures underwent phase-contrast microscopy in order to discover the morphology of the L-elements forming L-colonies. Two-week old cultures containing a considerable amount of L-colonies were selected for innoculation (Figure 1).

The feeding material containing L-form colonies together with the medium was placed in a test tube containing 10 ml of freshly prepared medium. The freshly recultured L-form cultures were grown for 3 days at 37 degrees and for 4 days at room temperature. Thereupon, the cultures were stored under the corresponding temperature conditions (4, 20, and 37 degrees) for 2-6 months.

Changes in the character of L-form growth were estimated through phase-contrast microscopy (to discover the morphological changes in structure of L-elements) and innoculation on the following media: a) a medium containing penicillin and the stabilizers to establish optimal conditions for L-colony growth; b) a medium without penicillin but containing stabilizers to keep account of intermediate forms of reversion; c) medium without penicillin and stabilizers, but containing serum to reveal forms of streptococcal reversions; d) the same medium, but without serum to review forms of reversion of typhus abdominales bacteria and Proteus vulgaris. After seven days of cultivation the subcultures of the L-forms being tested were investigated for the presence of typical L-colonies. The latter were then studied with a phase-contrast microscope. The revertants sound were isolated into a pure culture. The subcultures of L-forms of each series of experiments were placed under observation for not less than two months in order to discover possible subsequent changes (delayed reversions, lysis, etc.).

During storage of L-cultures under temperature conditions noted above, the following changes in their microstructural elements were observed. Homogenous bodies of different sizes and different optical densities, large vacuolized and granular spherical forms constituting the main bulk of the L-colonies of the original cultures, varied significantly: their homogeneity was eliminated, optical density reduced, they became more pale, they were transparent, surrounded about the periphery by dark granules (Figure 2). The quantity of homogeneous bodies was sharply reduced. The number of freely lying granules was considerably increased (Figure 3). These changes were recorded for all strains of L-forms independently of their species origin. The extent of morphological changes depends on their storage conditions.

Studies of morphological changes of L-colonies for different periods and conditions of storage using the example of the No 409 strain has shown (Figure 4) that minimal structural changes in the trace elements of L-colonies have been observed at 4 degrees. The L-elements are diverse spherical bodies (from large to small) vacuoles and granules. The increase of granular masses in the L-colonies occurred only after six months. More pronounced morphological changes set in when the L-cultures were stored at 20 degrees. After six months of storage the L-elements were chiefly granules, light-refracting bodies, and separate fragile light-grey spheres of small and moderate size. Deformed pale-colored bodies surrounded by granules appeared only by the

fourth-month of storage. The L-elements of colonies stored at 37 degrees underwent the most changes. Even after two months granular masses with moderate amounts of deformed spherical elements predominated in the L-colonies. After 6 months the L-colonies consisted almost entirely of light-refracting bodies and granules.

Capacity of cultivation in L-subcultures and reversion to bacterial forms as related to conditions and duration of storage

(a) № L-культуры	(b) Режим	(c) Результат высева после разных сроков (в месяцах) хранения					
		2		4		6	
		(d) рост L-форм	(e) рост форм реверсии	(d) рост L-форм	(e) рост форм реверсии	(d) рост L-форм	(e) рост форм реверсии
190	4°	+++	+	+++	+	++	+
	20°	+++	+	+++	+	+	+
	37°	+	−	+	−	+	−
196	4°	+++	−	+++	+	+++	−
	20°	++	−	++	−	+	−
	37°	++	−	+	−	+	−
409	4°	+++	−	+++	−	+++	−
	20°	+++	−	+++	−	+	−
	37°	++	−	+	−	+	−
152	4°	+++	−	+++	−	+++	−
	20°	+++	−	+++	−	++	−
	37°	++	−	+	−	+	−
(f) Протей	4°	+++	+	++	+	++	+
	20°	+++	+	++	+	++	+
	37°	++	+	++	+	++	−

Remark: +++ uncounted number of L-colonies; ++ 10 100 L-colonies; + individual colonies (<10); − lack of growth.

LEGEND: a) No of L-culture; b) Conditions; c) Result of seeding after various storage periods (in months); d) growth of L-forms; e) growth of reversion forms; f) Proteus.

Presented in the table are data characterizing the ability of strains to be cultivated in L-subcultures and to revert to the bacterial form depending on conditions and duration of

storage. The ability to be cultivated in the L-form is well pronounced in strains stored at four degrees. It equally characterizes the L-cultures of different bacterial species and remains essentially unchanged for 6 months. To a lesser extent the capacity for passage in the L-form is manifest in strains stored at 20 degrees. It begins to decrease from the fourth month and drops considerably by the sixth month. The reduced ability to be passaged in the L-form is more pronounced for the cocci. L-forms stored at 37 degrees are least of all capable of subsequent passages. This ability is sharply curtailed in these forms even after two months of storage, while after four months it becomes still lower and remains at this low level. The L-forms of cocci were also passaged poorly.

Of 5 stable L-cultures 3 proved capable of reversion to the bacterial form. Strain No 409 (α-hemolytic streptococcus) and No 152 (S. typhosa) proved stable. Strain No 196, not undergoing reversion at room temperature when recultured in 21-28 days, did revert after more prolonged storage at 4 degrees. This is probably due to the fact that the culture consisted of L-colonies of the 3A type and in part of the 3B type, retaining to some extent the capacity to revert. The stability of culture No 190 and Proteus proved ordinary, since both reverted at 4 and 20 degrees, and Proteus -- also at 37 degrees. The frequency of reversion depended on storage conditions and duration. Thus, at 4 degrees three strains reverted (No 190, 196, and Proteus), at 20 degrees -- 2 (No 190 and Proteus) and at 37 degrees -- only the Proteus strain. Reversion was noted most frequently during 2-4 months. After 6 months the capacity to revert was lost by the strain No 196 (4 degrees) and Proteus (37 degrees).

Since for longer durations and at higher temperatures (20 and 37 degrees) microcolonies comprised chiefly of granular masses predominated in the L-subcultures, we had the grounds to assume that either extinction of colonies of the 3B type took place and because of this the capacity for reversion was lost, or else the transition of the 3B colonies to 3A lacking the capacity to reversion took place. By means of this same mechanism (extinction of the type 3B colonies) we can also explain the decrease with time of the capacity of L-cultures for subsequent passage, since factors of time, desiccation, temperature, evidently exert in their turn an unfavorable effect on elements of type 3B colonies.

We often detected reversion even in visual phase-contrast microscopy and microphotographs of L-colonies selected from the test cultures. In the reversion of the L-forms of streptococcus the presence of large masses of granules reminiscent of cocci (Figure 5) was often observed. When such colonies were seeded onto sugar or seral broth, after one-two days of

storage in the thermostat we usually observed the typical bottom growth of crumbling consistency comprised of minute ball clusters, small chain-like extensions, paired and solitary cocci. A sign of L-form reversion of Proteus was usually the appearance in the L-colonies along with spherical coma-granular elements and light-refracting bodies of long threads, often partly segmented. When such cultures were reseeded into simple or seral broth cultures developed comprised of typical motile bacilli.

Revertants of streptococcal L-forms appeared as round or slightly elongated gram-positive cocci forming chainlets. Colonies on blood agar were small, delicate, greyish, without a hemolysis zone. Streptococcal revertants broke down to the acid level sugars such as glucose, maltose, mannite, saccharose, and to a lesser extent -- lactose, and did not form O-streptolysin.

Revertants of Proteus vulgaris L-forms were motile gram-negative bacilli. When they were seeded into broth and on agar growth characteristic of H-forms was observed. Upon seeding into condensed water on leveled agar they quickly made it turbid and covered the entire agar surface. Revertants exhibited high saccharolytic and proteolytic fermentive activity. They broke down to acid and gas glucose, and slowly and less intensively (by the 7-14thday) to acid or acid and gas -- saccharose and maltose, they did not decompose mannite and lactose, they diluted gelatin, formed hydrogen sulfide and did not form indole. It must be noted that the original Proteus strain fermented (to acid and gas) only glucose and was inactive toward saccharose and maltose.

Conclusions

1. Stable L-forms of streptococcus, typhus abdominales bacteria, and Proteus vulgaris retained their viability under conditions of being stored without reculturing in a semi-fluid culture for six months at 4 degrees (most favorable conditions), 20 and 37 degrees (least favorable conditions). Changes in several L-forms under the effect of different temperature conditions were similar. The "yield" of the L-form subcultures depended on temperature conditions and in part on the duration of storage of the original L-forms.

2. Granular elements of L-cultures proved capable of reproduction, and small, slow-growing L-colonies consisting of similar elements /to the granular elements/ (type 3A) were observed in the subculture. During the storage of L-cultures the capacity to revert was detected at all temperature conditions chiefly for the 2-4 month period, a longer (6 month) storage under these conditions was accompanied by gradual loss

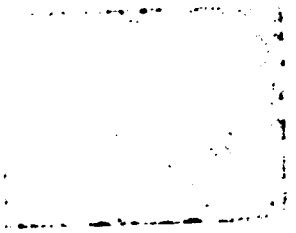

Figure 1. Str. haemolyticus No. 409. Microstructure of L-colonies of original culture, spheres of different sizes. Magnification: 1450.

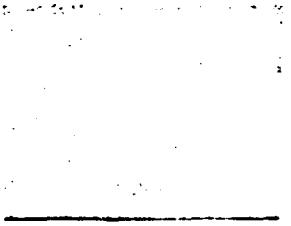

Figure 2. S. typhosa No. 152. Granular masses and spherical bodies with granules along periphery. Magnification: 1450.

Figure 3. Str. haemolyticus No. 1190. Submicroscopic granular elements, small spheres, light-reflecting bodies. Magnification: 1450

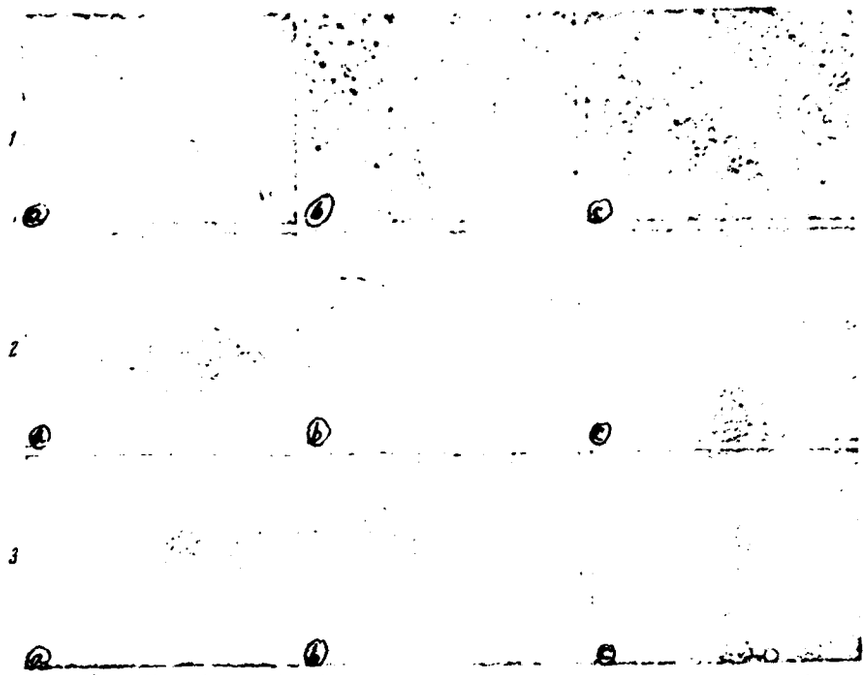

Figure 4. Morphological changes in L-colonies of strain No 409. 1 - Storage at 4 degrees for 2 (A), 4 (B), and 6 (C) months; 2 - Storage at 20 degrees for 2 (A), 4 (B), and 6 (C) months; 3 - Storage at 37 degrees for 2 (a), 4 (B), and 6 (C) months. Magnification: 1450.

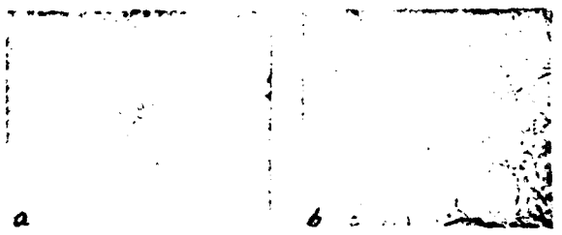

Figure 5. Progress of the reversion. a - Str. haemolyticus No 196 (formation of granular forms, stages preceeding coccal formation); b - Proteus vulgaris (long tortuous threads, segmentation of long forms, budding of bacilli). Magnification: 1450.

of the capacity to revert with retention of the capacity to be cultivated in the L-form. This fact is in all probability related to the high resistance to the effect of these temperature conditions of type 3A colonies capable of passaging in the L-forms and not capable of reverting in the bacterial form.

3. The revertants obtained were similar to the original bacterial strains and were characterized by high enzymatic activity. One of the revertants (Proteus) showed additional fermentive properties untypical for the original parental strain but similar to the properties of the L-form.

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