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Camp Detrick, Md.
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SPECIAL REPORT # 56

Reviewed By HTE
Date 7 Mar 60

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STUDIES ON THE DEVELOPMENT OF BIOLOGICAL DEFENSE AGAINST "UL"* (U)

(With a discussion of its status as a potential BW Agent)

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*Immunization Against Tularemia (See appendix VI-A)

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I INTRODUCTION

A **Object:**

To study the growth and characteristics of "UL" in order to determine the suitability of this organism as a potential BW agent and specifically to develop protective measures for defensive use.

B **Authority:**

The project was approved by the Chief of the Chemical Warfare Service 20 October 1943 and the project specifications forwarded to the Technical Director, Camp Detrick by letter of transmittal dated 3 November 1943. A copy of the original project specifications is included in Appendix 1. Specific authorization was given by the Technical Staff, Camp Detrick by letter to the Chief, B Division, dated 10 November 1943.

C **Historical:**

At a meeting in Dr. E.B. Fred's office at the National Academy of Sciences in Washington, D.C. on 1 September 1943(1) the potentialities of "UL" as a BW agent were discussed. The meeting was attended by Dr. E.B. Fred; Dr. W. Mansfield Clark; Dr. I.L. Baldwin; Dr. N. Paul Hudson; Dr. Gail M. Dack; Dr. Cora M. Downs; Dr. Lee Foshay; Dr. W. S. Nungester; Dr. Kenneth Goodner; Dr. Allen Abrams, OSS; Adm. R. E. Dyer, N.I.H.; Cmdr. L. D. Fothergill, USNR; Col. Martin B. Chittick, CWS; Lt. Col. O.C. Woolpert, CWS; Lt. Col. A. T. Thompson, CWS; and Lt. Max Bovarnick, CWS. A decision was made to have Dr. Foshay carry on investigative work under a WRS contract and as soon as it could be cleared to start additional studies at Camp Detrick. The suggested tentative division of work between Cincinnati and Camp Detrick was for Dr. Foshay to study stability, storage, and length of survival of "UL" under various environmental conditions with particular emphasis on conditions which might be expected to occur in bacterial warfare (B.W.) Dr. Foshay was also requested to seek to improve his vaccine. The studies at Camp Detrick were to be directed toward accumulation of data concerning all other phases of BW with particular emphasis on protective measures and development of an effective vaccine.

Room 8, Building D-4 was remodeled during November 1943 and in December "UL" work was initiated with Lt. L. L. Coriell, Dr. C. M. Downs and Pvt. Ruth Dorion all giving part time to "UL" studies while assisting with the "X" project, which was deemed very urgent at the time. In January 1944 Lt. T. L. Snyder of the O Division began studies on development of a suitable liquid medium and Lt. G. B. Pinchot joined the B Division workers. Subsequent expansion of the personnel and space assigned to this project is shown in Appendix B. By January 1945 sufficient new information had been accumulated to indicate that "UL" showed promise as a BW agent and in order to concentrate effort on the few remaining problems the project was moved to more spacious quarters and the O Division group was expanded.

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In March 1944 Dr. Downs returned to the University of Kansas where she continued "UL" studies in close cooperation with the Camp Detrick project under Contract # W-18-064-CWS-43.[2] She made frequent trips to Camp Detrick for consultation and spent a total of 7 months out of the next 19 months in the Camp Detrick laboratories. Correlation of the Camp Detrick and Kansas studies with Dr. Foshay's unit was also maintained by frequent consultation either at Camp Detrick or at Cincinnati.

D Status of Problem:

When the investigation of "UL" as a BW agent was begun at Camp Detrick the following facts were known: "UL" causes an infectious, septicemic, plague-like disease of wild rodents which is uniformly fatal to mice, guinea pigs and rabbits under experimental laboratory conditions. In nature it is transmitted from animal to animal by the bites of flies, lice, fleas, and ticks.

Man is usually infected while skinning wild rabbits, squirrels, gophers or other rodents. Other cases are traceable to wood tick or deer-fly bites. The incidence of infection in laboratory workers is very nearly 100 per cent. In man the disease is an acute febrile illness accompanied by chills, lymphadenopathy, prostration, headache, sweating, and sometimes a cutaneous rash. Local ulceration at the point of entry is common as is also bubo formation in the regional lymph nodes. The acute stage lasts about three weeks, while convalescence frequently takes 2 to 4 months. The mortality is about 5 per cent. Recovered cases are immune and in most of these specific agglutinins are demonstrable in the blood stream for many years. Authoritative reviews of the clinical disease and modes of transmission have been published by Francis[3] and Foshay[4].

Vaccination was first carried out by Foshay [5]. The vaccine used was a suspension of "UL" grown on solid medium and treated with nitrous acid, washed, neutralized, and resuspended in 0.1% formalinized saline. This vaccine had been given a clinical trial in man with encouraging results, but it failed to protect the highly susceptible guinea pig, rabbit or mouse[5].

Vaccination in man stimulated production of specific serum agglutins, the average titer being 1:320. Many persons became sensitized after vaccination, so that subsequent injections resulted in local or systemic reactions. Evaluation of the efficacy of the vaccine in man was difficult because it was not possible to know the degree of exposure in the vaccinated series. Foshay has developed a new vaccine (30) which is essentially a phenolized suspension of a culture of organisms grown in a simple gelatine hydrolysate medium.[6] This vaccine was made available by Dr. Foshay for vaccination of the personnel at Camp Detrick.

Growth of "UL" in the Laboratory has presented considerable difficulty in the past. In order to initiate growth on most media a relatively large inoculum was necessary. The presence of cysteine and some enrichment such as blood or egg yolk also seemed necessary. The usual media were

those described by McCoy and Chapin[7] and Francis.[8,9] In 1941 Berkman and Koser[10] and in 1942 Tamura and Gibby[6] described a liquid medium in which "UL" grew abundantly. The development of additional new and simple media seemed desirable.

Since "UL" did not grow well unless inoculated heavily on cystine blood agar, plate counts were not usually made and it was customary to match a suspension of organisms against a Fullers earth turbidity standard and estimate the numbers of organisms by determin tion of the mouse LD_{50}.

On the basis of clinical and experimental evidence available, "UL" was considered to offer promise as a potential BW agent. Two of the factors contributing to this view were: (1) the high infectivity of the organism as indicated by the frequency of laboratory infections, and (2) the multiplicity of portals of entry as attested by known cases of invasion via the skin and conjunctiva as well as by the gastro-intestinal and respiratory tracts.[11] The potentialities of "UL" as a BW agent are fully discussed in the Rosebury Report, 1942.[12] Intelligence reports to the effect that the Germans and Japanese were interested in "UL" as a possible BW weapon added to the interest in the development of defensive measures.

The following criteria essential for a good BW agent were laid down in the report by Downs, B Division, October 1943.[13]

1. It must be possible to cultivate the agent in large quantity and with relative ease.

2. The agent must be highly infective by more than one portal of entry.

3. The agent must survive under conditions of dispersion.

4. It should be possible to protect friendly troops against the agent by biological means.

5. Specific biological or chemotherapeutic methods of treatment should be available.

6. The agent should be difficult to detect.

A research program was planned by the "UL" group to answer the questions implied in the above criteria. The experiments herein reported follow the order suggested by these criteria.

II METHODS

Strains of "UL" employed in this study were obtained from Dr. Lee Foshay at the University of Cincinnati Medical School, from Dr. C. M. Downs at the University of Kansas, and two strains were isolated at Camp Detrick. Table I shows the designation, year of isolation, pathological source and virulence of the strains as determined here. "UL" strains are

usually designated by the source of the original isolation of the culture and no specific immunological or cultural distinction is implied. In referring to cultures the following abbreviations were frequently used: SM1 meaning strain S, mouse passage 1; S-M14, strain S, mouse passage 14; CYS-30, strain C, yolk sac passage 30; SME 10, strain S, chick membrane passage 10.

Cultures were routinely maintained on dextrose cysteine blood agar (DCBA) slants and transferred every three weeks with interval storage in the ice-box at 6° C. Some strains were lyophilized and stored dry. Virulence was maintained by mouse or rabbit passage at intervals of two to three months.

The media employed routinely were dextrose cysteine blood agar (DCBA) and Snyder's liquid and Snyder's solid medium [14]

In the early part of the work herein reported, initial suspensions of "UL" were prepared from solid or liquid media and standardized by comparison with a Fullers earth standard of 500 parts per million (referred to as T500 turbidity). Later a Coleman Spectrophotometer was used for this purpose. The arbitrary standard suspension adopted was 40% transmission at a wave length of 650 millimicrons. This standard was referred to as T40 and usually contained 1 to 2 billion, viable organisms per ml. The T500 suspension also contained an average of approximately 1 to 2 billion organisms per ml but replicate preparations varied between rather wide limits.

Unless otherwise designated the minimal lethal dose for mice was determined by the intraperitoneal injection of 0.5 ml of serial ten fold dilutions of a 24 hour saline suspension having a T500 or T40 turbidity. The mouse 50 per cent endpoint was expressed as LD_{50} determined by the method of Reed and Muench.[15] The LD_{50} was expressed as the dilution of a standard "UL" suspension one ml of which killed 50 per cent of the animals. In similar titrations on rats, hamsters, dogs, guinea pigs, rabbits, cotton rats, and monkeys an inoculum volume of 1 ml was employed.

Untreated mice infected with "UL" generally died within 6 days. In each experiment a representative number of mice dying during this period were autopsied and the hearts blood and spleen cultured on DCBA. All mice dying after the sixth day were autopsied. Mice that survived 10 days were considered non-infected.

III EXPERIMENTAL

A Media

1. Cultivation on Artificial Media

None of the media previously employed for the cultivation of "UL" were entirely satisfactory from the BW point of view. The best liquid medium available was that developed by Gibby and Tamura,[6] using gelatin

Histories of Strains Isolated, 1944-45 Winter

Camp — Isolated by direct culture of pus aspirated from a bulbous primary lesion on finger tip of Mrs. Campbell, patient of Lt. Col. Thomas Rankin, at Fort Thomas, Ky., on December 27, 1944, by L. F., on the tenth day of disease.

Carr — Isolated from primary lesion of Ancil Carr, via mouse passage, on Dec. 3, 1944, the 8th day of disease.

Coll — Isolated via mouse passage from digital primary lesion of Mary Collins, on Jan. 6, 1945, approximately the 17th day of disease.

Dieck — Isolated from pus from axillary lymph node excised from Clem Dieckman, via mouse passage, on Dec. 27, 1944, the 10th day of disease.

Holt — Isolated from pleural fluid of T. J. Holton, Covington, Ky., via mouse passage, on Dec. 12, 1944, during second week of disease.

Scherm — Isolated from digital primary lesion of Carrie Schermbeck, via mouse passage, on Dec. 26, 1944, the 3rd day of disease.

Strains of Low Virulence

Culture	Year of Isolation	Pathologic Source	Geographic Source	Years Since Last Animal Passage
Jap	1926	Human lymph node	Japan	19
Ohara	1931	Unspecified	Japan	14
Russ	1928	Lymph node	Russia	17
Max	1928	Lymph node	Russia	17 ✓
HD	1935	Human eye	Austria	10
26	1921	Human blood	Utah	24
Pier	1938	Human blood	Ohio	1½

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TABLE I

"UL" STRAINS USED AT CAMP DETRICK

Designation of Culture	Year of original isolation	Pathologic source	Geographic source	Isolated by.	Virulence Mouse LD_{50} $
NIH-38	1920	Human lymph node	Utah	Francis	~~100~~
26	1921	Human blood	Utah	"	$10^{-1.0}$
Jap	1926	Human lymph node	Japan	Foshay[1]	$10^{-4.4}$
✓ LR	1927	Human ascitic fluid	Arkansas	Foshay#	
Russ	1928	Human lymph node	Russia	Francis[2]	$10^{-2.0}$
Max	1928	Human lymph node	Russia	"	$10^{-1.7}$
Ohara	1931	Unspecified	Japan	"	$10^{-1.0}$
✓ Ri	1932	Human pus	Virginia	Francis	
✓ Ll	1934	Human pus	Canada	"	
✓ HD	1935	Human eye	Austria	Francis**	$10^{-4.0}$
Fields	1936	Human lung	Ohio	Foshay	
✓ ~~Cor~~ Church ~~or O FO 38~~	1941	Human lung	Ohio	" *	$10^{-8.6}$
Chris	1937	Human pus	Ohio	"	
✓ Stoll	1937	Human blood	Ohio	"	
✓ DePue	1938	Human lymph node	Ohio	"	

\# Obtained by Dr. Foshay from E. Francis who obtained the culture from M. J. Kilbury.

** Isolated by Hans David who gave the culture to Dr. Francis.

* Isolated by R. H. Broh-Kahn from lung at autopoSy at Jewish Hospital Cincinnati, Ohio.

TABLE I (cont.)

"UL" STRAINS USED AT CAMP DETRICK (cont.)

Designation of culture	Year of original isolation	Pathologic source	Geographic source	Isolated by	Virulence Mouse LD_{50} *
Die	1938	Human ulcer	California	Francis	
Pier	1938	Human blood	Ohio	Foshay	$10^{-1.0}$
Fox	1940	Human pleural fluid	Ohio	Hesselbrock	
Hugh	1940	Human ulcer	Ohio	Foshay	
Gib	1941	Primary lesion	"	"	
S or Schu	1941	Human ulcer	"	"	$10^{-9.5}$
Camp	1944	Human lesion	Kentucky	"	$10^{-9.5}$
Carr	1944	Human lesion	USA	"	$10^{-9.5}$
Dieck	1944	Human lymph node	USA	"	$10^{-9.6}$
Holt	1944	Human pleural fluid	Kentucky	"	$10^{-10.6}$
Ince	1944	Human ulcer	Kansas	Downs	$10^{-9.5}$
Scherm	1944	Human lesion	USA	Foshay	$10^{-9.2}$
Coll	1945	Human lesion	USA	"	$10^{-8.8}$
Schad	1945	Human lesion	Maryland	Coriell	
Clapp	1945	Human sputum	Maryland	Schabel[3]	

* Expressed as the dilution (of a T-40 "UL" suspension) 0.5 ml of which killed fifty per cent of the mice.

1 Personal communication from Dr. Foshay.

2 Obtained from Dr. Foshay who received the cultures from Dr. Edw. Francis.

3 Personal communication from Lt. Schabel, Camp Detrick.

Subcultures of the Francis strains were obtained from Dr. Foshay.

TABLE II

Studies on Media

Liquid, Tissue Media.

1. Chick embryonic extract in Tyrode's solution.
2. Chick embryonic extract with tissue fragments in Tyrode's solution.
3. Embryonic chick blood in Tyrode's solution.
4. Chick embryonic extract plus cystine.
5. Embryonic mouse brain in Simms' solution.
6. Embryonic mouse tissue in Simms' solution.
7. Serum ultrafiltrate.
8. Serum ultrafiltrate plus embryonic mouse tissue.
9. Red blood cell extract (Foshay).

Solid Media. The following substances were added to plain nutrient agar.

1. Potato extract, 1 to 10%.
2. Carrot extract, 1 to 10%.
3. Brewer's yeast extract, 1%.
4. Saline extract of Virulent "UL".
5. Liver extract, 1 to 5%.
6. Embryonic mouse brain in Simms' solution.
7. Embryonic mouse tissue in Simms' solution.
8. Serum ultrafiltrate.
9. Red blood cell extract (Foshay).
10. Serum, (rabbit).

hydrolysate and red blood cell extract. The most satisfactory solid medium was dextrose cystine blood agar and it had the disadvantage of requiring a heavy inoculum in initiate growth.

At the outset two problems were investigated at Camp Detrick: (1) the development of a simple, cheap liquid medium for mass cultivation, (2) the development of a solid medium which could be used for quantitative plate counts.

Details of the preparation of a satisfactory simple liquid medium may be found in Lt. Snyder's Report.[14]

Table II lists the media and enrichments studied in an effort to find conditions which would support abundant growth and perhaps be applicable to quantitative work. None of the media listed in Table II have results sufficiently favorable to justify their substitution for the usual dextrose cystine blood agar.

A method of counting surface colonies was developed as follows, using virulent strains S and C. Fresh dextrose cystine blood agar plates were used. A suspension of organisms was matched to a T500 turbidity and tenfold serial dilutions were made. One tenth ml of these dilutions was put in the center of the plates and spread gently over the surface with a sterile bent glass rod. After drying the plates were inverted and incubated at 37° C for 48, 72, and 96 hours until maximum growth had taken place. Comparative studies were made to determine the conditions of maximum growth and the limits of accuracy of the methods.

When cysteine hydrochloride was added to the basic dextrose blood agar the counts were higher than when cystine was added. Table III shows the results of a comparison of the two media.

TABLE III

Comparative Counts of "UL" on Solid Media

Dilution plated	Dextrose Blood Agar plus 0.1% Cystine					Dextrose Blood Agar plus 0.1% Cysteine HCl				
	Days Incubation									
	2	3	4	6	10	2	3	4	6	10
10^{-2}	*4+	4+	4+	4+	4+	3+	4+	4+	4+	4+
10^{-3}	0	0	0	0	0	2+	3+	4+	4+	4+
10^{-4}	0	0	0	0	0	0	3+	4+	4+	4+
10^{-5}	0	0	0	0	0	0	1+	2+	2+	2+
10^{-6}	0	0	0	0	0	0	0	171	235	240

* Colonies too numerous to count accurately; 1+ to 4+ indicates increasing number of colonies; 0 indicates no colonies.

Using dextrose cysteine blood agar (DCBA), numerous variations were tried affecting the physical conditions of growth, as follows:

(a) Increased CO_2 tension was obtained by incubating plates in a sealed dessicator in which part of the oxygen was replaced by tank CO_2. No improvement of growth was noted over similar plates incubated in air.

(b) Poured DCBA plates of "UL" failed to show growth.

(c) Freshly poured moist plates and plates kept moist by sealing with parafilm did not support growth as well as plates which were dried by overnight incubation at 37° C before inoculation.

(d) Heating DCBA for 15 minutes at temperatures of 60, 70, 85, 100 and 120° C did not completely destroy its ability to support growth of "UL". In every case the growth was not as good as in unheated medium.

(e) Comparative growth on DCBA plates adjusted to different pH values and inoculated with the same "UL" dilution is shown below:

Initial pH of Agar before Autoclaving	Final pH in the Petri Dish	48 Hr. Growth of "UL"
7.8	7.3	*2+
7.2	6.95	10+
6.7	6.6	4+

These results and others shown in Tables IVa and IVb demonstrated that for maximum rapid growth, "UL" tolerated only a narrow pH range of about 6.7 to 7.2. A slight increase in growth occurred if the cysteine was added after autoclaving the medium as shown by the following:

		48 Hr. Growth
a.	Cysteine HCl added before autoclaving the medium; final pH 6.85	6+*
b.	Autoclaved cysteine HCl added after autoclaving the medium; pH adjusted to 6.85 with sterile normal NaOH:	7+
c.	Seitz filtered cysteine HCl added after autoclaving with medium; pH adjusted to 6.85 with sterile normal NaOH:	8+

* The colony count and rapidity of growth on plates expressed as a scale from 1+ to 10+. 10+ growth means maximum number of colonies appearing after 36 hrs. incubation. 1+ indicates a smaller number of colonies which did not reach their maximum number until 4 or 5 days of incubation.

The slight increase in growth obtained on adding cysteine after autoclaving the medium was considered insufficient to compensate for the additional time required for this manipulation.

For quantitative estimation of organisms in experimental work it was necessary to develop a medium which would support rapid growth of "UL" and at the same time be simple to prepare in a reproducible form, preferably from ingredients found in any bacteriological laboratory. Such a study was made by comparing DCBA with Snyder's medium over a pH range of 6.4 to 7.6. Both media were studied (1) without blood, (2) with blood added, (3) with 0.1% Wilson liver concentrate added, (4) with blood and liver added. The results are shown in Table IVa.

Table IVb shows a similar comparison of the two media made with Bacto peptone and with Bacto proteose peptone. This table also compares the effect of adjusting the pH with disodium phosphate buffers instead of NaOH solution. Conclusions regarding the optimal conditions for growth of "UL" as shown in Tables IVa and IVb were as follows:

1. Final pH of 6.7 to 7.2. Optimum: 6.9.
2. DCBA was superior to all other media tested.
3. 2.5% dextrose was superior to 1.25% in DCBA.
4. Growth was distinctly poorer when Na_2HPO_4 was used in place of NaOH to adjust pH.
5. Addition of liver concentrate stimulated growth in Snyder's medium but required a high initial pH before autoclaving.
6. Liver could not replace blood in DCBA, and interfered with growth when added to the whole medium.
7. Best growth in Snyder's medium was obtained with blood and liver at initial pH 7.4 or 7.6.
8. Bacto peptone was somewhat better than proteose peptone in DCBA.
9. Optimum conditions indicated by these findings were: DCBA with 2.5% dextrose at pH 6.9, made with Bacto peptone, titrated with NaOH.

In evaluating the above results rapidity of growth as well as total colony count were considered. On DCBA maximum colony counts were obtained within 48 hours incubation after which no increase in colonies was observed. On Snyder's medium growth was slower and maximum colony counts appeared after 3 to 5 days incubation of plates.

The final method of making up DCBA embodied all the information described above. This medium was used throughout the rest of the work

TABLE IV (a)

COMPARISON OF GROWTH OF "UL" ON DCBA AND ON SNYDER'S PLATE MEDIUM

MEDIA (1)	INITIAL pH*: 6.4		6.6		6.8		7.0		7.2		7.4		7.6	
	pH** AUTO-CLAVED	GWTH (2)	pH AUTO-CLAVED	GWTH	pH AUTO-CLAVED	GWTH	pH AUTO-CLAVED	GWTH	pH AUTO-CLAVED	GWTH	pH AUTO-CLAVED	GWTH	pH AUTO-CLAVED	GWTH
DCBA WITHOUT BLOOD	6.38	-	6.6	-	6.72	-	6.90	-	6.88	-	6.95	-	7.1	-
DCBA (2.5% DEXTROSE)	6.5	-	6.6	2+	6.72	7+	6.85	9+	6.95	5+	7.12	7+	7.2	6+
DCBA (1.25% DEXTROSE)	6.4	-	6.6	-	6.7	4+	6.8	6+	6.92	5+	6.85	5+	7.0	-
DCBA+(LIVER(3)	6.05	-	6.2	-	6.3	-	6.45	-	6.75	+	6.85	+	7.05	2+
DCBA+(LIVER(3)BLOOD)	6.2	-	6.3	-	6.3	-	6.4	-	6.58	3+	6.6	4+	6.82	5+
S (4)	6.45	-	6.65	-	6.82	+	7.10	+	7.20	-	7.40	-	7.56	-
S + (BLOOD) (4)	6.43	-	6.70	+	6.80	5+	7.02	5+	7.19	5+	7.35	5+	7.4	-
S + (BLOOD)+(LIVER)(3)	6.20	-	6.50	-	6.75	3+	6.90	6+	6.92	7+	7.05	8+	7.08	8+

(1) ALL MEDIA WERE MADE WITH BACTO PROTEOSE PEPTONE.
(2) ALL PLATINGS DONE IN TRIPLICATE. READINGS AFTER 40-48 HOURS INCUBATION, EXPRESSED ON BASIS OF 1+ TO 10+ SCALE. - MEANS NO GROWTH.
(3) LIVER CONCENTRATE (WILSON) ADDED TO FINAL CONCENTRATION OF 0.1%.
(4) S = SNYDER'S 2% PEPTONE PLATE MEDIUM

* - pH OF MEDIUM BEFORE AUTOCLAVING
** - pH OF MEDIUM IN THE PETRI DISH READY FOR USE.

TABLE IV (b)

EFFECT OF pH, PROTEOSE, OR BACTO PEPTONE AND PHOSPHATE BUFFERS ON THE GROWTH OF "UL"

MEDIA	INITIAL pH* 6.6: pH** AUTO-CLAVED	6.6: GROWTH (1)	6.8: pH AUTO-CLAVED	6.8: GROWTH	7.0: pH AUTO-CLAVED	7.0: GROWTH	7.2: pH AUTO-CLAVED	7.2: GROWTH	7.4: pH AUTO-CLAVED	7.4: GROWTH	7.6: pH AUTO-CLAVED	7.6: GROWTH
DCBA (BACTO)(NaOH)	6.62	4+	6.80	8+	6.80	9+	6.92	8+	7.00	3+	7.02	-
DCBA(BACTO)(PHOSPHATE(2) BUFFERED)	6.40	-	6.55	-	6.72	-	6.92	+	7.15	-	7.38	-
DCBA(PROTEOSE)(NaOH)	6.60	3+	6.78	6+	6.80	7+	6.90	8+	7.02	+	7.12	-
DCBA(PROTEOSE)(PHOSPHATE BUFFERED)	6.40	-	6.60	-	6.75	-	6.75	-	7.18	-	7.48	-
DCBA(PROTEOSE)+(LIVER(3) NaOH)	6.25	-	6.32	-	6.45	-	6.45	+	6.70	5+	6.81	4+
DCBA(PROTEOSE)+(LIVER) (PHOSPHATE BUFFERED)	6.25	-	6.45	-	6.65	+	6.65	+	7.10	+	7.40	-
S-(BACTO)-(NaOH) (4)	6.68	-	6.88	-	7.05	-	7.05	+	7.38	-	7.58	-
S-(BACTO)(PO_4)	6.48	-	6.72	-	6.82	-	6.82	-	7.42	-	7.62	-
S+BLOOD-(BACTO)(NaOH) + (Liver)	6.32	-	6.40	-	6.50	-	6.50	-	6.90	3+	7.09	3+
S+BLOOD(BACTO)(PHOSPHATE BUFFERED)+(LIVER)	6.28	-	6.40	-	6.73	-	6.73	-	7.30	-	7.50	-

(1) ALL READINGS MADE AFTER 40-48 HOURS INCUBATION AND EXPRESSED ON BASIS OF A 1+ TO 10+ SCALE. ALL PLATINGS DONE IN TRIPLICATE. - MEANS NO GROWTH.
(2) ENOUGH DISODIUM PHOSPHATE ADDED TO BRING TO PROPER pH.
(3) LIVER CONCENTRATE (WILSON) ADDED IN CONCENTRATION OF 0.1%.
(4) S = SNYDER'S 2% PEPTONE MEDIUM.
* - pH OF MEDIUM BEFORE AUTOCLAVING. ** - pH OF MEDIUM IN PETRI DISH READY FOR USE.

wherever plating technics were employed. The method of preparation is given below:

Prepare a solution of double strength meat extract broth (0.6% meat extract, 2% Bacto-peptone, 1.0% NaCl) and a solution of 3% agar in water. These two solutions may be auto-claved and kept tightly stoppered or they may be used immediately. With the broth at a temperature of 30° C or less add 0.1% cysteine hydrochloride and adjust the pH to 7.1 with N/1 NaOH. The pH should be carefully checked on the Beckman potentiometer. The freshly autoclaved hot agar solution is then added to and mixed with an equal volume of double strength broth. The warm single strength cysteine agar is then placed in screw capped bottles containing 100 ml each. The bottles are autoclaved at 15 lb. for 20 minutes and then cooled to 45° C in a water bath; 3-5% of fresh sterile, defibrinated rabbit or human blood and 2.5% sterile dextrose solution are added to each bottle of melted agar before pouring. The plates are incubated overnight at 37° C and should be uniformly bright red and freee from surface moisture. They may be stored in an ice-box and used for a week after preparation. These plates are inoculated as previously described and incubated at 37° C. Maximum growth of "UL" should occur within 48 hours.

Nine virulent strains tested have given satisfactory plate counts on this medium. Eight strains of reduced virulence grew more slowly and plate counts were lower, irregular, and were not reproducible. Air contaminants grew rapidly on this medium, therefore it was necessary to observe good bacteriological technic when pouring and inoculating DCBA plates. A full 20 ml of agar should be poured into each plate to prevent excessive drying during the incubation period. After cysteine has been added, preparation of the medium should be completed and plates poured because the finished plates can be stored in the cold without loss of their growth promoting ability. If the plain cysteine agar is stored in screw capped bottles for several days it becomes discolored near the top and when subsequently remelted and made into plates growth of "UL" is usually poor. If the agar is too warm when the blood is added the resulting chocolate agar does not support maximum growth of "UL". Oxalated blood cannot be used and citrated blood is not desirable. However, packed red cells from citrated human blood have been found very satisfactory. Disregard of these technicalities may often entail experimental errors of considerable magnitude.

2. Quantitative Methods of Counting Viable "UL" Organisms

Comparison of the sensitivity of the DCBA plate count and the mouse LD_{50} titration as methods of estimating the number of "UL" organisms in a given "UL" suspension is shown in Tables Va and Vb. The methods of carrying out mouse titrations and plate counts are described in Section II (Methods) of this report. The plate counts are expressed as organisms per ml of the original suspension and the figures shown represent an average of three or four replicate plates. The LD_{50} titration in mice was converted to LD_{50} doses per ml of original suspension as

TABLE V (a)

Statistical Analysis of
DCBA Plate Counts and Mouse LD_{50} Titrations on 20 "UL"
Suspensions in Saline

LD_{50}	LD_{50}/ml x 10^9	Plate Count /ml x 10^9	$\frac{\text{No. Org./ml}}{LD_{50}\text{/ml}}$ = No. Org./LD_{50}
$10^{-8.88}$*	1.52	2.80	1.84
-8.54	.70	.95	1.36
-9.27	3.72	9.50	2.55
-8.00	.20	.278	1.39
-8.75	1.12	.720	.64
-9.62	8.32	8.99	1.08
-9.50	6.30	4.20	.67
-9.66	9.12	2.76	.30
-9.50	6.30	2.03	.32
-9.59	7.92	2.17	.27
-9.50	6.30	4.42	.70
-8.66	.92	1.10	1.20
-9.00	2.00	1.05	.52
-9.61	8.12	1.72	.21
-9.90	15.86	1.42	.09
-9.66	9.12	2.24	.25
-9.00	2.00	.74	.37
-8.71	1.02	2.39	2.34
-9.16	2.88	1.71	.59
-8.83	1.52	1.01	.66
		Mean:	0.867
		Standard Deviation:	0.7115
		Standard Error of Mean:	0.1591

* Expressed as the dilution of a standard suspension 0.5 ml of which killed fifty per cent of the mice.

TABLE V (b)

Statistical Analysis of
DCBA Plate Counts and Mouse LD_{50} Titrations on 20 "UL" Infected
Embryonated Chicken Eggs

LD_{50}	LD_{50}/mlx10^9	Plate Count/ml x10^9	No. Org./ml / LD_{50}/ml = No. Org./LD_{50}
$10^{-9.62}$*	8.32	3.50	.42
-9.70	10.00	5.70	.57
-9.50	6.32	2.09	.33
-7.38	.048	.047	.98
-6.75	.011	.039	3.54
-9.10	2.50	3.79	1.51
-8.80	1.32	2.04	1.55
-8.56	.72	.398	.55
-9.10	2.50	2.191	.88
-7.58	.076	.180	2.37
-8.52	.66	1.65	2.50
-8.21	.32	.336	1.05
-9.87	14.80	3.76	.25
-8.87	1.48	.61	.41
-8.86	1.44	2.59	1.80
-8.75	1.42	.905	.64
-9.14	2.76	.330	.12
-7.74	.118	.066	.56
-7.66	.092	.069	.75
-9.23	3.40	2.80	.82

Mean: 1.08
Standard Deviation: 0.8869
Standard error of Mean: 0.1983

* Expressed as the dilution of a standard suspension 0.5 ml of which killed 50% of the mice.

shown in column 3 of the Tables. The last column shows the number of organisms per LD_{50} dose and was obtained by dividing the number of organisms per ml as determined by plate counts by the number of LD_{50} doses per ml as determined by mouse titration. In all the titrations presented the virulent Schu strain was employed. Preliminary titrations done during the developmental stages of the plate count medium were not included, otherwise there was no selection of data. Table Va shows the results of 20 titrations on "UL" suspensions in saline carried out over a period of one year and Table Vb shows similar data on 20 "UL" infected, minced, embryonated chicken eggs.

The results indicate that when virulent Schu strain was used the DCBA plate count gave an accurate indication of the number of infectious units in a given "UL" suspension. A dose of one organism as determined by the DCBA plate count killed 50% of the mice with a standard error of 0.18.

Snyder's peptone medium[14] modified for plate counting by adding agar and cysteine HCl, may also be used for making plate counts. A comparison of this medium and DCBA medium during cloud chamber studies involving hundreds of plates demonstrated certain advantages of the DCBA medium[16], namely, greater uniformity of different lots, greater stability on storage, and in some cases greater accuracy and higher counts as shown by replicate plates.

Lt. T.L. Snyder[14] had previously shown that static cysteine peptone broth supported growth when inoculated with small number of organisms and incubated without shaking. To compare the growth promoting properties of this peptone medium with DCBA plates the following experiment was carried out.

A T40 saline suspension of a 24 hour culture of strain Schu was prepared and serial decimal dilutions made in gelatin diluent. These dilutions were used for inoculating DCBA plates (7 replicate plates per dilution), for a mouse LD_{50} titration (10 mice per dilution) and for inoculation of peptone broth flasks (10 flasks per dilution). The peptone broth consisted of 2% peptone, 1% N Cl, 0.1% glucose and 0.1% cysteine hydrochloride adjusted to pH 7.0 and dispensed in 25 ml amounts in 250 ml Erlenmeyer flasks. After inoculation the flasks were held static at 37° C for three hours and then continuously shaken. The growth in the flasks was determined by the appearance of turbidity and checked by Gram stain. The plate count was determined by direct counting and the results from the mouse titration were computed by statistical analysis. As a check on the method of Reed and Muench, which was used for the data previously reported, these data were analyzed by the method of Stevens[17] and also by the method of Halvorson and Ziegler.[18]

Results shown in Table VI indicate a very close correlation between the DCBA plate count, the mouse LD_{50} titration and the peptone broth titration. If the DCBA plate count and mouse titration give an accurate count of the number of "UL" organisms, then on the basis of this experiment it may be postulated that an inoculum of one "UL" organism is sufficient to

start growth in Snyder's peptone cysteine broth.

TABLE VI

The Number of "UL" Organisms per ml as Determined by DCBA Plate Counts, Mouse LD_{50} Titration and Peptone Broth Titration.

Technic	Organisms per ml of Original Suspension	
DCBA Plate Count	1.91×10^9	
Mouse LD_{50} Titration	1.04×10^9*	1.08×10^9**
Peptone Broth Titration	1.4×10^9*	1.5×10^9**

* As determined by the Stevens Method of Statistical Analysis.
** As determined by the Halvorson and Ziegler Method of Statistical Analysis.

3. "UL" Growth Curves

Since no investigation of "UL" viability growth curves has been previously reported, it seemed desirable to obtain data on this subject for the following reasons:

To determine whether gelatin hydrolysate of Snyder's peptone medium supported better growth.

To find the optimum time to harvest "UL" for vaccine and fractionation studies.

To study the effect of shaking and over-aeration as well as that of shaking alone.

To determine whether the increase in turbidity and/or plate count was paralleled by an increase in mouse infective units.

To determine conditions of growth in anticipation of plant production.

The two media used were Foshay's gelatin hydrolysate medium and Snyder's peptone broth. The composition of the two media is given below.

a. Snyder's Peptone Broth

Bacto Peptone	20 gms
Glucose	1 gm
NaCl	10 gms
Distilled water to make	1000 ml

b. Gelatin Hydrolysate Medium[6]

NaCl	7.0 gms
Glycerine	5.0 ml
Cystine hydrochloride	0.1 gm
HCl hydrolysate of Gelatin	15.0 gm (Original gelatin)
Hot Water extract of 100 ml packed red blood cells	
Distilled water to make	1000.0 ml

The reaction was adjusted to pH 7.1 with NaOH and checked on the Beckman potentiometer. The media were distributed in six liter Florence flasks, three liters per flask, and sterilized at 10 lb. pressure for twenty minutes. They were then brought to a temperature of 37° C and inoculated with the entire 24 hour growth from two large DCBA slants of strain Schu emulsified in saline. In one instance (Fig. 4) the temperature of the medium was 20° C when inoculated. All flasks were shaken at 70 oscillations per minute with an excursion of 1 3/4 inches. Preliminary results had shown that shaken cultures grew much faster and reached a higher final count than stationary cultures. In some cases over-aeration was accomplished by a continuous exchange of sterile air over the medium at the rate of 5 liters per minute. The incubation temperature was 37° C. At 0, 1, 2, 3, 4, 8, 10, 12, 16, 20, 24, 28, 36, 40, 48, 72, and 168 hour intervals 10 ml samples were withdrawn and decimal dilutions made in physiological saline. The dilutions were used for counts on DCBA plates and for animal inoculations. Turbidity readings were made on a Coleman Spectrophotometer.

The results of three representative experiments are presented in Figures 1, 2, 3, and 4. The numbers of organisms are expressed as logs of the numbers counted per ml. The mouse titrations are expressed as the log of the LD_{50} per ml and the turbidity readings in per cent of light absorption.

In general the form of the growth curves was similar to that of E. coli when grown in a favorable medium.[19]. The lag phase was short when grown at 37° C as shown in Figures 1 and 3. This may be accounted for partially by the large size of the inoculum (4 to 10 million per ml) and by the fact that the 24 hour DCBA slant growth used for inoculum probably contained a large percentage of recently generated organisms. The short lag phase probably also indicates that the media as constituted presented nearly optimal growth conditions for this organism. When the inoculum was added to the medium at 20° C a lag phase of 8 hours was observed followed by the usual 12 hour logarithmic phase.

In four experiments the logarithmic phase lasted for 11 to 12 hours and in one experiment it lasted for 8 hours, (Figure 1, gelatin hydrolysate medium). The stationary phase persisted beyond 48 hours and the phase of decline was rapid after 72 hours (see Figure 3.) If it were desirable for vaccine production, a maximum number of young cells could be obtained by harvesting near the end of the logarithmic phase, 12 to 16 hours after inoculation.

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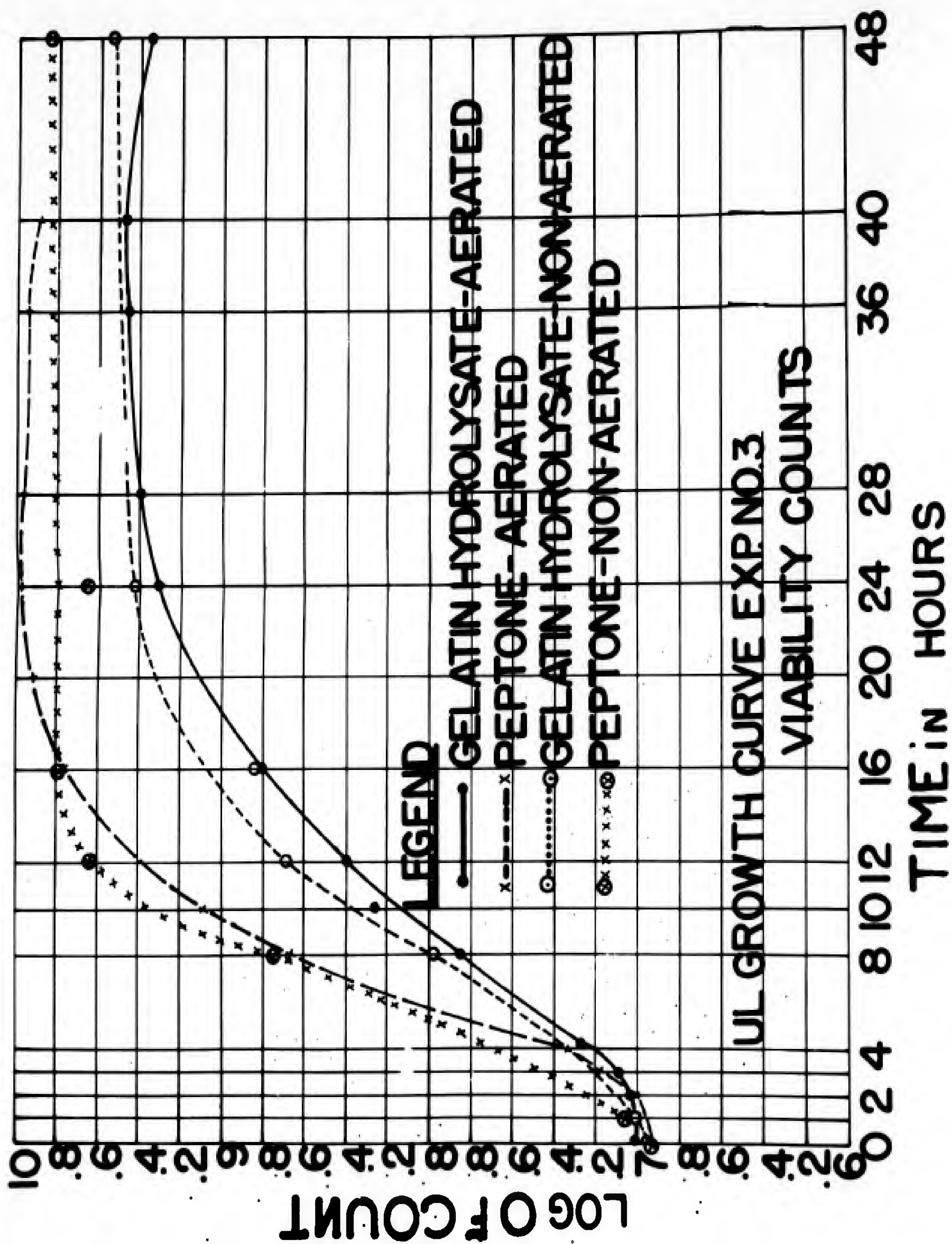

FIG. 1

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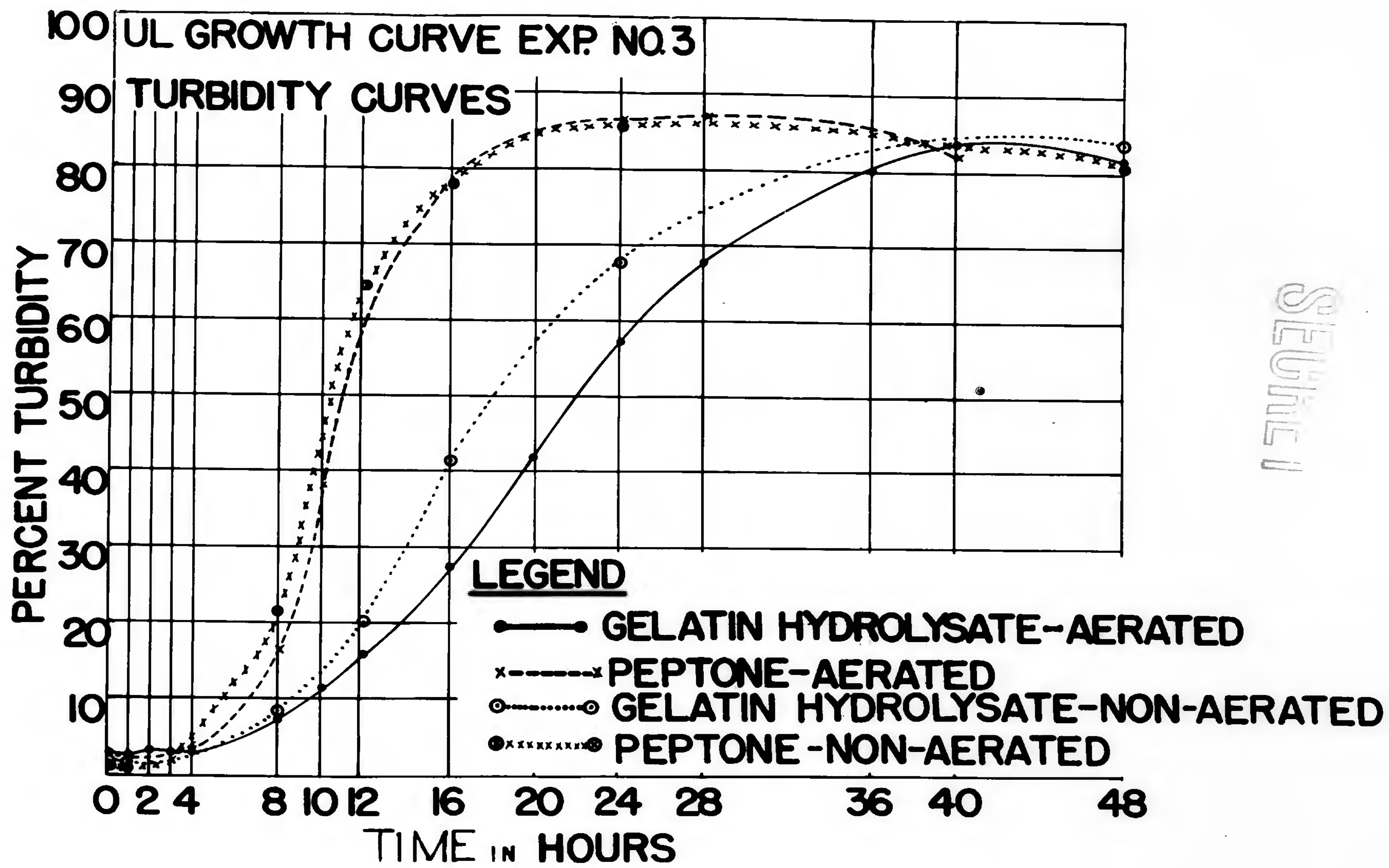

FIG. 2

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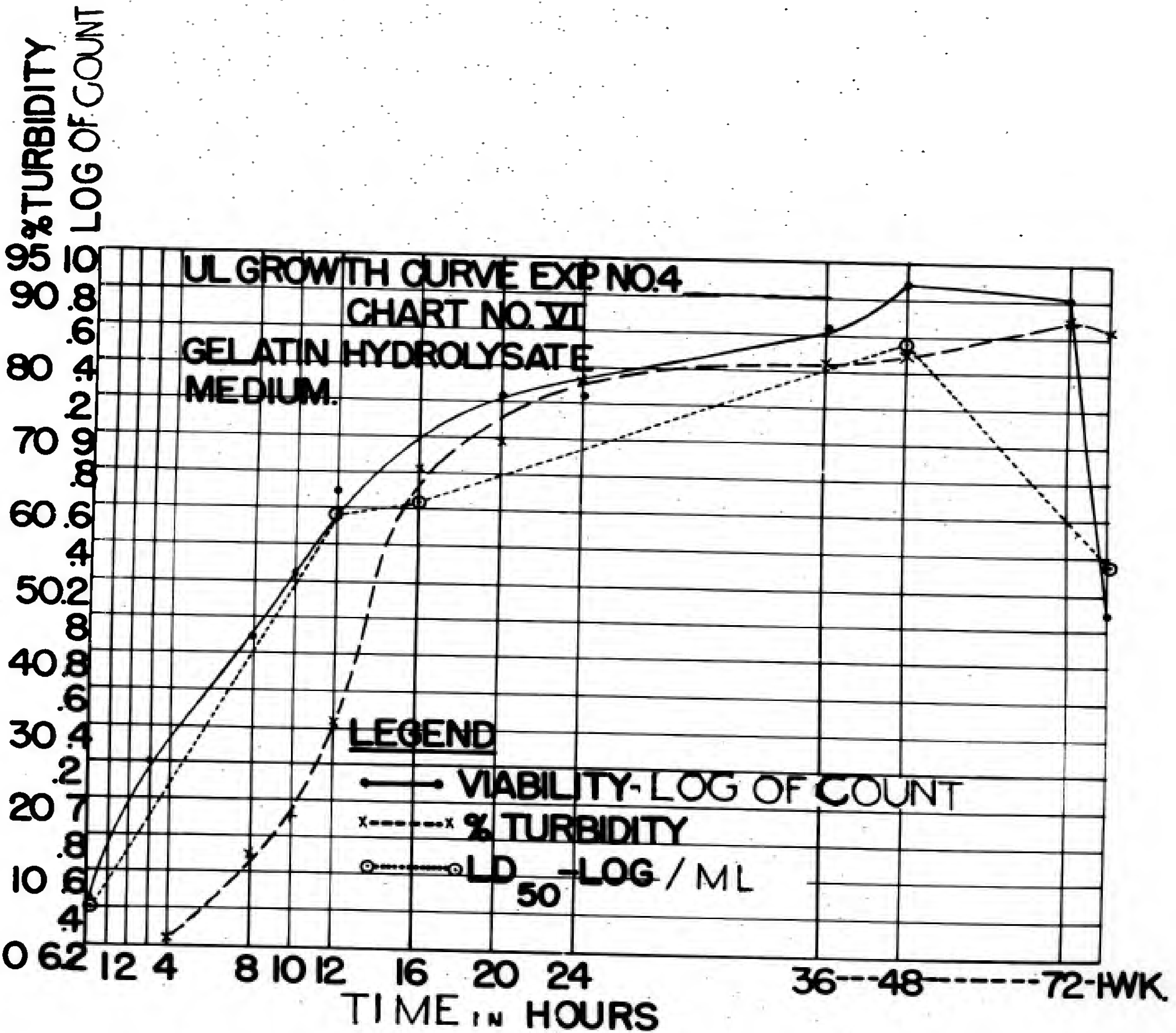

FIG. 3

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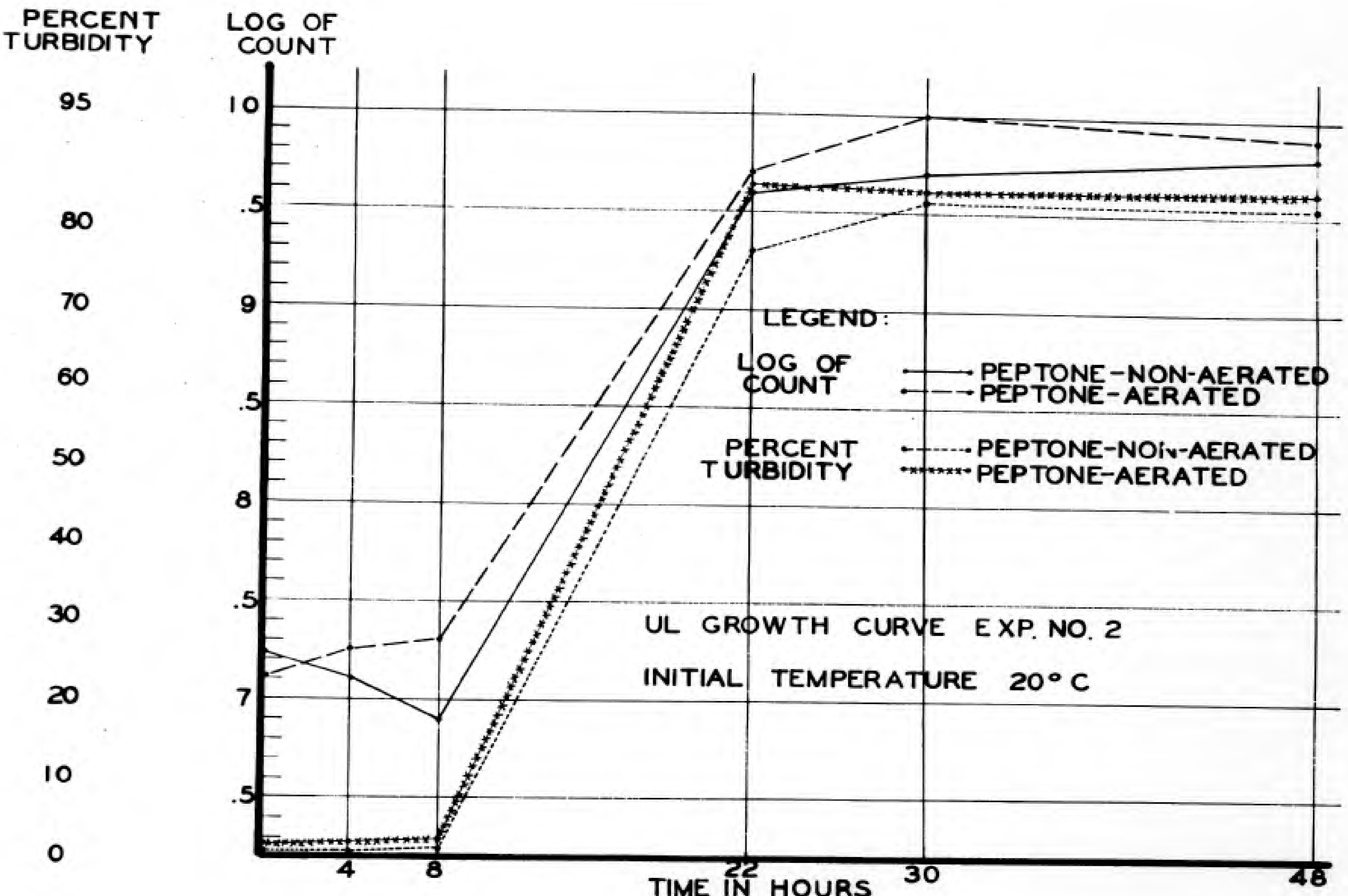

FIGURE 4

Snyder's medium supported better growth than did gelatin hydrolysate medium under the conditions of the experiments. These results were obtained several times and were confirmed using red blood cell extract prepared in Foshay's laboratory. The shape of the growth curves from the two media varied slightly, the main difference being a steeper logarithmic phase exhibited by cultures grown in Snyder's medium.

Over-aeration did not significantly alter the growth curve from that observed in cultures which were shaken only.

As shown in Figure 3 the number of organisms per ml as determined by DCBA plate count and by mouse LD_{50} titration closely paralleled each other throughout all phases of the growth curve. The close correlation in the pase of decline would indicate no loss in virulence of those organisms which remained.

The turbidity curve closely approximated the viability count and the LD_{50} curve; see Figures 2, 3 and 4. During the lag phase and the early hours of the logarithmic phase the turbidity curve was unreliable but near the end of the logarithmic phase and throughout the stationary phase it gave an accurate index of the number of viable cells. Turbidity measurements can be made quickly with a minimum of equipment and trained personnel, and results are available immediately. For these reasons turbidity measurements could probably be adopted to control production and to determine optimum time of harvest in pilot plant production of "UL".

Foshay (98) carried out some growth curve studies in which he compared the following methods of measuring growth of "UL" in gelatin hydrolysate medium: (1) turbidity measured on a reflecting type of photelectric comparator, (2) volume of packed bacteria, and (3) total bacterial nitrogen. He found that turbidity measurements gave results similar to those obtained at Camp Detrick. Bacterial volume and total nitrogen values gave a more accurate index of total bacterial substance, and the values for these two methods agreed fairly closely. Counts of viable cells were not made by Foshay but it is probable that they would have paralled the turbidity curve rather than the bacterial volumeor total nitrogen curve.

B Infectivity of "UL"

A study of the susceptibility of embryonated eggs and of various species of animals to "UL" infection was undertaken as part of the fundamental investigation necessary to the evaluation of "UL" as a BW agent and the development of biological protection against it. It was also desirable to know the LD_{50} for experimental animals as determined by various routes of infection.

1. Studies in Eggs

a. Growth of "UL" in Embryonated Eggs

The growth of "UL" in embryonated eggs, after heavy inoculation, was reported by Buddingh.[20] He observed that the organisms grew in-

tracellularly in the membrane and that death usually took place 72-80 hours after inoculation.

The work reported herein was done with the object of determining (1) the degree of multiplication of "UL" in various tissues of the embryonated eggs; (2) the length of time the organism survived in the harvested egg; and (3) the suitability of the infected embryonated eggs as a source of organisms for the preparation of vaccines.

The eggs to be inoculated were prepared in the usual manner and 0.2 ml of the culture was inoculated by the various routes designated in the tables. The eggs were sealed with collodion-iodine and incubated at 35-37° C for the designated length of time. The various tissues and allantoic fluid were harvested under aseptic conditions and the membranes and yolk sacs were broken up by shaking with sterile beads in a shaking machine. Plate counts of serial dilutions were made in the usual manner. In many cases mouse titrations were made on the dilutions used for plate counts in order to compare the accuracy of the plate counts and mouse titrations.

Both virulent and avirulent "UL" strains were studied. Yolk sac and chorio-allantoic membrane inoculation of embryonated eggs showed that avirulent strain 38 neither grew nor survived. Neither did it survive in infertile eggs.

Virulent strains S and C grew in embryonated eggs following yolk sac or membrane inoculations and caused death of the embryo in one to five days depending on the size of the inoculum. Within 24 hours large numbers of organisms were demonstrable in all parts of the egg and in the embryo. The greatest multiplication of organisms occurred just before death of the embryo. Some typical plate counts on membraneor yolk sac inocul ted eggs harvested 72 hours after inoculation are given below.

Membrane Inoculated Eggs

Tissue	Count /ml
Yolk sac	59×10^6
Embryo	10×10^6
Fluids	11×10^6
Membrane	200×10^6

Yolk Sac Inoculated Eggs

Tissue	Count /ml
Yolk sac	$10,890 \times 10^6$
Fluid	$3,280 \times 10^6$
Membrane	$1,370 \times 10^6$

The organism grew intracellularly in the membrane, in fibroblasts of the mesoderm and in liver cells as described by Buddingh.[20] These observations were confirmed in this laboratory and in addition large numbers of organisms were demonstrated in the cells of the yolk sac. Greatest multiplication of "UL" was obtained in the yolk sac of yolk sac inoculated eggs.

b. Comparison of Strains of "UL" in Embryonated Eggs

As a part of the study of the cultivation of "UL" in embryonated eggs a comparison was made of 8 virulent strains and 7 strains of diminished virulence by titration in 8 day embryonated chicken eggs. An attempt was made (1) to determine their relative virulence for the chick embryo; (2) to correlate the size of the inoculum with the time of death and with the viable count; and (3) to determine the effect of continued storage at incubation temperature on the "UL" count in eggs in which the embryos died as a result of infection.

Eight day embryonated chicken eggs were used throughout. "UL" cultures were grown for 24 hours on DCBA and the growth emulsified in physiologic saline to T40 turbidity. From this suspension logarithmic dilutions were made in saline for the inoculation of the eggs. Twenty eggs were inoculated with each dilution. A total volume of 0.2 ml was injected into each yolk sac with a 21 gauge $1\frac{1}{2}$ inch needle. Eggs were candled daily and the number of dead embryos recorded. Ten eggs from each lot which had received a particular dilution of inoculum were harvested on the day of maximum embryo death. The remaining ten eggs were left in the incubator at 37° C and harvested 8-10 later. Harvesting was done by mixing the entire contents (shell and shell membranes removed) of 10 eggs in a Waring Blender for 3 minutes. The foam was allowed to settle for 5 minutes, then logarithmic dilutions of the liquid portion were made in saline and counted on DCBA plates. The results are shown in Tables VII and VIII.

Chick embryos were killed by inocula of the eight virulent strains, through the 10^{-8} dilution. The day of maximum number of embryo deaths occurred on the 3rd to 4th day with the heavy inoculum and on the 6th to 9th days with the light inoculum. Of the eight virulent strains studied, six showed greater multiplication of "UL" in eggs receiving the heaviest inoculum, while two stains, Holt and SM21, showed approximately the same degree of "UL" multiplication with each dilution of inoculum. In practically all cases storage of dead embryos in the incubator at 37° C for 2-5 days after death resulted in a drop in viable "UL" as determined by plate counts on DCBA.

The seven less virulent strains (Table VIII) killed chick embryos more slowly. Multiplication of "UL" was not so great as with virulent strains and was about the same for all dilutions of inoculum. The data on strain 38 were obtained several months previously and are included in Table VIII for comparison.

TABLE VII

TITRATION OF EIGHT VIRULENT "UL" STRAINS IN EIGHT DAY EMBRYONATED CHICKEN EGGS

THE EGGS POOLED AND COUNTED ON THE DAY OF MAXIMUM NUMBER OF EMBRYO DEATHS AND AT COMPLETION OF TITRATION

STRAIN	DIL. OF INOCULUM	NUMBER OF DEATHS BY DAYS										PLATE COUNT OF THE WHOLE EGGS				CHICK EGG LD_{50}*
		1	2	3	4	5	6	7	8	9	10	COUNT/ML. $X10^{8}$	DAY HARVEST	COUNT/ML. $X10^{8}$	DAY HARVEST	
CARR	10^{-2}	-	1	4	15	-	-	-	-	-	-	15.8	4	10.3	9	$10^{-8.5}$/
	10^{-4}	1	1	1	9	8	-	-	-	-	-	5.5	5	.45	9	
	10^{-6}	2	-	-	3	14	-	-	-	-	-	7.4	5	1.52	9	
	10^{-8}	-	1	-	1	7	10	-	-	-	-	2.4	6	3.8	9	
DIECK	10^{-2}	-	-	-	19	1	-	-	-	-	-	34.5	4	.85	10	$10^{-8.5}$/
	10^{-4}	-	-	1	2	6	9	2	-	-	-	17.9	6	1.85	10	
	10^{-6}	-	-	-	-	2	8	3	3	4	-	10.8	7	2.0	10	
	10^{-8}	-	-	-	-	-	2	2	6	7	1	3.2	9	3.1	10	
CAMP	10^{-2}	-	-	1	18	1	-	-	-	-	-	41.2	4	.97	9	$10^{-8.5}$/
	10^{-4}	1	-	1	4	8	6	-	-	-	-	8.9	6	.4	10	
	10^{-6}	-	-	-	2	-	5	7	4	1	1	7.4	7	.8	10	
	10^{-8}	-	-	-	-	-	7	6	2	3	2	13.2	7	.8	10	
INCE	10^{-2}	-	-	2	10	6	2	-	-	-	-	21.4	5	C	10	$10^{-8.5}$/
	10^{-4}	-	-	-	6	7	7	-	-	-	-	24.5	6	C	10	
	10^{-6}	-	-	-	1	6	11	-	-	-	-	21.5	6	3.94	10	
	10^{-8}	-	-	-	1	-	4	7	3	1	-	8.0	8	——	10	
HOLT	10^{-2}	1	-	-	8	-	11	-	-	-	-	12.7	6	7.1	8	$10^{-8.5}$/
	10^{-4}	1	-	1	7	-	10	1	-	-	-	14.3	6	9.9	8	
	10^{-6}	1	-	1	4	-	10	4	-	-	-	13.5	6	10.7	8	
	10^{-8}	-	-	-	-	-	11	4	3	-	-	15.2	6	11.9	8	
SCHEEN	10^{-2}	-	-	15	5	-	-	-	-	-	-	7.5	4	.95	8	$10^{-8.5}$/
	10^{-4}	-	-	3	17	-	-	-	-	-	-	5.6	4	1.8	8	
	10^{-6}	-	-	1	7	8	4	-	-	-	-	1.1	5	2.6	8	
	10^{-8}	-	-	-	-	2	4	9	2	1	-	2.3	7	3.3	8	
COLL	10^{-2}	1	-	7	10	2	-	-	-	-	-	7.1	5	1.4	8	$10^{-8.5}$/
	10^{-4}	1	-	2	9	8	-	-	-	-	-	6.0	5	1.0	8	
	10^{-6}	-	-	-	8	9	2	1	-	-	-	2.7	6	1.0	8	
	10^{-8}	-	1	-	-	3	8	3	1	-	1	——	-	4.6	8	
BH21	10^{-2}	-	-	10	8	2	-	-	-	-	-	3.4	5	1.3	8	$10^{-8.5}$/
	10^{-4}	-	1	1	9	6	2	1	-	-	-	4.0	6	.9	8	
	10^{-6}	1	2	2	4	5	3	2	1	-	-	3.9	6	1.0	8	
	10^{-8}	-	-	-	3	6	5	-	1	-	3	——	-	4.0	8	

C: CONTAMINATED CULTURE
-: NO COUNT MADE
*: EXPRESSED AS DILUTION (OF A 2-40 "UL" SUSPENSION) WHICH KILLED FIFTY PERCENT OF THE EMBRYOS INJECTED.

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TABLE VIII

TITRATION OF SEVEN "UL" STRAINS OF LOW VIRULENCE IN EIGHT DAY EMBRYONATED CHICKEN EGGS

TEN EGGS POOLED AND COUNTED ON THE DAY OF MAXIMUM NUMBER OF EMBRYO DEATHS AND AT COMPLETION OF TITRATION

STRAIN	DIL. OF INOCULUM	NUMBER OF DEATHS BY DAYS										PLATE COUNT OF 10 WHOLE EGGS				CHICK EGG LD_{50}*
		1	2	3	4	5	6	7	8	9	10	COUNT/ML X10^8	DAY HARVEST	COUNT/ML X10^8	DAY HARVEST	
JAP	10^{-2}	1	-	1	-	18	-	-	-	-	-	19.4	5	C	9	
	10^{-4}	-	1	-	-	10	9	-	-	-	-	13.2	6	10.2	9	$10^{-8.5+}$
	10^{-6}	2	1	-	-	3	9	3	-	-	-	20.4	6	20.5	9	
	10^{-8}	1	-	-	-	1	-	1	5	8	1	----	-	24.0	9	
PIER	10^{-2}	-	-	-	7	-	13	-	-	-	-	6.0	6	4.2	8	
	10^{-4}	-	-	1	1	-	17	1	-	-	-	7.1	6	3.0	8	$10^{-8.5+}$
	10^{-6}	-	-	-	2	-	18	-	-	-	-	5.4	6	5.4	8	
	10^{-8}	-	-	-	2	-	8	8	-	-	-	5.8	7	8.0	8	
RUSS	10^{-2}	-	-	-	1	1	5	4	4	-	1	3.4	7	1.5	10	
	10^{-4}	-	-	-	1	-	1	2	7	4	2	4.0	10	4.0	10	$10^{-8.5+}$
	10^{-6}	-	-	-	-	1	1	3	6	3	2	2.2	10	2.2	10	
	10^{-8}	2	-	-	-	2	2	2	7	-	1	4.6	8	---	--	
MAX	10^{-2}	-	-	3	11	-	7	-	-	-	-	3.6	6	C	10	
	10^{-4}	1	1	-	-	-	4	6	8	-	-	3.2	8	1.2	10	$10^{-8.0}$
	10^{-6}	1	-	1	-	-	1	2	4	11	-	10.6	9	8.0	10	
	10^{-8}	-	1	-	-	-	2	-	2	3	2	---	-	4.3	10	
OHARA	10^{-2}	1	-	-	1	-	17	1	-	-	-	1.5	6	.025	10	
	10^{-4}	-	-	-	-	-	1	12	7	-	-	.6	8	.01	11	$10^{-7.2}$
	10^{-6}	-	-	1	1	-	-	-	7	4	6	1.6	9	.09	11	
	10^{-8}	-	-	-	1	1	-	-	-	-	2	---	-	.08	11	
26	10^{-2}	1	-	1	-	12	5	1	-	-	-	.4	5	.03	10	
	10^{-4}	1	1	1	-	-	1	2	6	5	1	1.5	9	---	--	$10^{-6.26}$
	10^{-6}	2	-	-	-	1	-	-	5	1	2	.01	10	---	--	
	10^{-8}	-	-	1	-	1	-	-	1	-	-	---	--	---	--	
38	10^{-1}	-	-	-	-	-	-	-	-	-	-	0	3	0	9	
	10^{-2}	-	-	-	-	-	-	-	-	-	-	0	3	0	9	0
	10^{-3}	-	-	-	-	-	-	-	-	-	-	0	3	0	9	
	10^{-4}	-	-	-	-	-	-	-	-	-	-	0	3	0	9	

C: CONTAMINATED
-: NO COUNT MADE
*: EXPRESSED AS DILUTION (OF A T-40 "UL" SUSPENSION) WHICH KILLED FIFTY PERCENT OF THE EMBRYOS INJECTED.

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Mixing eggs in the Waring Blender was a convenient method of pooling and homogenizing 10 eggs. It was found that blending for more than 3 minutes resulted in a marked fall in the number of viable "UL" organisms. This could probably be explained on the basis of the heat generated as shown by the following temperature readings obtained on 10 sterile embryonated eggs.

TABLE IX

Temperature Rise in Waring Blender During Homogenizing

Time in Blender in Minutes	0"	3"	5"	7"	10"	15"	20"
Temperature (in degrees C)	33	40	46	51	56	63	68

Separate counts made on foam which rose to the top of the blender and on liquid material at the bottom showed the following concentration of organisms in the foam.

TABLE X

Count per ml in Foam and Liquid from Waring Blender

	Foam	Liquid
Expt. 1	5.48×10^8	4.48×10^8
Expt. 2	11.58×10^8	8.58×10^8

These values are not remarkedly different but serve to emphasize the necessity of employing standard procedure in work of this kind.

These results on strains of varying virulence indicate that inoculation into embryonated eggs does not furnish a very delicate method, for detecting strain differences. In general the more virulent strains kill check embryos more quickly and give a higher final count, but the difference is not so great as that observed when mouse titrations are used. Continued storage for 2-5 days at incubation temperature caused a significant drop in viable "UL" counts.

c. Growth of "UL" in Living and Dead Chick Embryos

Although "UL" incoulation of embryonic membranes or yolk sacs seems to result in their parasitization and in abundant multiplication of the organism, it is possible to grow the later in the test tube on a medium of coagulate egg. It seemed of some interest to determine how well, if at all, "UL" would grow in an embryonated egg if inoculated after the embryo was killed. Pilot plant production of "UL" in embryonated eggs was being considered and from this standpoint it was desirable to know

whether embryos which were killed as a result of the trauma and manipulation of inoculation would still support "UL" growth.

Forty-five eggs (7 day embryos) were inoculated with 0.2 ml of 10^{-2} dilution of a T-40 standard saline suspension of "UL". The culture strain SM16, was 24 hours old. This experiment was repeated twice.

The eggs were divided into 2 lots of 20 and 25, respectively. Twenty were inoculated as usual (21 gauge 1½ inch needle inserted through air sac at an angle to avoid hitting the embryo). This group lived until killed by "UL" growth. In experiment 1, 10% died on the first day, 15% on the second day and 75% on the third day. In experiment 2, 2% died on the first day, 4.4% on the second day, and 93% on the third day. On the third day, all eggs were placed in the ice-box. Twenty-five eggs were killed at the time of inoculation by inserting the needle 3 times at an angle directed toward the embryo. The "UL" suspension was injected on the third stroke. These eggs were kept in the incubator for 3 days (the same length of time as those allowed to die as a result of "UL"), after which time they were placed in the ice-box until harvested. All eggs of this set which contained viable embryos 18 hours after inoculation were discarded. After 1 and 2 days in the ice-box (Experiment 1 and 2 respectively) the contents of the eggs were harvested, placed in 250 ml centrifuge bottles with glass beads and shaken 30-40 minutes. The shells were discarded. Serial dilutions in 0.85% saline were made from the shaken material and plate counts were made in triplicate on DCBA plates. Three eggs were pooled for each count. The average count in experiments 1 and 2 were as follows.

TABLE XI

Multiplication of "UL" in Living and in Dead Embryonated Chicken Eggs

	Organisms/ml of Whole Egg	
	Experiment 1	Experiment 2
Embryo killed at time of inoculation		
Group 1 (3 eggs per group)	449×10^7	44.2×10^7
Group 2	426×10^7	132.0×10^7
Group 3	423×10^7	136.2×10^7
Embryo allowed to die of "UL" infection		
Died 1st day	277.5×10^7	155.7×10^7
Died 2nd day	484×10^7	153.5×10^7
Died 3rd day	420×10^7 395×10^7 322×10^7	105.2×10^7
Embryo living after 3 days	None	107.0×10^7

It appeared from these results that there was no significant difference between the "UL" growth promoting properties of living versus dead chick embryos.

This knowledge has practical application in cultivation of "UL" on embryonated eggs in the laboratory or plant. Embryos that are killed by the manipulation and trauma of inoculation should not be discarded. If kept in the incubator the "UL" growth in these eggs will be equal in amount to that in eggs containing viable embryos.

d. Growth of "UL" in Embryonated Duck Eggs

Previous work in the "SI" group has shown better growth of the agent in duck eggs than in chicken eggs.(21) Since "UL" had not been grown in duck eggs, the following three experiments were set up. In the first, yolk sacs of 16 nine-day embryonated duck eggs were inoculated with 0.2 ml of 10^{-2} dilution of a standard suspension of "UL" strain SM21R3. Twenty 11 day embryonated chicken eggs were similarly inoculated with the same suspension. All eggs were incubated at 37° C and harvested on the 4th day after overnight storage in the ice-box. Yolk sacs, membranes and allantoic fluid were harvested separately. Giemsa stains were made on each yolk sac to detect contamination. Yolk sacs from 14 duck eggs were pooled for a plate count. Membranes and allantoic fluids were also pooled before counting.

Because of the fact that the duck eggs seemed to survive about one day longer than the chicken eggs and because of the high counts obtained in the yolk sac two additional experiments were set up, in which varying dilutions of "UL" used as the inoculum were injected and the day of death was recorded. The results of these three experiments are tabulated in Table XII.

These results showed that duck embryos were susceptible to "UL" infection and that they tended to survive longer than chicken embryos. The yolk sac counts were not consistent but in general the yolk sac counts on duck eggs were higher than those on chicken eggs. In duck eggs as in chicken eggs the yolk sac contained a larger number of organisms than the membranes or fluids. As duck eggs are 40% larger than chicken eggs the total yield of organisms would be larger than in chicken eggs, an advantage which might be taken into consideration in vaccine preparation.

e. Serial Passage in Chicken Eggs

Since serial passage in eggs in the case of some viruses and the rickettsia may alter their virulence for eggs and mice, it seemed worth while to study the effect on virulence of serial passage of "UL" through embryonated eggs.

A virulent strain of "UL", SM-1 (S strain-mouse passage 1) was passaged through yolk sac and membrane and the LD_{50} for mice and hhick

TABLE XII

Multiplication of "UL" in Embryonated Duck and Chicken Eggs Inoculated Via the Yolk Sac

	Dil. of Inoculum	No. of Eggs	Day of Death 1	2	3	4	5	6	7	8	9	Count/ml x 10^9 Yolk Sac	Membrane	Allantoic Fluid
Experiment I														
Duck Eggs (9 day)	10^{-2}	16	-	1	14	2	-	-	-	-	-	22.60	3.72	2.14
Chick Eggs (11 day)	10^{-2}	20	-	9	11	-	-	-	-	-	-	3.30	2.58	0.35
Experiment II														
Duck Eggs (9 day)	10^{-2}	7	-	-	5	2	-	-	-	-	-			
	10^{-4}	10	-	-	1	9	-	-	-	-	-	2.06		
	10^{-6}	10	1	-	1	8	-	-	-	-	-	0.78		
	10^{-8}	10	-	-	-	5	2	-	-	-	-	5.38		
Chick Eggs (11 day)	10^{-2}	7	-	7	-	-	-	-	-	-	-			
	10^{-4}	10	1	-	9	-	-	-	-	-	-	0.01		
	10^{-6}	10	-	2	6	2	-	-	-	-	-			
	10^{-8}	5	-	-	1	3	1	-	-	-	-			
Experiment III														
Duck Eggs (9 day)	10^{-2}	10	-	-	1	8	1	-	-	-	-	5.76		
	10^{-4}	10	-	-	-	3	7	-	-	-	-	7.88		
	10^{-6}	10	-	-	-	-	6	4	-	-	-	2.38		
	10^{-8}	6	-	-	-	-	-	2	2	1	1	0.10		
Chick Eggs (9 day)	10^{-2}	10	-	2	8	-	-	-	-	-	-	4.74		
	10^{-4}	10	-	1	3	6	-	-	-	-	-	2.46		
	10^{-6}	10	-	1	-	7	2	-	-	-	-	0.57		
	10^{-8}	9	-	-	-	1	6	2	-	-	-	4.78		

"UL" Strain SM21-R3 was used throughout these tests.

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embryos was determined. Table XIII shows the results of these experiments. No significant change in virulence for mice could be detected after yolk sac passage. The virulence for the chick embryo appeared to be slightly increased. The organisms tended to die out on repeated membrane passage but virulence titrations showed no significant change for mice or chick embryos. These experiments did not indicate that passage through embryonated eggs altered the virulence or growth capacity materially. Some of the eggs hatched and the blood of the apparently healthy chicks contained "UL".

2. Determinations of the LD_{50} Dose of "UL" for Laboratory Animals by Various Routes of Inoculation

The various portals of entry for infection of laboratory animals which might be naturally operative or which were useful experimentally were determined by the techniques listed below.

Percutaneous: In the case of mice and rats hair was removed with barium sulfide or electric clippers and 48 hours later .05 ml of culture was dropped on the skin and rubbed in gently with a smooth glass rod or gloved finger. Under these conditions the exact number of organisms penetrating the skin could not be measured, therefore the LD_{50} is given in terms of the dilutions which caused death in 50% of the animals treated with this dilution. Cutaneous: serial dilutions from a standard suspension of "UL" were made and 0.1 ml was injected into the skin of mice, rats and rabbits. Subcutaneous and Intraperitoneal: serial dilutions as above were made, mice received 0.5 ml, and rats and other animals, 1.0 ml. Intranasal: the animals were anesthetized and 0.05 ml of the appropriate dilution was gently dropped into one nostril. Conjunctival: An attempt was made to determine the sensitivity of the ocular portal of entry of "UL" in rabbits. It seemed logical that an inoculum of "UL" placed in the cul-de-sac of the eye might produce an infection in the animals by one of two routes: (1) by absorption through the ocular mucous membrane, or (2) by passage down the lacrimal canal and then to the respiratory tract. The lacrimal puncta of four rabbits were therefore destroyed with a cautery thus eliminating the possibility of respiratory absorption of the agent via the lacrimal canal. These four animals werekept for a month to allow all the reaction in the conjunctiva to subside before challenging them with "UL". An inoculum of 0.05 ml of 10^{-2}, 10^{-4}, 10^{-6} and 10^{-8} dilutions of "UL" was dropped into the right eye of four rabbits with open puncta and four rabbits which had had their puncta closed with a cautery. Intravaginal: In order to test the penetration of the organism on another mucous membrane four female rabbits were given vaginal inoculation of 0.05 ml of dilutions of 10^{-2}, 10^{-4}, 10^{-6}, and 10^{-8} of "UL".

The results of these various modes of inoculation are shown in Table XIV.

Mice, rabbits, guinea pigs, and hamsters seemed to be equally susceptible to "UL" when challenged subcutaneously, 1-3 organisms sufficing to kill. Mice and rabbits succumbed just as easily to intracutaneous injection as they did to subcutaneous injection. Guinea pigs and mice succumbed

TABLE XIII

Effect of Serial Egg Passage on Virulence of "UL"

Strain	Count/ml of Suspension Titrated	LD_{50}* Mice	LD_{50} Chick Embryo
SM1	2200×10^6	$10^{-9.23}$	$10^{-7.17}$
SYS26	5840×10^6	$10^{-9.30}$	$10^{-9.33}$
SME10	9500×10^6	$10^{-9.27}$	$10^{-9.0}$

* LD_{50} expressed as the dilution (of a T-40 suspension) which killed one LD_{50} per ml.

SM1 : Mouse passage 1 (original culture)
SYS26 : Yolk sac passage 26
SME10 : Membrane passage 10

with equal ease to intrapeitoneal injection. All animals were less susceptible to percutaneous application of virulent "UL". Applied percutaneously, a dose one million times greater than the subcutaneous dose was required to kill rabbits and guinea pigs, while in the case of mice the percutaneous dose was 100 times greater than the subcutaneous, indicating a species difference in the degree of protection afforded by the skin.

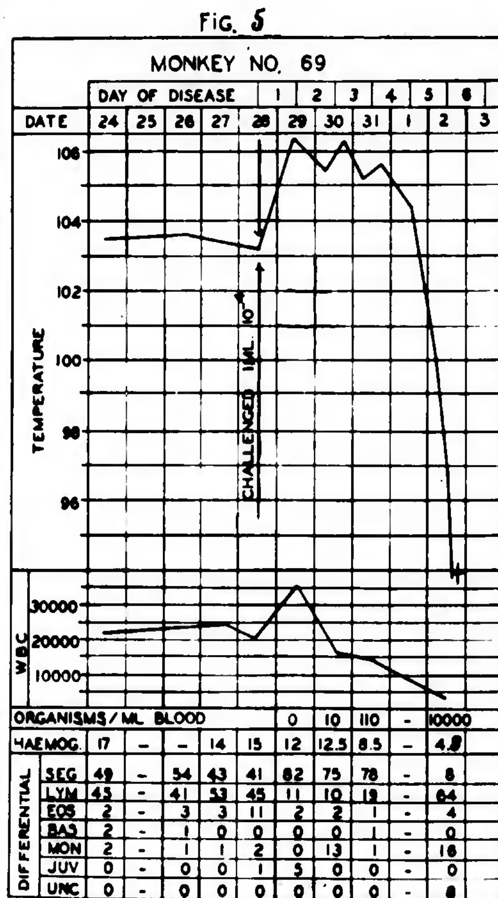
The intranasal dose for mice and guinea pigs was approximately the same and was about 100 times greater than the subcutaneous dose. Respiratory challenge in the cloud chamber indicated that the LD_{50} dose was 11 organisms for guinea pigs, 1 to 2 organisms for mice and 1000 to 10,000 organisms for rats. The figure for rats is only approximate as it is based on one experiment.

Rabbits: Rabbits were the only animals on which intraocular and intravaginal titrations were made and the LD_{50} by both these routes was about 1000 x greater than by the subcutaneous or intraperitoneal route. The animals inoculated on the conjunctiva with 10^{-2} and 10^{-4} dilutions of "UL" developed a watery discharge from the inoculated eye on the 2nd and 3rd day after inoculation. The two animals given the 10^{-2} dilution developed small yellow nodules in the conjunctiva. "UL" was isolated from smears taken from the conjunctiva of these two animals. The four animals inoculated with 10^{-2} and 10^{-4} dilutions of "UL" died between four and six days after inoculation. All of them had lesions of "UL" in the spleen and liver. The animals challenged with 10^{-6} and 10^{-8} dilutions of "UL" showed no evidence of infection. The above experiment strongly suggested that the route of infection by "UL" was through the conjunctiva. It was possible, however, that there could have been a minute opening in the lacrimal puncta which had not been occluded with the cautery. The animals inoculated intravaginally with the 10^{-2} and 10^{-4} dilutions developed nodular yellowish lesions in the vaginal wall and died within 6 days after inoculation. Both rabbits showed typical "UL" lesions in the spleen and liver.

Cotton Rats: One subcutaneous titration was carried out on 20 cotton rats which seemed to indicate that they were almost as susceptible as the guinea pig, rabbit or mouse. They were not as resistant as white rats.

Monkeys: A virulence titration of "UL" in Macacus rhesus monkeys was carried out to determine the susceptibility of this species. The data obtained were to be used later in studies on vaccinated and streptomycin treated monkeys challenged with "UL". The animals were young M. rhesus monkeys from India weighing 4½ to 5½ lbs. They were in a poor state of nutrition but had no apparent illness. They were injected subcutaneously with 1 ml of the suspensions as indicated. Table XV summarizes the results and Figure 5 shows the blood picture, blood culture, and temperature record of one representative animal.

All monkeys showed elevated temperatures 24 hours after challenge. Objectively the monkeys all showed the same series of clinical signs consisting of a stage of irritability characterized by vigorous physical and vocal resistance to handling. Later they showed subnormal activity and sat in



	24	25	26	27	28	29	30	31	1	2	3
DAY OF DISEASE					1	2	3	4	5	6	
ORGANISMS/ML BLOOD						0	10	110	-	10000	
HAEMOG.	17	–	–	14	15	12	12.5	8.5	–	4.[illegible]	
DIFFERENTIAL SEG	49	-	54	43	41	82	75	78	-	8	
LYM	45	-	41	53	45	11	10	19	-	64	
EOS	2	-	3	3	11	2	2	1	-	4	
BAS	2	-	1	0	0	0	0	1	-	0	
MON	2	-	1	1	2	0	13	1	-	16	
JUV	0	-	0	0	1	5	0	0	-	0	
UNC	0	-	0	0	0	0	0	0	-	[illegible]	

Figure 5

Normal M. Rhesus Monkey Challenged Subcutaneously With 10,000 LD_{50} of Virulent "UL" Strain S

TABLE XIV

Relative Susceptibility of Various Animal Species to "UL"

All animals challenged with dilutions of T-40 suspensions prepared from 24 hr. DCBA cultures

Route of Infection	Mice	Rats	Rabbits	Guinea Pigs	Hamsters	Monkeys	Cotton Rats	Chicks (3 Weeks)
Percutaneous	$10^{-6.8}$*	10^{-1}	$10^{-3.25}$	$10^{-3.5}$				
Intracutaneous	10^{-9}	10^{-3}	10^{-9}					
Subcutaneous	10^{-9}	10^{-4}	10^{-9}	10^{-9}	10^{-9}	10^{-7} to 10^{-8}	10^{-8}	
Intraperitoneal	10^{-9}	10^{-7}		10^{-9}				10^{-1}
Intranasal	$10^{-6.83}$	$10^{-2.36}$		10^{-7}				
Conjunctival			$5x10^{-5}$					
Intravaginal			$5x10^{-5}$					
Respiratory (cloud chamber)	10^{-9}	10^{-5} to 10^{-6}		10^{-8}				

* Numbers indicate calculated dilution of T40 suspension of virulent "UL" which killed fifty percent of the animals (LD_{50}).

The standard (T40) suspension contained approximately 10^9 organisms/ml.

TABLE XV

Subcutaneous Challenge of 5 M.rhesus Monkeys with "UL"

Monkey No.	Challenge Dilution	Viable Organisms in Challenge	Day of Death
33	10-0	2,030,000,000	3
70	10-2	2,030,000	4
69	10-4	203,000	5
68	10-6	2,030	6
72	10-8	20	7

their cages with their heads between their knees. This was followed by noticeable weakness progressing to extreme prostration, which sometimes persisted for 12 hours before death. During this latter stage the temperature and blood pressure fell to subnormal levels. Frequent chills occurred throughout the disease. Most animals did not eat during the last 24 to 48 hours of life, although some did pick up food and nibble at it half heartedly. The heart beat remained forceful up to the end. Respiration became slow and irregular before death. No convulsions were noted. All monkeys developed grey pallor of the face about 48 hours before death. This occurred at a time when the monkeys were still active and eating well. This sign proved to be the first clinical evidence of impending prostration and fatal outcome. Monkeys #33 and 69 developed multiple petechial hemmorrhages in the skin over the face, shoulders, hind legs, and abdomen before death. A prolongation of the clotting time was observed in the latter stages of the disease. The hemoglobin level dropped to 4.8 gms per 100 cc in Monkey $69. This was lower than the level in the other four animals which dropped to only 7 to 8 gms per 100 cc. Bile from Monkey #69 cultured at death contained 1800 "UL" organisms per ml. Total blood lipid determinations a few hours before death gave elevated levels in three out of four animals. The significance of this finding has been investigated further by Lt. G. S. Pinchot. (personal communication).

Gross pathological findings were quite uniform and consisted of local skin necrosis without involvement of the muscles of the belly wall. The local lesion was not at the site of inoculation but just to the left of the umbilicus in every case. Regional lymph node enlargement was present in all animals and liquefaction occurred in the two animals which survived 6 to 7 days. Spleens were enlarged 2-3 x, were firm on cut section, and in animals which lived more than four days there were multiple small yellowish-white areas of focal necrosis. The livers were pale and yellowish in color with a few scattered areas of focal necrosis visible in those animals which lived 6 to 7 days. Kidneys and brain showed no unusual gross findings. The intestines were not abnormal except for 3 to 20 small black cysts containing clotted blood and a small threadworm in the wall of the colon. The adrenals were pale.

Histopathological findings on sections stained with H and E and Giemsa were as follows:

Liver: The architecture was obliterated except for the central veins. Liver cords were swollen and parenchymal cells showed extensive fatty degeneration. Small areas of focal hepatitis containing polymorphonuclear cells, nuclear remnants and fibrin were found in all animals except #33 which died on the third day of the disease. A few typical pseudocysts filled with pale blue granules averaging 0.5 microns in diameter were seen in all monkey livers. They were located in or at the periphery of an area of focal hepatitis.

Spleen: Sections showed massive coagulation necrosis with much fibrin and tissue debris. Only the capsule and connective tissue retained their staining properties in some sections. A few pseudocysts were observed.

Kidney: Glomerular tufts were swollen and the intracapsular space contained granular debris. Convoluted tubules showed cloudy swelling and slight fatty degeneration. No necrosis was observed.

Lymph nodes showed all stages of involvement from frankly normal glands to proliferation of the germinal centers, through infiltration with inflammatory cells, focal necrosis and finally complete destruction and liquifaction.

Adrenals: Section of the adrenal gland showed loss of cortical lipoid. Scattered arease of polymorphonuclear and mononuclear infiltration were seen in the cortex and in a few animals small areas of hemorrhage.

The local lesion showed necrosis of the skin and subcutaneous tissues with scattered infiltrations of polys and mononuclear cells in the surrounding area. The underlying muscle layer was not involved.

Sections of the brain and meninges were similar to those seen in normal monkeys.

Lung sections showed no evidence of tuberculosis. A few areas of chronic inflammatory reaction were observed consisting of blood pigments, histiocytes, a few polys and very little fibrosis. The picture was suggestive of a reaction to protozoan infestation. A small mite was later seen in some of these areas.

It was apparent from these results that the monkey is one of the more susceptible animals although the number of animals used was too small to enable one to ascertain the minimal lethal dose on a statistical basis. Later studies with more mature monkeys showed them to be more resistent. Some survived the acute phase and went on to develop a subacute or chronic form of the disease in which a granulomatous pneumonia was a prominant finding. The disease in monkeys had more the appearance of the human disease than is observed in other small laboratory animals. There was an ulcerative local lesion, regional lymphadenopathy with occasional suppuration of the glands, delayed appearance of a positive blood culture

and a late "UL" pneumonia in animals which received a small challenge. Most animals developed a panleucopenia just before death.

Chicks: Young chicks, 9 days old, were quite resistant, succumbing to a dose of 0.5 ml 10^{-2} dilution of a T40 suspension. Chicks which had received 0.5 ml of 10^{-2} and 10^{-4} dilutions were sacrificed 17 days after challenge and the heart's blood, spleen and liver tissue yielded positive and typical "UL" cultures. Serum agglutinins were present in low titer, 1:20 and 1:40 respectively. A similar test was done on 28 day chicks. Three chicks were injected, one with 0.5 ml of 10^{-0}, one with 10^{-2} and one with 10^{-4}. The results are as follows: the 10^{-2} chick died of "UL" on the 4th day; the two remaining chicks survived 42 days at which time they were sacrificed. No evidence of infection was present and cultures were negative.

Dogs: Five five-week old pups were challenged intradermally with 0.1 ml of 10^{-0}, 10^{-2}, 10^{-4}, 10^{-6}, and 10^{-8} dilutions of T500 suspension of SM14. The pup receiving 10^{-0} died on the 9th day and the one receiving the 10^{-8} dilution died on the 14th day. Positive blood and spleen cultures were recovered. The remaining pups survived the challenge dilutions of 10^{-2}, 10^{-4}, and 10^{-6}. Thirty-eight days after challenge the serum from these pups had titers of 1:320, 1:640, and 1:2560 respectively. They were re-challenged by subcutaneous injection on the 38th day with 0.5 ml of a T500 suspension of strain SD2. All of the pups developed local lesions which were filled with a greenish gelatinous mass having no cellular elements. One dog developed a large bubo in the right groin with edema of the flank. This dog was sacrificed 4 days after the rechallenge. Postmortem showed an enlarged inguinal node from which a positive culture was obtained. The parenchymatous organs showed no gross lesions. Cultures of the heart's blood and spleen were negative. The serum agglutination titer was 1:160. The two remaining dogs absorbed the local lesions without suppuration. Twenty-four days after challenge they were skin tested with 0.1 ml of "UL" skin test antigen and a 10^{-5} dilution of living "UL" and living "US". Necrotic lesions appeared at the site of injection of the "UL" skin test antigen. No reaction to the living "US" was observed and minute pinpoint areas of erythema appeared at the site of the living "UL" injection. These two animals were sacrificed 31 days after the above challenge. There was no gross pathology evident at autoposy. Blood cultures were negative. Agglutination titers were 1:5120 and 1:1280 respectively.

Three normal 8-9 week old pups were challenged intradermally with 0.1 ml of T500, 10^{-2}, and 10^{-4} dilutions of "UL" respectively. A papule which developed into a punched out ulcer appeared at the site of inoculation. The regional lymph nodes were enlarged in all of the animals. Suppuration of the regional lymph nodes occurred in one animal. All became emaciated. One developed convulsions on the 13th day and was sacrificed. Postmortem examination showed multiple foci of necrosis in the liver, spleen and lung. Another pup was sacrificed after 18 days with gross findings as above and a positive heart's blood culture. The third pup which had survived 27 days was then rechallenged with a T500 suspension. At the time of rechallenge

pus was aspirated from the old local lesion. This pus killed a guinea pig, and characteristic lesions were apparent. The pup did not become ill but developed a large swelling filled with greenish gelatinous pus at the site of rechallenge. This pup was sacrificed 4 days after rechallenge. "UL" was not recovered by culture or animal inoculation from the local lesion or the internal organs. Microscopic examination of sections of spleen and liver showed small areas of mononuclear and ephelioid cells surrounded by normal parenchymal cells suggesting healing process. Agglutination was positive at 1:1280 at the time of sacrifice.

One small adult dog which received 1 ml of a T500 suspension intravenously gave a positive blood culture 5½ hours after injection. The blood culture was positive after 23 hours also and the animal died 46 hours after challenge. At autopsy there was slight swelling of the spleen. The kidneys bulged on cutting and were pale. The organisms were multiplying in this animal as shown by the quantitative blood cultures: 180 organisms/ml at 5½ hours and 2.5×10^6 organisms/ml at death.

One large adult dog was injected subcutaneously with 1 ml of heart's blood from a rabbit which had died of "UL" a few minutes before the dog received the injection. This experiment was done in order to check on a G2 report to the effect that the Germans were studying "UL" in dogs injected with rabbit blood.(22) This dog developed no local lesion or regional lymphadenopathy and did not appear ill at any time. Blood cultures made 27 days after injection were negative. The serum gave agglutination of "UL" at 1:80 and of "US" 1:160. Six days later this dog was rechallenged subcutaneously with 1ml of a T500 suspension of strain SD-2. No evidence of systemic debility appeared but he developed a large subcutaneous abscess at the site of injection which became fluctuant after 2 weeks. This dog was sacrificed 16 days after rechallenge. Fifty ml of greenish serous fluid was aspirated from the abscess which was sterile by culture and guinea pig inoculation. Spleen cultures and animal inoculation were negative. The internal organs showed no gross pathology. Serum agglutination was positive at 1:1280.

A third adult dog, the mother of the 5 week old pups was bled 32 days after her pups were challenged. At this time her serum agglutinated "UL" at 1:640. Her blood culture was negative and it was presumed that she had become infected while suckling her infected pups. She was therefore rechallenged by the subcutaneous injection of 1 ml of T500 SD-2, 37 days after challenge of the pups. She was sacrificed 7 days later and no gross pathology was evident at autopsy. Blood cultures were negative and the serum agglutination was positive at 1:320.

The data presented above shows the dog to be relatively resistant to "UL" infection with considerable individual variation in susceptibility.

Rats: Since it was proposed to use rats extensively for various tests and expecially for testing the efficacy of vaccines it was necessary to determine the susceptibility of this species to infection by various

routes. They showed greater resistance to all routes of infection tested than did mice, guinea pigs or rabbits, but were more susceptible than dogs or chicks. They were most susceptible when injected intraperitoneally and became progressively less susceptible as the organisms were administered by the subcutaneous, intracutaneous, intranasal and percutaneous routes. Although animals injected with "UL" frequently survived they usually showed evidence of illness after challenge. The coat was ruffled, they were sluggish and lost weight. The minimal infective dose was less than the LD_{50} dose and although it has not been determined exactly it is probably between 1.0 ml of 10^{-8} and 10^{-9} dilution of the T40 suspension. The presence of infection was ascertained in a group of rats which were injected with the dilutions shown in Table XVI. Twelve rats per dilution were used, 2 to 6 out of each dilution being sacrificed on the 3rd, 6th, and 15th day as shown in Table XVI. The remaining animals died or were sacrificed on the 28th day. Rats sacrificed at the various intervals were tested for the presence of "UL" infection by culturing spleen and hearts blood on DCBA and by injecting hearts blood and supernatant from macerated spleen into each of four mice. Mouse injection of the macerated rat spleens was the most sensitive method of detecting "UL" and these data are shown in Table XVI. The results indicated that the rats challenged with 10^{-5} through 10^{-8} dilutions were all infected when examined on the 6th day. In dilutions of 10^{-5} through 10^{-7} the spleens still contained organisms on the 15th day. On the 28th day 25 rats which had received 1 ml of 10^{-5} dilution were sacrificed. "UL" was not recovered by culture or mouse injection from any of the rats, indicating that they had recovered. The LD_{50} dose in this rat titration was 1 ml of $10^{-4.6}$ dilution, and since the minimal infective dose (MID) was less than 1 ml of 10^{-8} dilution of LD_{50} dose was at least 1000 times the minimal infective dose. The comparative data on the rat MIB, rat LD_{50}, mouse LD_{50} and DCBA plate count are shown in Table XVI.

After rats have recovered from an infection they resist 10,000 to several million LD_{50} doses. This protection lasts at least 54 days. Almost 100% of rats recovering from a challenge dose continue to harbor organisms in the spleen for 15 days as shown by mouse injection of the ground rat spleen. Four weeks after challenge the presence of latent infection could not be demonstrated.

Normal rats challenged with a moderate dose of organisms had a very low titer or no titer of agglutinins when bled 3 days after challenge, but at the end of 6 days 8 out of 10 animals had titers from 1:180 to 1:640 while two challenged with the smallest number of organisms had a titer of 1:20 and 1:40 respectively. After 15 days 6 of 8 animals had a titer of 1:640 and two of 1:80 and one of 1:160. At the end of 28 days the titers had dropped to a level of 1:160 in 3 animals and 1:80 in 2 animals. (See Figure 11, Section F).

Summary: The results of the infectivity studies indicated that "UL" grew abundantly in embryonated duck or chicken eggs and in dead embryonated chicken eggs; that the virulence of different strains of the organism

TABLE XVI

Determination of the Infective Dose (MID_{50}) and the Lethal Dose (LD_{50}) of "UL" in White Rats

(All rats challenged subcutaneously with 1 ml of serial dilutions of Strain SM21R4)***

Challenge Dilution	MID_{50} Days after infection sacrificed: Number Rats	3	6	15	Total	LD_{50} Number Rats	LD_{50} Total Deaths on 15th Day
10^{-4}	0	0	0	0	0	6	6
10^{-5}	16	6/6*	5/5	5/5	16/16	20	4
10^{-6}	6	1/2	2/2	2/2	5/6	6	0
10^{-7}	6	2/2	2/2	2/2	6/6	6	1
10^{-8}	7	3/3	2/2	1/2	6/7	6	1

* Numerator indicates number of rats from which positive "UL" cultures were obtained. Denominator indicates total number of rats sacrificed.

MID_{50} : 10^{-8} LD_{50} : $10^{-4.68}$

Rat MID_{50} dose 1 ml 10^{-8} number of organisms = 9.0

Rat LD_{50} dose 1 ml $10^{-4.68}$ number of organisms = 18,000

Mouse LD_{50} dose 0.5 ml $10^{-9.35}$ number of organisms = 0.4

*** Original suspension contained 1,710,000,000 organisms per ml by plate count.

for eggs was of the same general order as for mice or rabbits; that serial passage in embryonated eggs did not materially affect the virulence of the strains used; that the organisms multiplied most abundantly in the yolk sac.

The results on the various routes of infection of animals showed that "UL" was infective by all the routes tested and that in general the smallest dose was required for subcutaneous or intraperitoneal infection.

The susceptibility of the animals used varied with the species, the dog, chicken and white rat being most resistant; the mouse, hamster, guinea pig, and rabbit being least resistant and the cotton rat and monkey being somewhat more resistant than the rabbit but less resistant than the white rat.

C. Survival Experiments

1. Cloud Chamber Studies

Cloud chamber studies were carried out to determine whether "UL" would withstand the oxidation and drying of nebulization, and whether animals, could be infected by exposure to such a cloud.

Preliminary studies were done by the B Division workers with apparatus designed and assembled by Lt. Pinchot (Chamber A). Later studies were done with Dr. Henderson using the British cloud chamber (Chamber B). When Dr. Rosebury's cloud chamber (Chamber C) building was ready for use the cloud chamber studies were taken over by tha A Division "UL" group.

a. The Pinchot Apparatus (Chamber A)

Preliminary work by the B Division was carried out as follows: A Darby type atomizer was employed to nebulize the test culture, suspended in a solution of 10% gelatin, egg yolk or physiological saline. The cloud was drawn into a drying chamber and mixed with atmospheric air, then passed into the animal box or by-passed into bubbling samplers containing saline or peptone broth. The number of organisms caught in these samplers was determined at the end of the experiment. The exhaust was passed into an incinerator. The whole apparatus was operated under a negative pressure of 2 cm of water. By measuring the air flow per minute, the number of organisms recovered in the sampler, and the time of exposure of animals, an approximation of the CT (concentration in mgm per cubic meter x time) in the animal box was calculated. In these calculations it was assumed that 10^9 organisms would weigh 1 mgm.

When the above mentioned fluids were used as suspending media, gelatin in a final concentration of 3.3% proved to be the best. Recovery was 3-5% of the number nebulized, as determined from the volume and count per ml of the suspension sprayed. Avirulent strain 38 could not be recovered under similar conditions of dispersal and sampling.

Mice placed in the cloud chamber died following exposure to CT values as low as 0.0018, or calculated to 1-2 organisms per mouse. Mice exposed to greater cloud concentrations developed multiple foci of infection beneath the visceral pleura and scattered throughout the substance of the lung, indicating that respiratory infection had taken place. One immune mouse which had survived challenge after vaccination and subsequent re-

TABLE XVII

Cloud Chamber Experiments (Chamber A)

Original Count x 10^6/ml	Number Nebulized x 10^5	Percent Recovery	Time	Animal Exposure Data: Mice Exposed Dead/Tested	Animal Exposure Data: Rats Exposed Dead/Tested	Animal Exposure Data: Immune Animals Dead/Tested	C.T.
Nebulizing Fluid Gelatin 3.3%							
1) 4910.0	14730	3.0%	15 sec.	4/4	2/2	Mouse 1/1	0.132
2) 7980.0	15960	1.4%	5 min.	0	1/2	Rat 0/3	0.5
3) 0.443	1300	3.6%	45 sec.	5/8	0		0.0018
4) 0.260	0.780	5.0%	15 sec.	3/8	0		0.0022
Nebulizing Fluid Dog Serum 3.3%							
5) 1680	5040	6525×10^6 **	30 sec.	8/8	0		4.0

* S was strain used.

** Numbers of organisms are given rather than per cent because the numbers of organisms were unexpectedly large.

TABLE [illegible]

CLOUD CHAMBER EXPERIMENTS IN BRITISH APPARATUS (CHAMBER B)

NEBULIZING DATA:
COMPARISON OF SUSPENDING MEDIA AS SHOWN BY PERCENT SURVIVAL IN THE RESERVOIR

EXPERIMENT NUMBER	SUSPENDING MEDIUM	PERCENT SURVIVAL IN RESERVOIR AFTER NEBULIZATION	IMPINGER FLUID	SPRAY FACTOR***	COUNT OF SUSPENSION X10^? ML: RESERVOIR BEFORE NEBULIZING	RESERVOIR AFTER NEBULIZING
1*	0.?% NORMAL RABBIT SERUM IN DISTILLED WATER	[illegible]	DISTILLED WATER	[illegible]	11.5	[illegible]
2A	0.67% NORMAL RABBIT SERUM IN DISTILLED WATER	68	DISTILLED WATER	0.71x10^-6	8.17	[illegible]
2B	0.67% NORMAL RABBIT SERUM IN 0.85% SALINE	48.8	N-SUPERNATANT - 0.85% SALINE	0.2x10^-6	18.7	9.6
3	0.5% NORMAL HUMAN SERUM IN 0.85% SALINE	63	N-SUPERNATANT - 0.85% SALINE	0.24x10^-6	10.9	6.9
4	2% DEXTRIN IN 0.5% SALINE	106	4A) 2% DEXTRIN IN 0.5% SALINE & N-SUPERNATANT	0.43x10^-6	14.7	15.6
			4B) 2% DEXTRIN IN 0.5% SALINE	0.27x10^-6		
5	2% DEXTRIN IN 0.5% SALINE	7.2	5A) 0.85% SALINE & N-SUPERNATANT	0.18x10^-6	2.22	0.16
			5B) 0.85% SALINE	0.4x10^-6		
6	2% DEXTRIN IN 0.5% SALINE	78.2	2% DEXTRIN & N-SUPERNATANT	0.23x10^-6	3.58	2.8
7	2% DEXTRIN IN 0.5% SALINE	50	2% DEXTRIN & N-SUPERNATANT	0.06x10^-6	0.276	1.39
8A	2% DEXTRIN IN 0.5% SALINE IN 0.5% NORMAL HUMAN SERUM	98	2% DEXTRIN & N-SUPERNATANT	0.10x10^-6	0.026	0.025
8B	2% DEXTRIN IN 0.5% SALINE IN 0.5% NORMAL HUMAN SERUM	69	2% DEXTRIN & N-SUPERNATANT	0.05x10^-6	0.047	0.033
9	2% DEXTRIN IN 0.5% SALINE IN 0.5% NORMAL HUMAN SERUM	95.6	2% DEXTRIN & N-SUPERNATANT	0.21x10^-6	0.037	0.035
10**	2% DEXTRIN IN 0.5% SALINE IN 0.5% NORMAL HUMAN SERUM	98.8	2% DEXTRIN & N-SUPERNATANT	0.05x10^-6	0.036	0.035

* SOURCE OF CULTURES FOR EXPERIMENTS 1-9 WAS 24 HOUR CULTURES SCRAPED FROM DCBA SLANTS.
** SOURCE OF CULTURES FOR EXPERIMENT 10 WAS 24 HOUR AERATED PEPTONE BROTH CULTURE.
*** SPRAY FACTOR: NUMBER OF ORGANISMS PER LITER OF SPRAY / NUMBER OF ORGANISMS PER LITER OF SUSPENSION — A LARGER NUMBER INDICATED GREATER STABILITY OF THE ORGANISM

TABLE XIX

Guinea Pig Exposure to Respiratory Challenge in Chamber B

Experiment Number	Dead/Tested	Estimated Challenge Dose of Organisms per Guinea Pig	Organisms per liter of Cloud	CT**
5	20/20	1000-6000	7200-40,000	0.006
6	10/10	4000	28,000	0.002
7	10/10	95	188	0.0005
9	7/10	45	312	0.00024
8A	8/9	20	130	0.00013
8B	8/10	12	82	0.000085
10***	1/9	10	67	0.000062

* The numbers of the Experiments correspond to those of Table XVIII

** CT: Mg x min/M^3 , assuming that 1×10^8 organisms weigh 1mg.

*** "UL" recovered from spleen of one guinea pig sacrificed ten days after exposure.

challenge, remained well after exposure in the cloud chamber to CT values of 0.5. These results are summarized in Table XVII.

b. The Henderson Apparatus (Chamber B)

In Table XVIII and Table XIX we have summarized experiments done with the British Cloud Chamber apparatus (Chamber B). Throughout these experiments the apparatus was operated by Dr. Henderson, Lt. N.A. Johnson and their co-workers. A description of the apparatus and details concerning its operation are to be found in BDP reports #10[23] and #13[24]. The safety features of this apparatus deserve favorable comment. The absence of "UL" infection among the operators during 10 separate trials speaks for itself.

Throughout these cloud chamber experiments the following factors were investigated; (a) the fate of the organisms within the nebulizer during its operation; (b) what factors influence the viability of "UL" when dispersed as an aerosol; (c) what information can be obtained regarding the infection of animals with "UL" via the respiratory tract.

It is well known that a certain amount of destruction of bacteria takes place within the nebulizer during operation. This is influenced to some degree by the kind of liquid in which the organisms are suspended. The cell counts of "UL" suspension prepared in several media before and after nebulizing, together with the per cent survival of "UL" in each instance, are tabulated in Table XVIII. The latter are seen to range from 7.2 to 98.8 per cent. The best results were obtained when "UL" was suspended in 0.5% saline containing 2% dextrin and 0.5% normal human serum. On three occasions, using the above solution, over 95% survival of "UL" was recorded.

In Table XVIII are included data relating to the question of stability of "UL" aerosols. Most information here is gained from inspection of the column of spray factors. These range from 0.05×10^{-6} to 0.78×10^{-6}. Since the spray factor of a more durable agent, such as "N", is in the neighborhood of 3×10^{-6}, it is evident that a large number of organisms in a "UL" cloud die quickly upon dispersal. (The estimated time necessary for an organism to pass from the nebulizer to the impinger is about 3 seconds.) One point which may require attention in the future is that the highest spray factors, 0.78×10^{-6} and 0.71×10^{-6}, were obtained when "UL" was suspended in a medium in which sodium chloride was absent (Experiment 1 and 2a column 5, Table XVIII).

Guinea pigs were exposed to "UL" clouds in five of the chamber experiments. From the results compiled in Table XIX it is evident that a fatal infection in guinea pigs may be induced by as few as 12 organisms. In experiment 10, Table XIX, no deaths attributed to "UL" occurred in the ten day period following exposure. The animals appeared well and accordingly were sacrificed. "UL" was cultured from the spleen of one of these animals and the pathogenicity of the recovered culture was confirmed by mouse inoculation. In view of the high susceptibility of guinea pigs to "UL" this pig was counted as a fatality.

Since the CT is the standard US method for expressing the cloud concentration of a BW agent we have calculated the CTs of "UL" in these particular experiments in order to provide easy comparison with the CTs of other agents. We do not feel that the CT is a very accurate method of expressing the effectiveness of a BW agent as an aerosol. The calculation of CT is based on the assumption that 1×10^{8} organisms weigh 1 mg. Inasmuch as a particular CT is arrived at by first actually measuring the number of viable cells per given volume of aerosol, it is just as easy and perhaps more accurate to speak of the effective concentration of a BW aerosol in terms of organisms per liter of cloud.

These findings suggest that a low salt content of the suspending fluid favors survival in the cloud. The best stability in the suspending media studied was obtained with 2% dextrin in 0.5% saline plus 0.5% normal human serum. Guinea pigs inhaling as few as 12 organisms succumbed to "UL" with pneumonic lesions and septicemia.

These cloud chamber studies were exploratory and the results obtained are not considered final. They do, however, indicate that the respiratory infectivity of "UL" for mice and guinea pigs is of the same order as that observed following parenteral challenge. These preliminary results further indicate that "UL" is relatively unstable when nebulized under the conditions studied, and emphasize the need for a thorough study of suspending media, types of nebulizers, improved impingers and impinger fluids, and the development of standardized equipment which will permit study of the effect of relative humidity, temperature, oxidation, and time on survival of organisms in the cloud.

2. Storage

The studies on the survival of "UL" on storage were begun by the B Group before the A Group was activated. Eventually the A Group took over this part of the work and the details of their work are given in Special A Report.[16] The results reported below may be considered as preliminary and exploratory.

Certain specifications were recommended by Lord Stamp regarding the bacterial count, stability under certain conditions of storage, and physical state of the material which was to be dispensed. A minimum count of 10^{10} virulent organisms per ml was considered necessary for effective use in a BW munition. The material should be stable at this count for at least two to three months.

According to the Munitions Division of Camp Detrick it was desirable that the material be stable in the dry state and at the fluctuating temperature of outdoor air. Dispersal in the moist state was considered possible but was contingent on the development of suitable munitions. On the other hand the difficulties of loading munitions with highly infectious agents in the dry state would probably be very great. Obviously an agent which was stable only under lowered and constant temperatures was less desirable than one stable under fluctuating temperatures.

Our experiments were therefore directed primarily toward studies, (a) of methods which would give very large numbers of organisms per ml; and (b) of conditions for storage which would preserve the maximum number of organisms.

Virulent organisms were cultured in embryonated eggs and in liquid culture media under varying conditions of aeration. In eggs, the average count attained as shown by a great many experiments was usually $10^{9.5}$/ml.

The highest counts reached in culture media under the conditions outlined below were usually 10^{10}/ml. These were as follows: peptone broth medium distributed in 3 liter or 10 liter lots in 6 liter Florence blasks or in 20 liter carboys; these were inoculated with young cultures containing sufficient organisms to provide an initial count of 10 million per ml; the flasks were shaken at 37° C for 24 hours. Although these counts were near the minimum required it seemed desirable to explore the stability of such cultures with the hope of improving the initial numbers after we knew the best conditions for survival.

a. Lyophilization.

The lyophilizing apparatus used in all the experiments reported below was a manifold type with hypervac pump efficient enough to lower the pressure to 100 micra within 2 minutes. The material was dispersed in vials, shell frozen under the conditions indicated, and after lyophilization sealed under vacuum, under nitrogen or in air as indicated in the respective tables.

The first experiments were done with a variety of strains grown on DCBA. The bacteria were scraped off, and suspended in sterile skim milk before lyophilization. The data on the counts before and after lyophilization and stability under storage at 6° C are shown in Table XX.

The per cent survival after 20 days was poor with all strains, Dieck Showing the best survival and Carr and Scherm the poorest. It was of interest that on continued storage in the lyophilized state the viable count progressively decreased. Under identical conditions of preparation a culture of "US" showed 100% survival. It seems likely from these results that the lack of stability of "UL" was traceable to its inherent fragility and not to the technic used. After 45 days storage the survival was so poor that it was considered unnecessary to continue the experiment. There was not enough difference between the strains to make it worth while to continue study on any one of them.

Table XXI gives the results on one strain, S, grown under the conditions outlined for the organisms in Table XX and suspended in skim milk as before. However, in this experiment duplicate vials were shell frozen in liquid air at -180° C, and in CO_2-methyl cellosolve at -75° C, and were lyophilized at room temperature and at -10° C. The vials were

TABLE XX

Survival of Six Virulent "UL" Strains After Lyophilization
In Skim Milk at pH 6.5

Culture*	Original Count/ml	% Survival after Lyophilization		
		5 days	20 days	45 days
Scherm	17.4×10^9	0.04	0.003	
Carr	25.1×10^9	0.05	0.000004	
Holt	18.0×10^9		0.001	
Coll	33.2×10^9		0.01	0.000001
Dieck	43.2×10^9		0.18	0.004
Camp	38.6×10^9		0.09	0.0002

* Vials were vacuum sealed and stored at 6° C after lyophilization.

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Table XXI

Survival of "UL" Strain SM21R3 Grown for Twenty Four Hours on DCBA, Suspended In Sterile Skim Milk at pH 6.5 and Lyophilized

Treatment	Count After Days of Storage*				
	0	6	28	42	73 Days
None (Control)	52.3×10^9/ml		30.7×10^9/ml 58%**		
Frozen Liquid Air Melted and counted	32.7×10^9/ml 62%				
Frozen Liquid Air Lyophilized at Room Temperature		0.55×10^9/ml 1.0%	0.32×10^9/ml 0.6%	0.38×10^9/ml 0.7%	
Frozen Liquid Air Lyophilized at -10° C		0.65×10^9/ml 1.2%	0.007×10^9/ml 0.001%	0.03×10^9/ml 0.05%	0.05×10^9/ml 0.08%
Frozen CO_2 Melted and Counted	8.3×10^9/ml 16%				
Frozen CO_2 Lyophilized at Room Temperature		0.06×10^9/ml 0.1%	0.006×10^9/ml 0.01%	0.21×10^9/ml 0.04%	
Frozen CO_2 Lyophilized at -10° C		0.114×10^9/ml 0.21%	0.013×10^9/ml 0.02%	0.013×10^9/ml 0.02%	

* All Vials stored at +6° C after processing.
** Percent survival.

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then stored at 6^{0} C. The liquid air froze the preparations much more rapidly than did the methyl cellusolve-CO_2 bath. Counts made immediately after shell freezing showed a 38% destruction of organisms frozen in liquid air and 86% destruction of organisms frozen in the CO_2 mixture. The survival after lyophilization was much better in the material frozen in liquid air and lyophilized at room temperature than when lyophilized at -10^{0} C. The material frozen in CO_2 maintained the numbers of organisms better when dried at -10^{0} C than when dried at room temperature. In all cases survival was not more than 1 per cent after lyophilization and therefore entirely impractical on the basis of B" standards. In contrast to the results of lyophilization, one control vial, not frozen nor sealed, and placed in the ice cabinet at $+6^{0}$ C gave 58 per cent survival when counted after 28 days storage.

Table XXII gives the results of a comparison of the survival of "UL" grown in eggs and in Snyder's medium and stored frozen or in the lyophilized state. These results confirm those obtained in other experiments, namely, that "UL" survives better when stored frozen than when lyophilized. Other experiments done at Kansas and here have failed to show that peat or ascorbic acid-gelatin influenced survival favorably. Numerous experiments have confirmed the results of Stamp[25] that "US" survives well when dried in ascorbic acid-gelatin mixtures but all attempts by the A and B divisions to use this method for "UL" survival showed that "UL" did not survive well under the conditions that were favorable for "US" survival.

b. <u>Survival in the Moist State.</u>

Sixty embryonated eggs were inoculated by way of the yolk sac with the virulent S strain of "UL". All eggs in which the embryos died on the third day were divided into 3 lots and stored in the shell at -40^{0}C, at -4 to -6^{0} C and at room temperature. Two whole eggs were harvested before storage and shaken in a bottle with glass beads for 30 minutes. Plate counts and mouse titrations done on these two eggs served as controls on the stored eggs. At intervals 2 eggs were harvested from each stored lot and counts made as above. Figures 6, 7, and 8 give the survival time expressed as colony counts on DCBA plates and as mouse LD_{50} per ml. These results show that when tested after 35 days and after 56 days the numbers surviving at -40^{0} C and at -4^{0} to -6^{0} C were about equal. There was a rapid and progressive decrease in the numbers of organisms in eggs stored at room temperature. The decrease in numbers of organisms on storage as shown by plate count paralleling the numbers of organisms required to give a mouse LD_{50}, indicating that the virulence of the surviving organisms was maintained.

When "UL" organisms were stored in the frozen egg for a period of time there was a progressive drop in total number of living virulent organisms as shown by the experiment above. Is the rate of death accelerated by thawing the frozen egg? This question could not be answered by any technic known to the authors. How stable is "UL" upon continued storage in

Table XXII

Survival of "UL" From Infected Eggs and From Peptone Broth Cultures When Lyophilized or Stored Frozen in CO_2

"UL" Cultures	Original Count/ml	Count/ml Frozen in CO_2 -28 Days	% Survival	Count/ml Lyophilized 31 days	% Survival
Whole Egg	5390×10^6	1.7×10^6	0.31	2.4×10^3	0.00004
Yolk	390×10^6	1.74×10^6	0.45	19.7×10^3	0.005
Whole Egg & Gelatin Ascorbic Acid*	6000×10^6	6.2×10^6	0.10	1.22×10^6	0.02
Egg Plus Basic Peat**	3100×10^6	88×10^3	0.003	Sterile	0
Snyder's Medium	162×10^6	2.3×10^3	0.002	0.95×10^3	0.0007
Snyder's Medium & basic Peat***	1620×10^6	Sterile	0	Sterile	0

* Equal volumes minced egg and 10% gelatin ascorbic acid mixture. pH 7.1.
** 3 ml minced egg added to 1 gm of powered basic peat.
*** 3 ml Snyder's Medium added to 1 gm of powered basic peat.

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the thawed egg? To answer this question the following investigation was undertaken:

When "UL" counts and LD_{50} titrations were completed on frozen eggs represented in Figures 6, 7, and 8 the minced specimens were put away in a cold box at $+4$ to $+6^{\circ}$ C. At intervals thereafter these same specimens were removed from the cold box, shaken and recounted. The mouse LD_{50} values are given in Table XXIII.

Data in Table XXIII indicate that in 4 out of 5 eggs the numbers of virulent "UL" decreased to about 1 per cent within 29 to 49 days after being thawed and stored further at $+4$ to $+6^{\circ}$ C. One out of 5 eggs showed no decrease in count over the same period. On the basis of these findings the death rate of virulent "UL" did not appear to be accelerated by thawing and continued storage in the liquid state.

Minced infected eggs, whose embryos had been removed, and a heavy suspension of "UL" in 10% gelatine were each mixed with the following materials and stored at room temperature and at $+4$ to $+6^{\circ}$ C: Glycerine, buffered glycerine, gelatin and ascorbic acid,(25) brass filings, asbestos, Fullers earth, glass dust. Counts made at once showed a large number of organisms to be present. Counts made after storage for 3 weeks at <u>room temperature</u> showed no survivals in any carriers. Counts made after 3 weeks storage at <u>ice-box temperature</u> showed that the best survival of "UL" in egg material occurred when the latter was suspended in gelatin and ascrobic acid, in brass filings and in Fullers earth. A pure culture of "UL" in glycerine survived best alone, or when suspended in basic peat. None of these preparations sufficiently stable to meet BW standards and were not investigated further.

It was felt that some fatty substance might stabilize "UL" by forming a protective coating around each organism. Storage in egg yolk appeared to improve stability and this may have been because of the fat content of the yolk. The organisms were known to have a high content of ether-soluble and alcohol-soluble materials. When placed on the skin of an experimental animal "UL" was not destroyed by application of strong disinfectants within 10 seconds after exposure. This suggested that the organisms penetrated the skin within 10 seconds, which seemed very unlikely, or else they were protected from the killing action of the disinfectant by some secretion of the skin. An attempt was made to survey the effect of fatty substances on the survival of "UL".

A 24 hour peptone broth culture was mixed in equal portions with various fat and oil emulsions and stored at room and at ice-box temperature. Counts were made at weekly intervals. The following substances were tested: soya bean, cottonseed, coconut, mineral and castor oils, lard lanolin, and alcohol-ether extracts of guinea pig muscle, liver and adipose tissue.

A sample survival chart of the results of these experiments is shown in Figure 9 in which the results of "UL" storage in 50% soya bean oil

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FIGURE 6
SURVIVAL OF "UL" IN INFECTED EMBRYONATED CHICKEN EGGS STORED AT ROOM TEMPERATURE

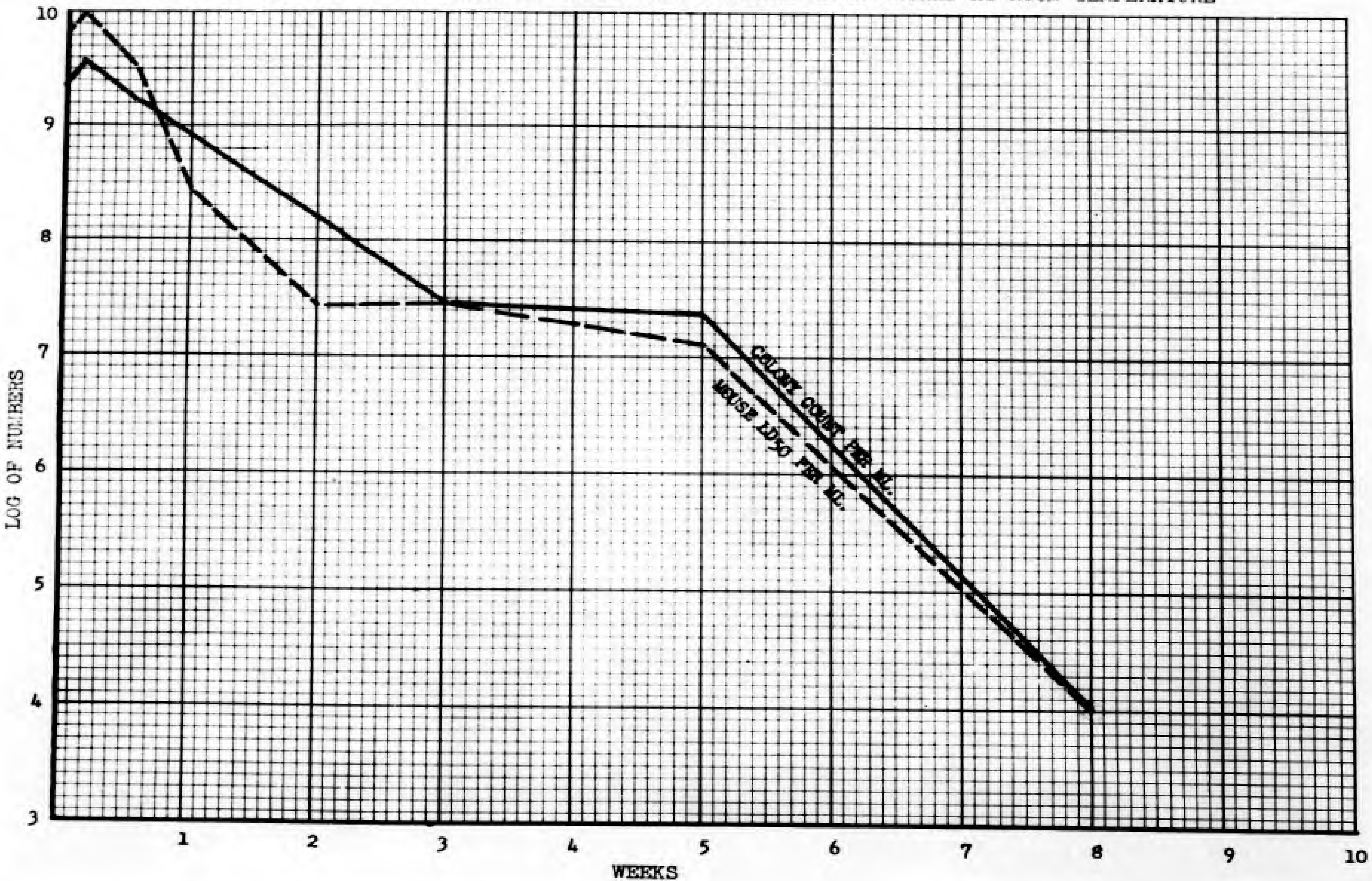

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FIGURE 7

SURVIVAL OF "UL" IN INFECTED EMBRYONATED CHICKEN EGGS STORED AT -4° to -6°C

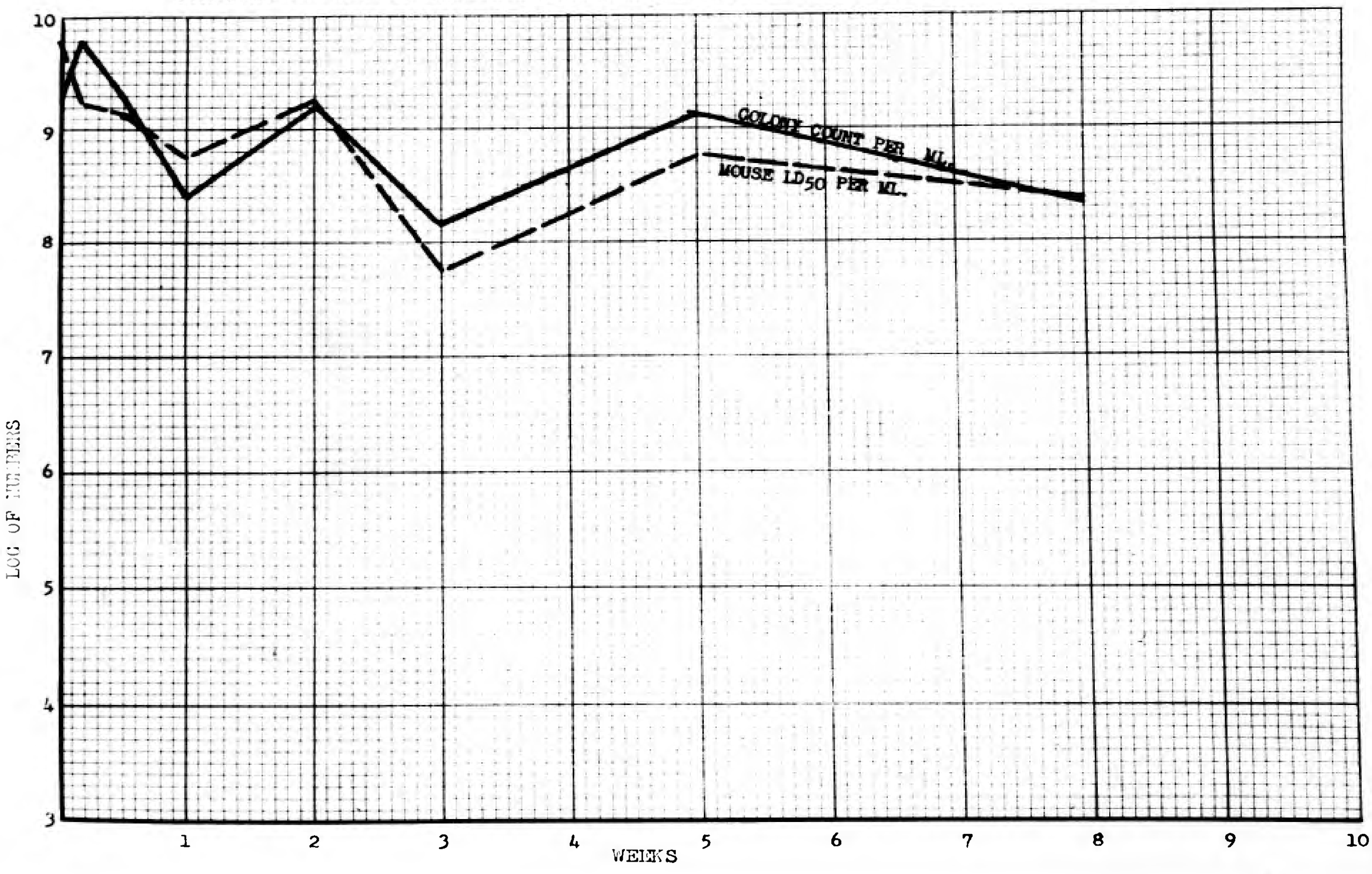
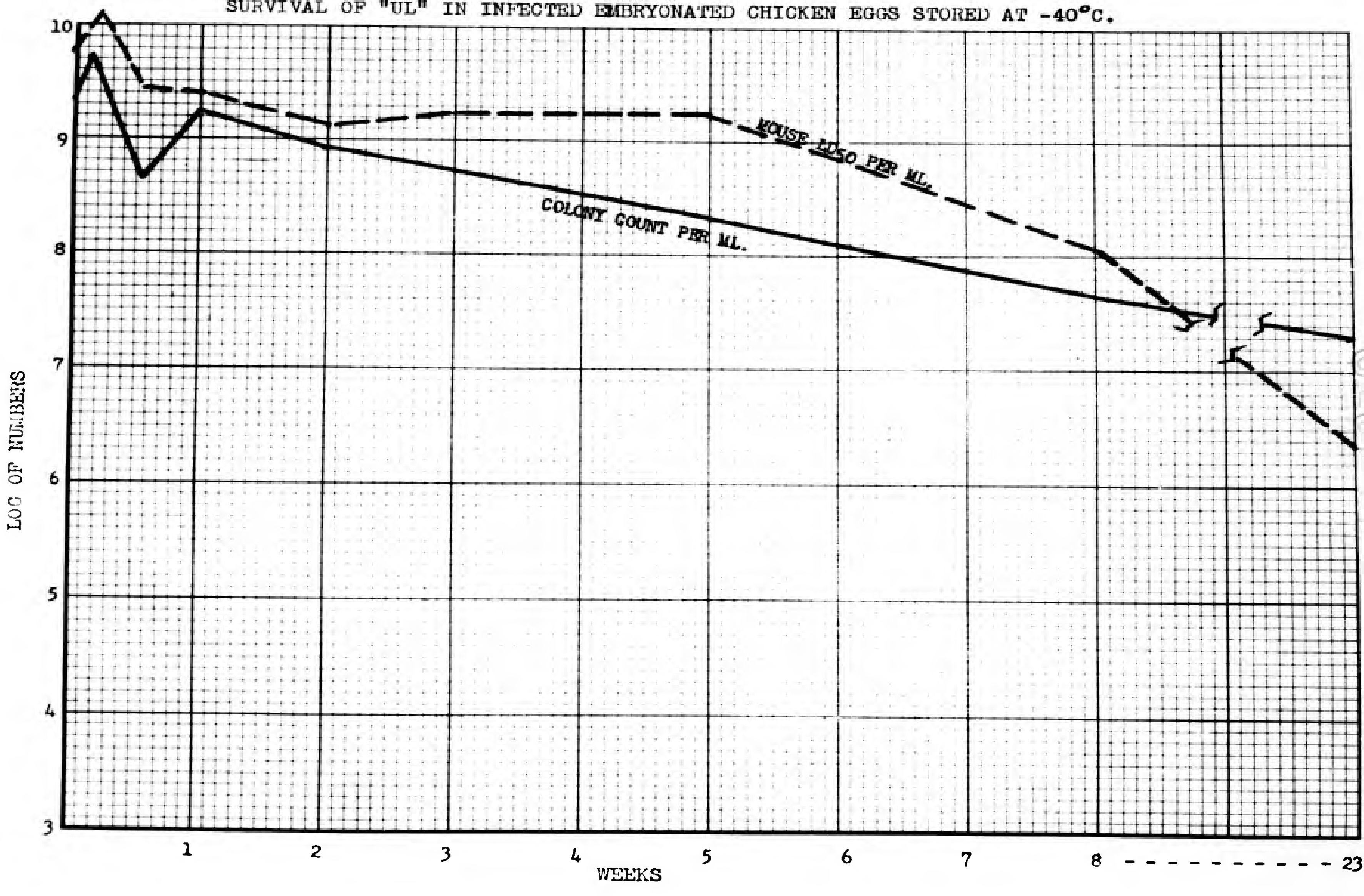


FIGURE 8

SURVIVAL OF "UL" IN INFECTED EMBRYONATED CHICKEN EGGS STORED AT -40°C.



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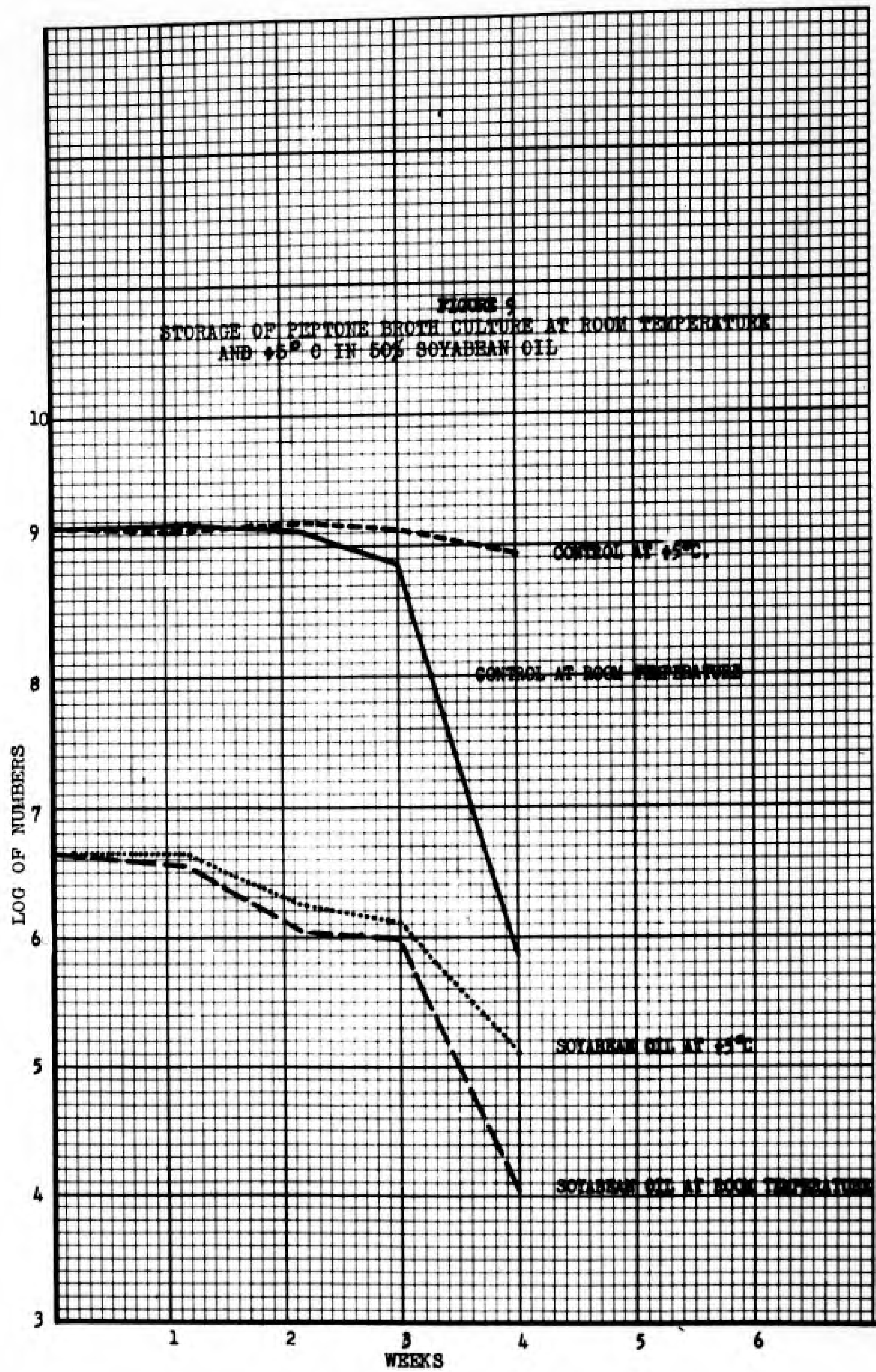

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Table XXIII

Effect of Thawing and Continued Storage at Temperatures above Freezing on Survival of "UL" in Embryonated Eggs

Serial Number	Days Frozen	Mouse LD_{50} When Thawed	Days Stored at +4 to +6°C After Thawing	Mouse LD_{50}	Total Days Since Inoculation
50	38	$10^{-8.6}$ *✓	49	$10^{-6.4}$	87
51	38	$10^{-8.45}$	49	$10^{-6.74}$	87
52	59	$10^{-8.23}$	29	$10^{-7.74}$	88
53	59	$10^{-8.19}$	29	$10^{-7.55}$	88
73	59	$10^{-7.48}$	29	$10^{-7.74}$	88

* Survival figures are expressed as dilution containing one LD_{50} per ml for mice

✓ Simultaneous plate counts on all eggs gave close correlation between numbers of organisms and LD_{50}/ml

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are shown. Of all the substances tested best results were obtained with soya bean oil and with lanolin although neither of the "carriers" promoted better survival than that observed in the control, (peptone broth only). It would be of interest to pursue further studies with lanolin because of the information it might give on the mechanism of skin penetration. The storage and survival experiments were taken over by the A Division and no more work was carried out by the B group.

In summary of the preliminary B Division work on storage of "UL" the following points can be stated: (1) "UL" did not survive the lyophilization process in sufficient numbers to be practical; (2) quick and slow freezing both caused a drop in numbers of organisms, although rapid freezing appeared to be less destructive in one experiment; (3) "UL" survived poorly when stored in the moist state at fluctuating room temperatures; (4) best survival was obtained in liquid peptone broth culture or in embryonated egg cultures stored at ice-box temperatures; (5) no method of storage tested gave survival adequate to meet minimum BW munitions standards.

c. Electron Microscopy of "UL"

A recent report on the morphology of "UL" by Hesselbrock and Foshay (75) suggested that a study carried out with the aid of the electron microscope would be of great value in the interpretation of the multiple morphological features of this organism. The resolving power of the electron microscope would permit precise observation of the cell wall and would be of value in the examination of forms whose size and shape are not discernible by ordinary microscopic techniques.

The extremely high mortality and complete fragmentation of "UL" during sonic vibration as well as the high mortality incurred through the lyophlization process, suggested that this organism might possess some unusual morphological feature to account for its extreme susceptibility to these procedures. This work was carried out in port at the Johnson Foundation, U. of Pennsylvania by Dr. L. A. Chambers, Camp Detrick consultant in Biophysics.

1. Method

For the present investigation, three varieties of liquid media were used for the cultivation of the organism, namely; (1) peptone broth (14), (2) gelatin hydrolyzate, Tamura and Gibby (6), (3) soya bean hydrolzate, Foshay (30), as well as a solid medium, dextrose cysteine blood agar. The incubation period was varied to obtain cultures with a variety of morphological forms.

No differentiation between 43 strains, including both virulent and avirulent varieties, could be made on the basis of morphology described by Hesselbrock and Foshay (75). In our studies, two virulent strains, Schu and Memph were used.

Various procedures were followed in preparing cultures for electron microscopic study. Suitable suspensions were prepared from solid

medium by one of the following techniques:

a. A small amount of surface growth was removed and suspended in sterile physiological salt solution or in sterile distilled water. The suspension was usually incubated at 37° C for 5 hours.

b. A few ml of sterile physiological salt solution were pipetted onto the growth of a slant culture which was then allowed to incubate for a few hours at 37° C until a slight turbidity appeared.

c. A small amount of surface growth was removed with a sterile wire and passed three or four times through a wire loop containing a film of sterile distilled water until a fairly uniform suspension resulted.

d. Suspensions from liquid media were prepared by removing a small amount of culture with a capillary pipette and mixing it with sterile distilled water. Occasionally the culture was used without dilution.

e. A drop of the suspension prepared by the previously described techniques was placed upon the collodion electron microscope mount by means of a capillary pipette or wire loop and allowed to dry in air. (76)

2. Description of Electron Photomicrographs Presented in Plates I and II

1. Saline suspension prepared from 24 hr glucose cysteine blood agar culture and reincubated at 37° for 20 hours. Cluster of coccoid forms showing peripheral arrangement of chromatin.

2. Same. A. Form showing peripheral chromatin mass and protoplasmic streamer or false "flagellum"

B. Form suggesting binary fession

C. Small coccoid form with extremely delicate cell wall

3. Same. Large coccoid form with irregular chromatin arrangement and extremely delicate cell wall.

4. Saline suspension prepared from 96 hr glucose cysteine blood agar culture and reincubated at 37° for 5 hrs.

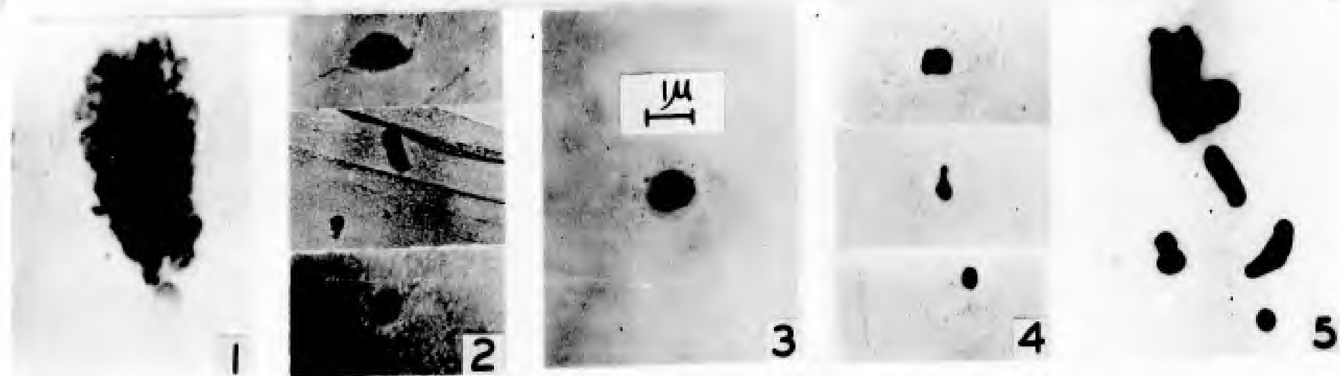
A. Bacillary form and adjacent coccoid form

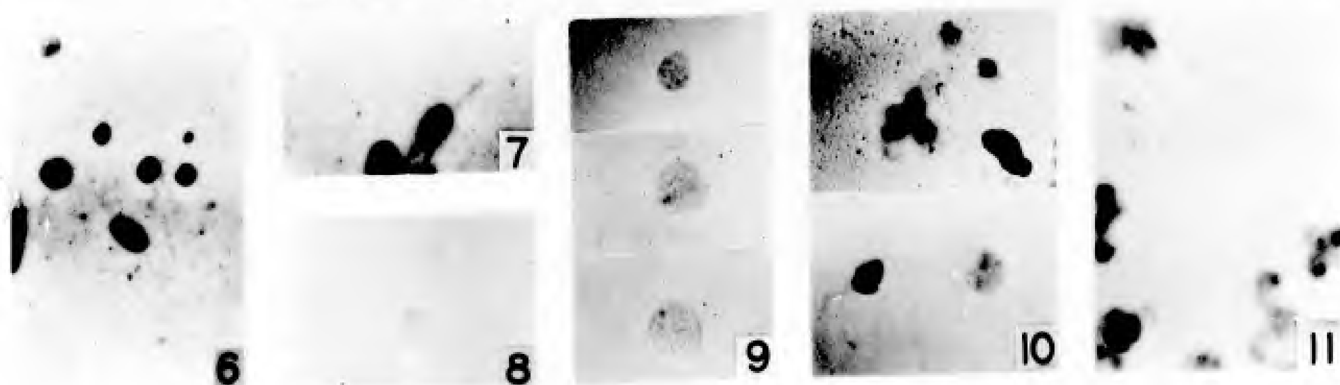
B. Filamented coccoid form with small spherical chromatin body at tip of filament

C. Typical small globule of most prevalent size; 0.45 to 0.5 y in diameter.

PLATE I

Electron Micrographs of "UL" Reproduced
with Slight Reduction from 6000 Magnification:


1μ
1
2
3
4
5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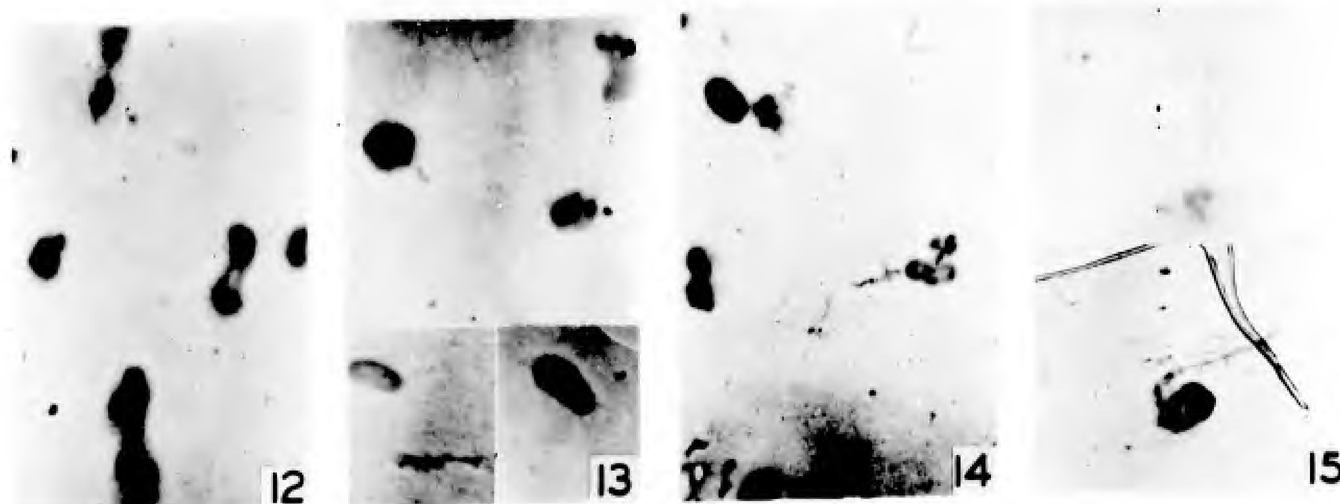
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13
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15

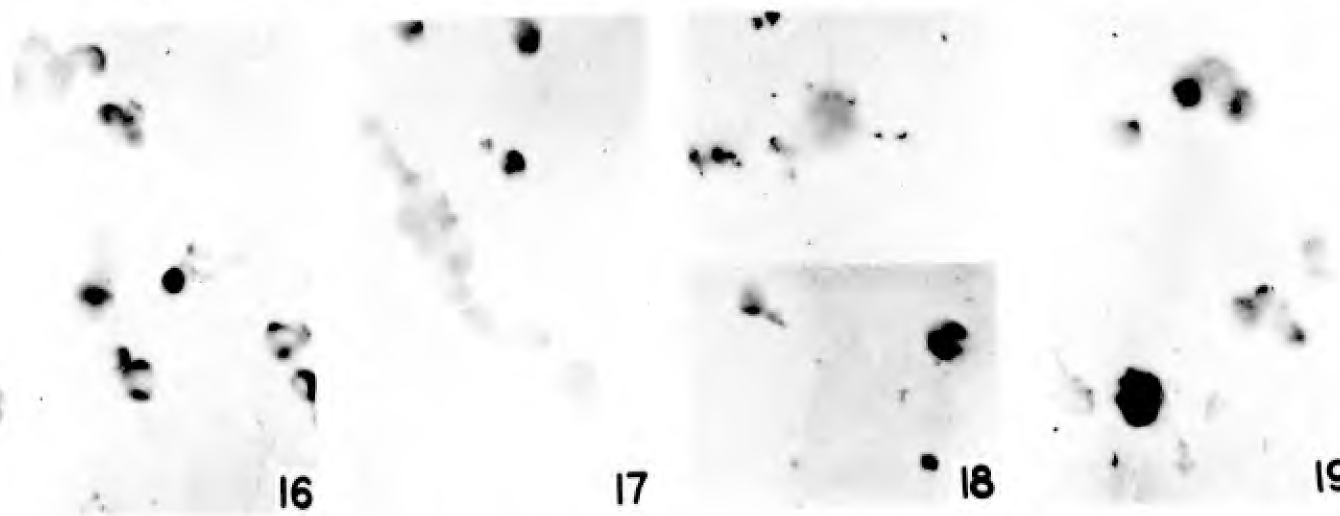
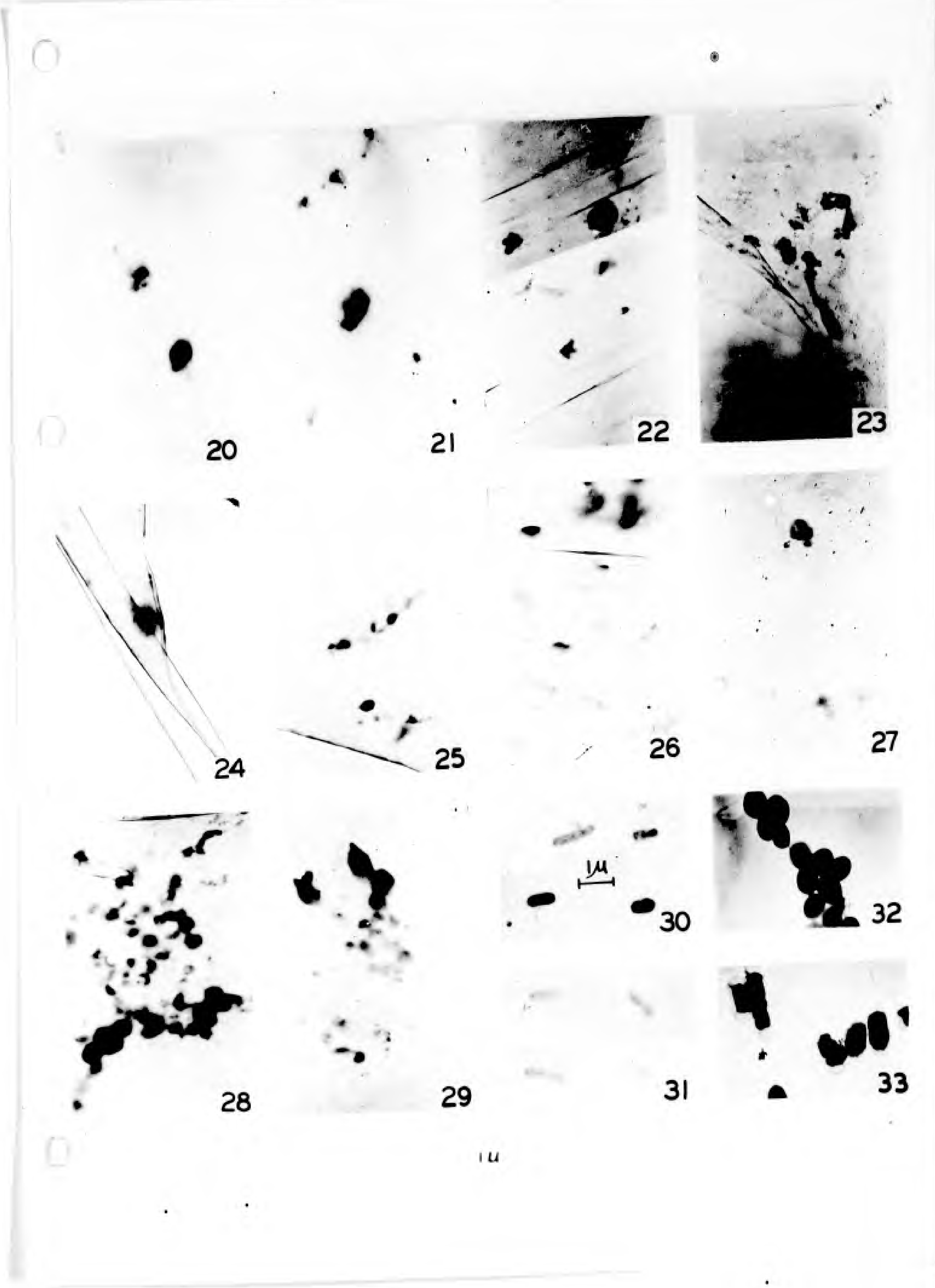

16
17
18
19

PLATE II

Electron Micrographs of "UL" 20-29 incl. (6000x); 30 (washed murine typhus, 6500x); 32 (staphylococcus aureus, 6500x); 33 (typhoid "R", 6500x) with approximately 60 percent reduction from initial magnification.


20
21
22
23
24
25
26
27
28
29
1μ
30
31
32
33

5. Peptone broth culture incubated at 37° for 24 hrs. Large and small coccoid and bacillary forms. Note so-called "involution" form at right.

6. Same. Note variation in size of coccoid forms. Smallest form at upper right is approximately 210 mu in diameter.

7. Same. Filamented bacillary form.

8. Distilled H_2O suspension prepared from 24 hr cysteine blood agar culture and reincubated at 37°C for 5 hrs. Coccoidal form with single peripheral chromatin mass.

9. Same. Disintegrating coccoid forms. Note minute granular chromatin concentrations and "punched out" appearance.

10. Same. A. Cluster of irregular coccoid forms with extremely delicate cell walls. Note another of the so-called "involution" forms.

B. Two coccoid forms. Contrast more dense form with disintegrating form at right.

11. Soybean hydrolzate culture incubated at 37°C for 4 days. Long delicate filament with rather dense chromatin granule near upper tip. Note small drumstick form at left.

12. Same. Large dumbbell shaped structure with spherical chromatin concentrations at terminal ends. Note possible early budding of form at left and filamented form at upper left.

13. Same. A. Note crescent shape of form at upper right and peripheral chromatin arrangement of form directly below.

B. Large Oval forms.

14. Same. Oval form with adjacent partially disintegrated cell. Suggestion of binary fission by cell at right. Note finely beaded filament near center of electronmicrograph and drumstick form at lower left.

15. Same.

A. Long delicate filament

B. Oval form with long branching filament. Note two minute forms directly above. The larger is approximately 110 mu in diameter.

16. Same. Large coccoid forms with peripheral chromatin concentrations. Note fine filament arising from cell at lower left.

17. Same. Chain of large bacillary forms. Note bipolar arrangement of chromatin within of a number of cells.

18. Same. A. Irregularly shaped coccoid mass with pseudopod projections. Structures of extremely low density such as this could be seen in most of our preparations. Suggests a ruptured cell with contents flowing into surrounding area.

B. Note crescentic chromatin arrangement of cell at right and irregular projection from cell at left.

19. Same. Comma shaped form with large concentration of chromatin at terminal ends.

20. Gelatin hydrolyzate culture incubated at 37°C for 8 days. Granular coccoid form with granular filamentous projection. Note apparent budding of form at right.

21. Same. Large coccoid form with extremely delicate cell membrane. Note chromatin granule in filament in form above.

22. Distilled H_2O suspension prepared from colony on glucose cysteine blood agar which had been incubated at 37°C for 3 days and allowed to remain at room temperature overnight. Folds in the collodion membrane are visible.

A. Large coccoid form with patchy chromatin distribution.

B. Rupture of cell and dispersion of granular contents into surrounding area.

23. Same. Group of so-called "involution" forms.

24. Same. Large coccoid form. Contrast sharpness of collodion film edge with the nebulous character of cell. Note that a part of the folded film edge can be seen through the semi-transparent cell.

25. Same. Chain of small coccoid forms. Note distribution of cellular debris in surrounding area produced by cellular rupture.

26. Same. Large coccoidal form in right center with protoplasmic streamer or "false" flagellum. Note evidence of sessile budding directly below. Many disintegrating forms are visible.

27. Same. Another coccoid form suggesting budding. Below: ruptured cell and protoplasmic streamer of "false" falgellum.

28. Same. Cluster of coccoid, crescentic and oval forms.

29. Same. Cluster of coccoid and oval cells of various sizes. Note the nebulous character of cells emphasizing lack of density of this organism.

30. Washed Rickettsiae; 8100 x. Note define morphology and cell wall.

31. Washed murine typhus rickettsiae, 6500 x. Contrast the cell walls of the organisms shown in Figs. 30 and 31 with the extremely delicate cell walls of "UL" shown in Figs 2, 3, 19, 20 and 21.

32. Staphlococcus aureus: 4000 x. Note density of these cells in contrast to low density possessed by cells of "UL" in Figs 1, 28, and 29.

33. Eberthella typhosa "H", 6500 x. Again contrast cell wall and density of this organism with the cell wall and density of "UL".

3. Observations

Most frequently observed during the study with both liquid and solid media was a small coccoid form 0.45-0.5 microns in diameter, although large coccoid, large and small bacillary, oval, minute, and filamented forms were often seen. Less frequently observed were bean shaped, dumbbell, bizarre and so-called "Involution" forms which usually represented but a small portion of the total population. Finely filamented forms were more frequently observed in 5 day broth cultures than in younger broth cultures or cultures on solid medium. Occasionally delicate filaments attached to the cell, or broken and free, were observed. Frequently these filaments contained minute cytoplasmic concentrations described and termed "minimal reproductive units" by Hesselbrock and Foshay (75). The electron micrographs demonstrated areas of greater electronic density in the concentrated periphery of many coccoid and bacillary forms. These were similar in size, shape and location within the cell to chromatin containing structures described by these authors. The various morphological forms appeared singly, in diploform or in short or long chain formation.

During the course of this study minute forms of 250 mu or less in diamter were observed. Foshay and Hesselbrock (1945) (74) reported that morphologic units of "UL" passed the 600 mu Elford gradecol membrane but not the 500 mu membrane and were therefore in the range of 300 to 350 mu in diameter. These investigators postulated that units of smaller size exist. A minute morphological form, approximately 110 mu in diameter, is shown in Plate 1, Figure 6. In Plate 1 Figure 15 a form approximately 210 mu in diameter is shown. No morphological differentiation with the exception of size could be demonstrated between the minute forms and the usual coccoid forms.

Examination of the elctron photomicrographs presented in Plates I and II revealed that although photomicrographs were sharp as evidenced by the edge of the collodion film seen in various preparations the cells generally presented a hazy nebulous appearance. "UL" seemed to possess an

extremely delicate outer limiting structure (cell wall) of very low electronic density in contrast to the cell walls of various other organisms described by Wamoscher (1930) (79) and reported to be "extremely solid, elastic, extensible, and enormously resistant to pressure." Usually the cell wall enclosed the cytoplasm so closely that the two were not discernible. The extremely delicate structure of the cell wall of "UL" is best illustrated in photomicrographs showing large coccoid and large bacillary forms whose cytoplasm is unevenly distributed allowing the cell wall to be more clearly visible. Usually it was difficult or impossible to differentiate the limiting edge of the delicate cell wall from the surrounding area. Protoplasmic streamers or "false" flagellar indicating a break in the cell wall were frequently observed. Although semi-transparent cytolyzed cells were often observed and carefully examined, jagged lines of fracture which would indicate the presence of a solid or rigid cell wall were not demonstrated. These "ghosts" exhibited less opacity to the electron beam than did the mechanically cytolyzed Bacillus subtilis and Bacillus anthracis cells described by Mudd and associates (77) and the mechanically cytolyzed streptococcal cells described by Mudd and Lackman (78).

From these observations, it seems likely that the delicate structure of the cell wall of "UL" might at least partially account for the high mortality rates incurred during sonic vibration or lyophilization processes.

4. Summary

In general "UL" possesses multiple morphological units including large and small coccoid and bacillary, oval, minute, filamented, bean-shaped, dumbbell, bizarre, and so-called "involution" forms. The suggestion of the existance of minute morphological units or less than 300 mu in diameter was confirmed. The typical cell possesses little opacity to the elctron beam and presents a semi-transparent nebulous appearance. Critical examination for a cell wall revealed an extremely delicate structure of very low electronic density which possibly accounts for the low survival rate when subjected to sonic vibration or lyophilization process.

D. Rapid Detection of "UL"

Since the problem of rapid detection is of prime importance in the event of suspected BW attack and also in the early diagnosis of suspected cases of "UL", attempts have been made to develop methods for the rapid identification of "UL".

The methods usually described for the isolation of "UL" from clinical material involve the injection of guinea pigs or rabbits. These animals usually die in 4-7 days and show typical gross pathological changes in the liver and spleen. The organisms may be isolated from the animals at autopsy by inoculatinn of suitable media with heart blood, spleen or liver. Typical "UL" colonies appear after 2 or 3 days incubation. A more rapid detection method would be very desirable.

Much progress has been made in the preparation of media which will give quicker and more abundant growth of "UL" than did the older media. If

means could be developed to eliminate or inhibit the growth of contaminants, such media could be used for the detection of "UL".

Three methods for elimination of contaminants have been tried with success, namely, (1) use of bacteriostatic agents harmless to the pathogens sought, (2) injection of contaminated material into animals and (3) filtration.

Experience has shown that "UL" colonies may be obtained with an inoculum of as few as 1-2 organisms on the DCBA media described in this report on page 19. Previous work done by the "UL" group had shown that penicillin and sulfadiazine did not inhibit "UL" in concentrations lethal for many common contaminants.

Tyrothricin was also tested but it was found to be lethal for "UL" in one hour at a concentration of 0.025 mg per ml. Agents "N", "BG", and "SM" survived when tested against this concentration of tyrothricin. Concentrations of 1-1,000,000 crystal violet inhibited the growth of "UL" and did not inhibit all contaminants. Tyrothricin and crystal violet were therefore considered unsatisfactory for the differential elimination of contaminants from specimens containing "UL".

Further work was done on the use of penicillin and on penicillin and sulfadiazine combined. It was necessary to determine the limits at which the above antibiotics could be used without inhibition of "UL", and the effect of the presence of contaminants on the isolation of "UL". The last point was of great importance since presumably most of the material to be examined in the field would be grossly contaminated. The results presented in table XXIV were obtained by exposing suspensions in saline of different organisms to various penicillin concentrations for 1 hour at room temperature. The suspensions were then plated out on DCBA plates and observed for growth. The data show that "UL" was not inhibited by 500 U of penicillin when exposed for one hour at room temperature, whereas 1 U of penicillin completely inhibited the growth of "N" and 10 and 100 U partially inhibited Staph. aureus.

Penicillin inhibited most but not all organisms found in samples of soil when the organisms were suspended in normal saline and exposed to 250 or 500 units of the drug for 1 hour. When tested in the same manner, sulfadiazine in concentrations of 50 to 250 mgm per cent also inhibited most of the contaminants in soil. When sulfadiazine or penicillin were incorporated in the DCBA medium they were not as effective in inhibiting contaminants and interfered more with the growth of "UL". A sample titration of the combined effect of penicillin and sulfadiazine is shown in Table XXV. One gram of soil was added to each of several tubes of saline containing 1.0 ml of 10^{-7} diltuion of "UL" and 500 units per ml of penicillin. Various amounts of sulfadiazine were added also as shown in the table. All tubes were incubated for 1 hour and then plated on DCBA. One tube was filtered through a Selas .01 filter (maximum pore diameter 7.5 microns) before being plated.

TABLE XXIV

Titration of "UL", Staphylococcus Aureus and "N" Susceptibility to Penicillin

Organisms	BCBA Plate Count After Exposure to the Following number of Units of Penicillin/ml						
	None	0.1	1.0	10	100	250	500
"UL" Colonies	149*	-	138	274	183	276	385
"N" Colonies	4+	4+	0	0	0	0	0
Staph. aureus	4+	4+	4+	2+	1+	0	0

* Number of colonies per plate or 1+ to 4+ indicating increasing number of colonies too numerous to count

TABLE XXV

Selective Inhibition of Soil Contaminants by Penicillin and Sodium Sulfadiazine

Tube #	Soil	Penicillin Units/ml	Sodium sulfadiazine mg%	Plate Count Contaminant	Plate Count "UL"
1	None	None	None	None	17
2	1 gm.	None	None	Numerous	None
3	"	500	50	10	2
4	"	500	250	6	7
5	"	500	500	9	15
6*	"	500	50	None	6

* Treated same as tube #3 then passed through Selas .01 filter before plating.

The results in Table XXV show that the combined effect of 500 units of penicillin and 50 mgm per cent of sulfadiazine effectively controlled soil contaimants and permitted recovery of "UL". For this particular soil sample fitlration through a Selas .01 filter removed all the contaminants not eliminated by penicillin and sulfadiazine. Since some of the organisms contained in soil are spreaders, one surviving contaminant may sometimes overgrow a plate and interfere with the growth of "UL". In such cases the added step of filtration might be necessary. Filtration alone, before treatment with penicillin or sulfadiazine, did not removed all contaminants.

In order to determine the smallest number of organisms detectable by mouse inoculation in a short time groups of white mice were injected with decreasing numbers of organisms. The animals were sacrificed 12, 18, 24, and 48 hours after inoculation and the hearts blood and spleen cultured on DCBA plates. The plates were scanned in 24 hours and at short intervals thereafter. When any indication of growth appeared gram stains were made and as soon as possible sufficient growth was scraped up to emulsify in immune serum for a rapid slide agglutination test. Table XXVI gives a summary of the results obtained on DCBA. Bacto cystine heart agar plus Bacto hemoglobin and Snyder's peptone agar were also used. They were as satisfactory as DCBA with heavily infected spleens but less so when the infection was light.

When barnyard soil was mixed with "UL" and injected into mice many of the latter continued to harbor soil organisms. These later grew out on spleen culture plates, thus masking the presence of "UL". This difficulty was overcome by adding 250 units of penicillin to each ml of soil suspension of "UL" 30 minutes before the mice were injected. The earliest time at which positive cultures could be obtained from the spleen of sacrificed animals appeared to be proportional to the total number of organisms injected. In other words the time necessary for the organisms to become established in the spleen was proportional to the size of the infecting dose. This has a practical value in detection work, since the speed of detection depends on the number of organisms injected.

Experimentally, if 2000 to 5000 organisms were injected into a mouse, the animal could be sacrificed in 12 hours and a positive spleen culture obtained after 18 to 36 hours incubation. If 15 to 50 organisms were injected, the highest percentage of positive cultures were obtained from animals sacrificed 36 to 48 hours after injection. If it were desirable to attempt detection of 1 to 2 organisms in an unknown specimen, one should wait 72 hours after injection before sacrificing the animal. Regardless of the number of organisms injected, however, animals always survived 2 to 4 days after spleen cultures became positive. Therefore approximately 3 days were gained by sacrificing the animals rather than waiting for them to die of infection.

Precipitin tests were performed on the peritoneal fluid collected from mice at the time of sacrifice, in experiments similar to those described above. The immune sera used were high titered human and rabbit sera. The rings of precipitate were weak after 2 hours at room temperature and further work indicated that the test was not specific nor delicate enough to justify its use.

An attempt was made to use the skin test for the rapid detection of "UL". This is reported in the B Division Report for May 1944.[26] Further studies on guinea pigs indicated that the test gave nonspecific and equivocal reactions. Other animals used were rabbits, rats, and mice, but the results were not any more clear cut in these animals.

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Table XXVI

Detection of "UL" by Mouse Injection and Subsequent Sacrifice for Culture of Spleen and Heart's Blood

No. of Organisms Injected I.P.	Hour Sacrificed	Hours of Incubation of Plates After Sacrifice*	Heart Culture	Spleen Culture
21,000	12	24	+	+
2,100	12	36	+	+
3,010	12	48	-	+
301	12	48	-	+
30	12	48	-	-
3,010	18	24	+	+
301	18	24	-	+
30	18	24	-	+
1,530	18	66	+	+
153	18	66	+	+
15	18	66	-	+
1,530	24	28	+	+
153	24	28	+	+
15	24	28	-	-
1,530	48	24	+	+
153	48	24	+	+
15	48	24	+	+
84 in soil	12	48	C	C
84 in soil**	12	48	+	+

* The earliest time at which the culture of "UL" could be certainly recognized.

** 250 U of penicillin per ml of soil and organisms added 30 minutes before injection of mice.

C Grossly contaminated.

Final recommendations for rapid detection of "UL" from clinical specimens or contaminated air, soil, water or fomites are as follows: Inject 1 ml of specimen or saline washings thereof intraperitoneally into each of 4 white mice. If the specimen is grossly contaminated with other organisms which might be pathogenic to mice, it may be incubated for ½ hour with 250-500 units of penicillin and/or 50 mgm per cent of sodium sulfadiazine before animal injection. The animals may be sacrificed 12, 24, 48 and 72 hours after injection and spleen cultures made on CBA (See section on media, this report). The cultures are incubated at 37° C, and when typical colonies of "UL" appear they are examined by the Gram stain and confirmed by the rapid slide agglutination test. If animals are not available, direct culture on CBA after treatment with penicillin and sulfadiazine may be substituted for the mouse injection. However, in inexperienced hands this procedure is apt to be less reliable than the mouse technique.

In diagnosis of cases, speed in isolation of the organism is also highly desirable because a specific antibiotic is now available for treating cases of "UL". The methods outlined above are applicable to sputum, exudates from local lesions and pus. For blood cultures it is recommended that guinea pigs or rabbits be used instead of mice and that 5 to 10 ml of fresh or defibrinated blood be injected intraperitoneally into each animal. Blood specimens should be taken at the time of chills or on a rising fever curve.

These recommendations were transmitted to the Safety Division which has confirmed and extended the results experimentally and in routine safety tests.

E. The Effect of Disinfectants on "UL"

Relatively little was known about the action of common disinfectants on "UL" when this work was started. Francis[27] stated that 0.1% formalin in saline suspension kills "UL" in less than 24 hours and that 1% tricresol sterilizes infected spleen material in 2 minutes. Downs [28] reported effective germicidal action of 70% alcohol, 5% phenol and 1% formaldehyde in less than 10 minutes using a "UL" suspension in saline and *in vitro* tests only. Foote and Steinhaus[29] reported that 0.35 ppm of available chlorine killed "UL" in 8 minutes when the organism was suspended in water. The TDP (Thermal Death Point) was known to be 55-60°C for 10 minutes when a saline suspension of organisms was under test.

It was necessary in view of the limited information available to find out what disinfectant would be most efficient and practical for laboratory use and what materials could be used as decontaminants in the field.

The recommended routine procedure in the laboratory was to autoclave or boil all contaminated glassware and equipment. In view of the report by Francis[27] that tricresol was effective we adopted the use of GI germicide (labeled 2-chloro-4-phenylphenol, ortho phenyl phenol, isopropylalcohol soap Phenol coefficient, FDA 5) in 1 or 2% solution as

a routine procedure.

The tests referred to in the literature have all been carried out with cultures suspended in water or saline. Since a high protein content is known to affect the action of disinfectants unfavorably, and since knowledge was desired concerning the action of disinfectants on contaminated egg material as well as in routine disinfection, the experiments reported below were done on both infected egg material and on suspensions of "UL" in saline.

In the search for a cheap and efficient disinfectant for laboratory use the following materials were tested on "UL" in infected minced egg and found to be ineffective in the concentrations shown.

Disinfectant	Concentration	Time of Exposure
Commercial soap flakes	1.0%	30 minutes
Diversol (Detergent)	10.0%	30 minutes
"Roccal"	10.0%	20 minutes

Table XXVII gives comparative tests on 4 disinfectants commonly used in the laboratory and on tincture of iodine which is frequently used as a skin disinfectant. Infected egg material shaken and containing 4.6×10^9 organisms per ml as shown by plate count was suspended in 9 parts of the disinfectant to be tested. The mixture was allowed to stand the designated length of time at room temperature then 1 ml was removed, diluted in 9 ml of saline and injected into 4 mice, 0.5 ml per mouse.

It is apparent that GI germicide, chlorox and HTH* were not effective. Phenol in 5% solution was effective and has been used routinely in this laboratory since these tests were made. Since Tr. of Iodine was effective it has been recommended for use as a skin disinfectant in case of laboratory accidents. It has been shown to be more effective than 5% phenol in preventing percutaneous infection in mice. (The studies in skin disinfection were done in part at Camp Detrick and in part at Kansas and to avoid repetition they are all reported in Final Report Contract #W-18-064-CWS-43, Kansas)(2).

While 5% phenol was an effective laboratory disinfectant, its use for large scale decontamination on the battlefield was not practical. The Chemical Warfare Service already had equipment, supplies and trained personnel in the field for using bleaching power, $Ca(OCl)_2$, to decontaminate areas exposed to mustard gas.

* HTH. High Test Hypochlorite, a refined bleach having twice as much available Chlorine (70%) as crude bleach (35%)

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Table XXVII

The Effect of Disinfectants on "UL" in Infected Minced Embryonated Chicken Eggs

After Exposure to the Disinfectant the "UL" Suspensions Were Injected into Mice.

Disinfectant		Minutes Exposure 1	10	30	% Efficiency
Germicide*	0.1%	4/4#	4/4	4/4	0
"	1.0%	4/4	4/4	4/4	0
"	5.0%	-	4/4	-	0
"	10.0%	-	1/4	-	75
Chlorox	1.0%	4/4	4/4	4/4	0
"	5.0%	4/4	4/4	3/4	8
H.T.H.**	0.5% Available Chlorine	-	3/4	-	25
"	2.0% Available Chlorine	-	1/4	-	75
Phenol	1.0%	4/4	4/4	2/4	16
"	5.0%	0/4	0/4	0/4	100
Tr. Iodine	7.5%	-	0/3	-	100

Control animals injected with disinfectant only remained healthy.

* Government Issue, labelled 2-chloro-4-phenylphenol, ortho phenyl phenol, isopropyl alcohol, soap. F.D.A. phenol coefficient 5.

** Freshly prepared and assayed for available chlorine by F.C. Division.

\# Fractions indicate the number of animals which died over the number injected.

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An experiment was set up to determine whether a solution of bleaching power would effectively kill "UL". Fresh 1.5% solutions of $Ca(OCl)_2$ were made up and tested at pH 12 and pH 8 by adding 1 ml of infected egg material to 9 ml of the disinfectant solution. At intervals shown in Table XXVIII sufficient 0.5 N Sodium thiosulfate was added to stop the action of the chlorine. One half ml was then injected intraperitoneally into each of 6 mice. Similar tests were done using a saline suspension of "UL" instead of the egg suspension. Control mice were injected with $Ca(OCl)_2$ and sodium thiosulfate only. All mice were observed twice daily and dead animals were autopsied and cultured to determine the cause of death. The results of the experiments are shown in Table XXVIII and indicate that 1.5% $Ca(OCl)_2$ solution killed all "UL" in a heavy egg suspension in three minutes at pH 12. At pH 8 five minutes were required to kill all the "UL". "UL" in saline suspension was killed more rapidly than "UL" in infected egg material.

In contrast to these findings with "UL", "N" spores were killed more rapidly at a pH value near neutrality (100). Since the "bleach" solution is normally strongly alkaline it could be used with maximal efficiency for "UL" decontamination without adjusting the pH.

Recommendations were made that if the enemy used "UL" against our troops available $Ca(OCl)_2$ could be employed for decontamination.

F. Immunization Against "UL"

At the beginning of the "UL" project it was evident that there was urgent need for the development of methods which could be used to compare the efficacy of various vaccines. In the original authorization one of the criteria for the acceptance of a vaccine for human use was that such a vaccine would immunize suitable laboratory animals. Foshay(5)(30) had prepared two vaccines and had used them extensively in human trials. Analysis of his results indicated that this vaccine was of some value in protecting man against "UL" infection but it did not immunize rabbits, guinea pigs or mice. The successful use of an improved vaccine, provided by Foshay, as an immunizing agent in man is reported elsewhere in this present report, page 122 . Downs (31) using a formalized suspension of "UL" and intensive immunization had prolonged the life of rabbits challenged with "UL" but had not prevented infection and ultimate death of the animals. Downs, in the report of Sept 1, 1943(1) had suggested the use of rats as test animals because they had a greater degree of natural resistance(31a) than rabbits, guinea pigs or mice. The degree of natural resistance of rats was first shown by means of a complete MLD titration at Camp Detrick reported in DDD Monthly Progress Reports of Feb., 1944(32) and March, 1944.(33) A high degree of immunity in recovered and in vaccinated rats was reported in April, 1944(34) extended in May(35) and confirmed in the DDD Monthly Progress Reprt for June, 1944(36) The following results show the development of numerous vaccines and the immunizing value of these vaccines on various experimental animals.

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Table XXVIII

Effect of 1.5% Solution of Bleaching Powder ($Ca(OCl)_2$) on "UL" in Infected Chicken Eggs or When Suspended in Saline

Suspension	pH of $Ca(OCl)_2$	Exp't.1. Min. Exposure 1	5	10	Suspension	Exp't.2 Min. Exposure 1	3	5
"UL" in Egg ($0.3x10^9$ mg/ml)	12	6/6*	0/6	0/6	"UL" in Egg ($2.6x10^9$ mg/ml)	3/6	0/6	0/6
	8	6/6	0/6	0/6		6/6	1/6	0/6
"UL" in Saline ($3.1x10^9$ mg/ml)	12	0/6	0/6	0/6	"UL" in Saline ($1.0x10^9$ mg/ml)	0/6	0/6	0/5
	8	2/6	0/6	0/6		1/6	0/5	0/6
Controls $Ca(OCl)_2$ plus Na Thiosulfate	12	0/6						
	8	0/6						

* After exposure to $Ca(OCl)_2$ solution, 0.5 N sodium thiosulfate was added to stop the action of the chlorine and 0.5 ml was injected I.P. into each of 6 mice. Fractions indicate number of deaths over total number of mice injected.

The vaccine preparations are listed and their methods of preparation are described briefly in Appendix D.

The first experiments were done on rabbits and guinea pigs more to confirm previous work than in the hope that these animals could be immunized.

1. Immunization of Laboratory Animals

a. Rabbits (lepus cuniculus)

Rabbits were injected with the vaccines listed in Table XXX. Three formalized culture suspensions were used, also one yolk sac-ether-extracted vaccine prepared by the method employed in making typhus vaccine.(38)

These vaccines were administered subcutaneously in amounts of 0.5 ml or intradermally in amounts of 0.2 ml, one dose being given every second day for a total of 3 doses. Some animals were later given single booster doses of vaccine at 2 to 3 week intervals after the first series. The fourth column in Table XXX shows the number of days elapsing between the first administration of vaccine and time of challenge. The animals were bled for the determination of the agglutinin titers 6 days after the last injection of vaccine and were challenged at the same time.

The animals vaccinated subcutaneously, when tested just before challenge showed agglutinin titers ranging from 1:40 to 1:1280, 50% giving titers of 1:160-640. One rabbit had the unusually high titer of 1:40, 960. In spite of adequate agglutinin response all of the animals succumbed with typical lesions. There was prolongation of life of 1 to 2 days in this group. All control rabbits were dead by the 6th day, 4 having died on the 4th and 5th days. In the vaccinated group one rabbit died on the 5th day, six on the 6th day, ten on the 7th day and one each on the 8th, 9th and 10th days. Animals vaccinated with formalized culture vaccines were protected as well as, and developed more agglutinin antibody than, rabbits vaccinated with vaccine #2 prepared from infected yolk sacs by the procedure used for making typhus vaccine.

Twenty-six rabbits vaccinated by the intradermal injection of 0.6 ml of the same vaccines given in 0.2 ml doses over a period of 4 days were bled for agglutinins and challenged as above. Agglutinin production was poorer than in rabbits vaccinated subcutaneously. Three-fourths showed agglutinins at 1:80 to 1:320; one at 1:20; two at 1:640 and one at 1:1280. One half of these animals died on the 6th day and all were dead by the 8th day.

There seemed to be less protection conferred by the intradermal injection of formalized vaccine than by the subcutaneous injection. The latter method prolonged the lives of some of the vaccinated animals by one or two days. All animals vaccinated with formalized culture vaccines developed indurated nodules at the injection site. The yolk sac vaccine did not protect any better than did the formalized vaccines, and did not stimulate good agglutinin production although it was prepared from yolk

Table XXX

"UL" Vaccination of Rabbits with Agglutinin Response and Results of Subsequent Challenge

Comparison of Subcutaneous and Intradermal Vaccination

All vaccines administered in dose of 0.5 ml subcutaneously or 0.2 ml intradermally

Vaccines No.	Type	No. of Rabbits	Route of Vaccination	Days Before Challenge	Challenge Dose in MLD 1	10	100	1000	Titers Agglutination
(1)	Formalized NIH-38	6	Subcutaneous (7 injections)	56		3/3*	3/3		(2)** (3) (1)
(1)	Formalized "S"	8	Intradermal (4 injections)	32		3/3	3/3	2/2	(1) (3) (2) (2)
		4	Subcutaneous (4 injections)	26		2/2	2/2		(2) (1) (1)
(1)	Formalized "C"	8	Intradermal (4 injections)	28		3/3	3/3	2/2	(1) (3) (1) (2) (1)
		4	Subcutaneous (4 injections)	26		1/1	3/3		(1) (1) (2)
(2)	OF038 YS#1	10	(3 injections) Intradermal	13		4/4	3/3	3/3	(5) (4)
		12	Subcutaneous (3 injections)	19		4/4	4/4	4/4	(5) (5) (2)
Controls		12	None		4/4	4/4	4/4		None

* Number of animals which died of "UL" / Total number challenged

** Number of animals showing this titer.

sacs containing a large number of "UL" organisms. Stained smears and precipitin tests with this vaccine likewise showed it to be poor in antigen and it was therefore postulated that somewhere in the processing the essential angigen had been destroyed or lost. In order to check this hypothesis, egg vaccines were prepared without ether extraction, purification being accomplished by freezing and thawing and differential centrifugation. These vaccines contained a considerable amount of egg protein and large numbers of "UL" organisms as demonstrated by stained smears. Rabbits were vaccinated with three of these crude vaccines (#'s 11, 12, 13) and with one pooled vaccine (#9 and 10) previously made by the other extraction procedure. Results are shown in Table XXXI.

Better agglutinin production was observed with the crude egg vaccines, titers being comparable to those obtained with the formalized pure culture vaccines. When challenged with 1, 10, 100, 1000, or 10,000 rabbit MLD doses of virulent "UL" the vaccinated rabbits showed an increased survival time of one to three days; however, all died with the exception of one animal which had been challenged with 1 MLD (see vaccine #12) There was no correlation between the agglutinin titer and the length of survival time. The animal which survived had a titer of 1:80 at the time of challenge and a titer of 1:80, 21 days after challenge. Twenty-eight days after challenge this animal was rechallenged with 1000 MLD and survived 33 days when he was accidentally killed. Post mortem examination showed no gross evidence of "UL" infection and cultures were negative. Apparently this animal had been sufficiently immunized by vaccination to withstand a challenge of 1 MLD (1.0 ml of 10^{-9} dilution) for when subsequently rechallenged with 1000 MLD he remained well.

All the rabbits vaccinated with crude egg vaccines showed marked Arthus reactions when given booster injections of vaccine 15 days after the original vaccination. (See DDD Monthly Progress Report April 1944)[34] Crude Vaccine #14 was also tested in guinea pigs and shown to be a potent sensitizing agent as shown by anaphylactic shock. The sensitization was due to the egg protein or some altered egg protein or "UL" metabolite. "UL" suspension scraped from DCBA did not cause anaphylactic shock as shown in Table XXXII. This antigen appeared to very active when administered intravenously. It was less [illegible] active on subcutaneous administration. Comparison with titers obtained after vaccination with formalized pure culture vaccines shows the acetone extracted vaccine to be a poorer antigen when measured by ability to stimulate agglutinin antibody production in the rabbit.

The results of rabbit immunization may be summarized as follows:

1. Rabbits were not protected by formalized pure cultures of "UL".

2. Good agglutinin antibody response was obtained with formalized pure culture vaccines administered subcutaneously. Less response was obtained following intracutaneous administration.

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Table XXXI

"UL" Vaccination of Rabbits with Agglutinin Response and Results of Subsequent Challenge

**Rabbits Vaccinated Subcutaneously - 0.5 ml x 3 at 2 day Intervals

Vaccines No. Type	No. of Rabbits	Days Before Challenge	Challenge Dose in MLD						Agglutinin Titer
			0.1	1	10	100	1000	10,000	
(13) S-YS LIV 44	26	25		6/6*	6/6	6/6	4/4	4/4	(1)*** (1) (7) (12) (5) (2) (1)
(12) S- Memb. F 8-IV 44	10	25		5/6		4/4			(2) (4) (1) (2) (1)
Challenge Controls	18		1/5	5/5	5/5	3/3			
(11) SYS 2nd Sup.	11	16		3/3	4/4	4/4			(1) (1) (4) (3) (1) (1)
(9&10) SYSM & C-YSM Mixed	2	125			2/2				(1) (1)
Challenged Controls	11			3/3	4/4	4/4			

* $\frac{\text{Number of animals which died of "UL"}}{\text{Total number challenged}}$

** (9 and 10) administered in 1.0 ml dose as above with three booster doses at one month intervals.

*** Number of animals showing this titer.

Table XXXII

Anaphylaxis in Guinea Pigs Sensitized by Crude Egg Vaccines

Sensitizing Antigen	Shocking Antigen	Result: Anaphylaxis	Result: Survival
#14	#14	Severe	Recovered
"	"	"	"
"	"	"	Died
"	"	"	"
"	"	"	"
"	"	"	"
"	"	"	"
"	"	"	Recovered
"	"UL" suspension	None	"
None	#14	"	"
None	#14	"	"

Antibody response in rabbits given intravenous and subcutaneous injections of acetone extracted vaccine #30 is shown in Table XXXIII.

Table XXXIII

Agglutinin Antibody Production in Rabbits Following Vaccination with Acetone Insoluble Antigen #30

Rabbit #	Route of Vaccination: Subcutaneous	Route of Vaccination: Intravenous	Highest Titer
697	2 ml	-	1:80
704	2 ml	-	1:40
696	2 ml	-	1:80
733	2 ml	-	1:640
689	2 ml	-	1:80
684	2 ml	-	1:40
195	-	*1.2 ml	1:2560
409	-	1.7 ml	1:1280
523	-	1.7 ml	1:1280
643	-	1.7 ml	1:1280

*Total quantity of vaccine given over a period of 1 month.

3. A high agglutinin antibody titer at the time of challenge was not followed by an increased survival time.

4. Considerable tissue reaction to formalized pure culture vaccines was observed.

5. Vaccines prepared from cultures grown in embryonated eggs and processed by the method used in preparing typhus vaccine contained few "UL" organisms, as shown by microscopic examination and precipitin tests. They did not stimulate good antibody production and did not protect rabbits as well as the formalized pure culture vaccines.

6. Crude embryonated egg vaccines contained large numbers of "UL" organisms and stimulated good agglutinin antibody production; however, the survival time of challenged animals was not significantly greater than for animals vaccinated with formalized pure culture vaccines.

7. One animal vaccinated with crude vaccine #12 survived challenge and was later shown to be immune.

8. The crude egg vaccines sensitized rabbits and guinea pigs.

9. In view of the failure of the egg vaccines, which contained "UL" organisms grown in vivo, further attempts to immunize the rabbit were temorarily discontinued.

b. Guinea Pig (Cavia cobaya)

Previous reported attempts to immunize guinea pigs with formalized culture vaccines had met with complete failure and the present work on these animals was limited to testing two vaccines prepared from "UL" infected embryonated chicken eggs. Both vaccines were prepared from yolk sacs of eggs infected with virulent "UL" cultures, one vaccine (#2) being made by the ether-extraction method used in preparing typhus vaccine, and the other (#15) by emulsifying the yolk sacs and centrifuging out the large tissue fragments. Animals were vaccinated and challenged as shown in Table XXXIV.

Guinea pigs vaccinated with the ether-extracted yolk sac vaccine and tested 18 days after vaccination did not develop any agglutinins in serum dilutions of 1:10.

When challenged with 10, 100 and 1,000 MLD doses of virulent "UL" all succumbed, with an average of one and a half days increased survival time.

Guinea pigs vaccinated with the crude yolk sac vaccine and challenged with 10 and 100 MLD doses of virulent "UL" survived an average of 3 days longer than controls, and one animal challenged with 10 MLD survived 31 days. This animal was rechallenged with 1000 to 10,000 MLD on the 31st day and survived 10 days, or 7 to 8 days longer than a normal animal would be expected to survive.

Table XXXIV

"UL" Vaccination of Guinea Pigs and Results of Subsequent Challenge

Comparison of Purified and Crude Yolk Sac Vaccines

No.	Vaccines Type	No. of Guinea Pigs	Route of Vaccination	Days Before Challenge	Challenge Dose in MLD 1	10	100	1000	Agg. Titers	Survivors
(2)	OFG38YS #1	9	Subcutaneous 0.5 ml x 3	19	-	4/4	3/3	2/2	9-0	0/9
		10	Intracutaneous 0.2 ml x 3	19	-	4/4	4/4	2/2	10-0	0/10
Controls		16	None		4/4	4/4	4/4	4/4	None	0/16
(15)	SYS 30% F	10	Subcutaneous 0.5 ml x 4	26		4/5	5/5	-	-	1/10
Controls		13	None		4/5	5/5	4/4	-	-	1/13

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From the evidence obtained the results of guinea pig vaccination were similar to those reported above for rabbits. A crude yolk sac vaccine raised the guinea pigs resistance when measured by increased survival time, but did not consistently protect against a challenge of 10 MLD.

In summary, the guinea pig did not appear to be a favorable animal for demonstration of active immunity induced by vaccination when using the technics and vaccine currently available.

c. Rats (Rattus Norvegicus albinus)

Preliminary MLD titrations on rats demonstrated that they were more resistant to "UL" than the mouse, guinea pig, or rabbit, and subsequent rechallenge of recovered rats from 14 to 90 days after their original challenge showed that they had developed a solid immunity as a result of the previous sub-lethal challenge. Recovered rats usually survived rechallenge with 10,000 to 1,000,000 LD_{50} doses indicating that they were capable of developing considerable resistance.

All vaccines previously reported had been made from "UL" cultures grown on artificial media, and it was believed that vaccines prepared from cultures grown in the presence of living cells might supply a more complete antigen. With the "UL" organisms actively parasitizing tissue cells they might produce unknown toxic substances which do not appear when the organism is grown on artifical media, and these unknown substances might be the essential immunizing antigens. Partly for this purpose and partly as another means of growing large numbers of "UL" for storage and stability studies, "UL" was grown in embryonated chicken eggs. A total of 26 vaccines was prepared from various portions of infected embryonated eggs, using various preservations and methods of extraction and purification. Fourteen vaccines were prepared from cultures grown in peptone broth, five from cultures grown on DCBA, and several lots of vaccine were made by the method described by Dr. Foshay[30] from cultures grown in gelatin hydrolysate red blood cell extract liquid medium. Tables XXXVI to XXXIX show the results of rat vaccination and challenge. Successive doses of vaccine were given at 48 hour intervals, following the procedure used for human immunization, and animals were challenged from two to three weeks after receiving the first dose of vaccine. The detailed method of preparing each vaccine is given in Appendix D and the vaccine number corresponds to that given in the tables of rat vaccination.

Table XXXV shows the combined data from a large number of individual MLD titrations in normal and vaccinated rats. In this table the results from all vaccinated animals are added together, with no attempt to differentiate them on the basis of type vaccine used. The data show that rats are more susceptible to intraperitoneal than to subcutaneous challenge, and that a significant degree of protection was conferred by vaccination. In a general way a higher percentage of survivors was observed following subcutaneous challenge, however, this was accompanied by a higher percentage survival in controls. Another point brought out by the table is the variation in susceptibility of rats to successive dilution of challenge suspension. No difference could be detected between three strains of white

Table XXXV

Susceptibility of Normal and Vaccinated Rats to Subcutaneous and to Intraperitoneal Challenge

All Rats Were Challenged with 0.1 ml of Serial Ten Fold Dilutions of "UL" Saline Suspensions Prepared from 24 Hour Cultures on D.C.B.A. and Matched to a Turbidity Standard. The Undiluted Standard Suspensions Contained Between 1 and 2 Billion Organisms per ml.

Challenge Dilution	Normal: Challenged Subcut # Dead / # Chall.	Normal: Challenged Subcut % Survival	Normal: Challenged I.P. # Dead / # Chall.	Normal: Challenged I.P. % Survival	Vaccinated: Challenged Subcut # Dead / # Chall.	Vaccinated: Challenged Subcut % Survival	Vaccinated: Challenged I.P. # Dead / # Chall.	Vaccinated: Challenged I.P. % Survival
10^{-1}	79/96	18			16/170	91		
10^{-2}	42/54	23	10/10	0	8/58	87		
10^{-3}	6/10	40	71/110	36			10/55	82
10^{-4}	19/37	49	68/82	18	3/59	95	12/104	89
10^{-5}			74/114	36	1/9	89	1/16	94
10^{-6}	16/47	66	69/86	20	7/92	93	91/333	73
10^{-7}	10/16	38	25/42	41				
10^{-8}	10/29	66	18/30	40				
10^{-9}	2/8	75	2/8	75				

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rats tested. This variability is in sharp contrast to the uniformity of results obtained in MLD titrations on the more susceptible mouse, guinea pig, rabbit, and hamster, and necessitates the use of larger numbers of rats if reliable results are to be obtained.

Preliminary experiments had suggested that the "UL" antigen present in embryonated egg tissues was at least partially destroyed by chemical or physical manipulation involved in processing by the ether extraction method. The use of crude egg vaccines had been more promising but had the drawback of containing a large proportion of egg protein which was responsible for sensitization of the animals and, it was postulated, might also interfere with the development of immunity by the process of antigenic blocking. To obtain a suspension of "UL" organisms grown in the presence of living chick cells containing a minimum of egg protein, and without resorting to chemical or physical purification, vaccines were prepared from the allantoic fluid of "UL" infected 10 day embryonated eggs. Greater numbers of "UL" per ml were obtained from eggs inoculated via the membrane (see section on Cultivation of "UL" in Embryonated Eggs, this report). Formalin, chloroform, and ultraviolet irradiation were compared as killing agents for the allantoic fluid vaccines. The results shown in Table XXXVI indicate that all allantoic fluid vaccines protected rats. Poorest protection was observed with formalin killed vaccines, while vaccines killed by chloroform and ultraviolet irradiation protected 90 to 100% of animals against challenge with 100% of animals against challenge with 100 to 1,000 LD_{50}. Vaccine #23 was attenuated by ultraviolet irradiation and did not kill white rats but still contained viable "UL" organisms which killed mice. This vaccine protected very well but not significantly better than a similar vaccine killed with chloroform, #26 in Table XXXVI. Apparently chloroform in the concentration used did not destroy the "UL" antigen present in allantoic fluid. Another ultraviolet-killed vaccine #28 was prepared in which all "UL" organisms were killed by 3/4 second exposure in a special apparatus made available by Parke-Davis and Co.[37] This vaccine protected rats as well or better than the previous attenuated vaccine as shown in Table XXXVI.

Table XXXVII shows that rats were portected as well by crude membrane and yolk sac vaccines as they were by the allantoic fluid vaccines. No difference was observed between formalin and chloroform when used as killing agents for vaccines. One yolk sac vaccine #25 was purified by substituting chloroform for ether and otherwise following the method used in preparing typhus vaccine. This vaccine conferred some protection but was not as active as the crude vaccines or the allantoic fluid vaccines.

Table XXXVIII shows the results of four experiments using Foshay's vaccine grown in gelatin hydrolysate-red blood cell extract medium and killed with 0.5% phenol. This vaccine also protected rats against virulent "UL" challenge. Where significant numbers of animals were used the protection afforded appeared to be somewhat less than that observed with allantoic fluid vaccines, especially when the number of "UL" organisms per ml of the respective vaccines was considered.

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Table XXXVI

Immunization of Rats with Vaccines Prepared from Allantoic Fluid Of Infected Embryonated Chicken Eggs

All Animals Challenged Subcutaneously with 1 ml of Decimal Dilutions of a T-40 Suspensions of Strain S

Vaccine #	Mode of Immunization	No. of Rats	Challenge Dilution 10^{-1}	10^{-2}	10^{-4}	10^{-6}	10^{-7}	10^{-8}	% Survival* Vaccinated	Control
17-AF-13-III F	0.5 ml x3 subcut.	8			-	3/8**			63	
18-AF-13-IIICH	0.5 ml x3 subcut.	8				0/8			100	
Control	None	8				5/8	4/8	5/6		39
26-MAF-CH	0.5 ml x3 subcut	40		2/15	0/15	1/10			87,100,90	
Control	None	46		11/15	6/15	1/16				27,60,94
23-MAF-UV	0.5 ml x1 Subcut.	42		1/13	0/14	0/15			92,100,100	
Control	None	45		9/15	7/15	5/15				40,54,66
28-AF-UV-3/4	0.5 ml x3 subcut.	40	3/40						92	
Control	None	34	31/34							9

* % Survival figures are given in order of ascending challenge dilution.
** Number died over number challenged.

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Table XXXVII

Immunization of Rats with Vaccines Prepared from Yolk Sacs and from Membranes of "UL" Infected Embryonated Chicken Eggs

All Animals challenged with 1 ml of dilutions of a T4C suspension of Strain S.

Vaccines #	Mode of Vaccination	No. of Rats	Route of Challenge	Challenge Dilution				% Survival
				10^{-3}	10^{-6}	10^{-7}	10^{-8}	
11 SYS 2nd Supt.F.	0.5 ml x3 subcut	8	Subcut.		0/8*			100
19 YS-13-III-F	0.5 ml x3 subcut.	8	Subcut.		1/8			88
22 M-14-III-Ch	0.5 ml x3 subcut.	8	Subcut.		1/8			88
Controls	None	8	Subcut		5/8	4/8	5/6	39
25 C-5	0.5 ml x3 subcut.	10	I.P.	5/10				50
Controls	None	10	I.P.	9/10				10

* Number dead over number challenged.

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Table XXXVIII

Immunization of Rats with Foshay's Vaccine
All Animals Challenged with 1 ml of Decimal Dilutions of a T40 Suspension of Strain S

Vaccine #	Mode of Immunization	No. of Rats	Route of Challenge	Challenge Dilution 10-1	10-2	10-3	10-4	10-6	10-8	% Survival*
# 16	0.5 ml x3 subcut.	45	Subcut.		2/15**		2/15	0/15		87,87,100
Controls	None	60	Subcut.		9/15		7/15	5/15	3/15	40,54,66
# 16	0.5 ml x3 subcut	20	Subcut.	4/20						80
Control	None	18	Subcut.	18/1c						0
# 16	0.5 ml x3 subcut.	10	I.P.			0/10				100
Control	None	10	I.P.			3/10				70
# 16	0.5 ml x3 subcut.	133	I.P.					45/133		66
Controls	None	20	I.P.					13/20		35

* % Survival figures are given in order of ascending challenge dilution.
** Number dead over number challenged.

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Table XXXIX shows the results of rat vaccination with vaccines prepared from "UL" cultures grown in peptone broth (see Media Section this report). Because preliminary experiments had indicated that young broth cultures made better vaccines than older cultures, we tested vaccines prepared from cultures grown for 10 hours, 24 hours, 72 hours and 10 days. No significant difference was observed and these findings have been confirmed and extended by Dr. Downs at Kansas.(2) In a preliminary experiment an alum precipitated peptone broth vaccine (Table XXXIX) gave good protection but had the drawback of causing considerable tissue reaction in rats at the vaccination site. This type of reaction has been one of the drawbacks to human vaccination in the past, especially when formalized vaccines were used. We therefore deferred further studies on alum precipitated antigens until purified fractions could be used.

Twenty-four hour peptone broth culture was extracted with acetone in an attempt to remove some of the nonessential waste products.(42,43,44,45) The acetone insoluble portion was retained and standardized as described under vaccine #30 and #31 Appendix D. Immunization of rats with these preparations as shown in Table XL indicated that the acetone insoluble portion contained the antigen responsible for immunization. No tissue reaction was observed following subcutaneous injection of this vaccine in rats. A low agglutinin antibody titer to 1:20 to 1:80 was observed in rats three weeks after vaccination.

The degree of antibody production following vaccination of the rat, guinea pig or rabbit does not parallel the degree of resistance to challenge. The rabbit produces much more antibody than the rat but succumbs to challenge with 1 MLD. Skin tests with Foshay's vaccine and with acetone extracted vaccine on persons hypersensitive to "UL" showed the acetone extracted vaccine to be much less irritating when used in comparable amount as determined by total nitrogen assay. These studies with the acetone extracted antigen were promising and indicated that further fractionation might be useful to (1) remove sensitizing material from the whole antigen and (2) to concentrate or purify the essential immunizing antigen.

Rats had been protected with vaccines prepared from cultures grown in three menstrua, namely, Foshay's gelatin hydrolysate-red blood cell extract medium, peptone broth medium, and embryonated chicken eggs. For future fractionation studies it seemed wise to concentrate upon cultures grown in only one of these media and to choose the one which protected best in its unmodified form. A comparison of three types of vaccine shown in Figure 10. All rats were vaccinated with 0.5 ml subcutaneously in three doses at 2 day intervals and challenged subcutaneously 17 days later with 1.0 ml of 10^{-1} dilution of a standard suspension of virulent strain S. All vaccines protected against this challenge of approximately 1000 rat LD_{50}. Foshay's vaccine gave slightly less protection than the other vaccines although the difference was not great. The peptone broth, acetone extracted peptone broth, and allantoic fluid vaccines all conferred about equal protection. The acetone extracted vaccine and Foshay's vaccine were standardized to contain the same amount of bacterial nitrogen. The ultraviolet killed peptone broth vaccine contained about one-third this amount, and the

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FIG. 10

SURVIVAL OF VACCINATED WHITE RATS CHALLENGED SUBCUTANEOUSLY WITH 1 ML. OF 10^{-1} DILUTION OF T 500 SUSPENSION OF STRAIN S

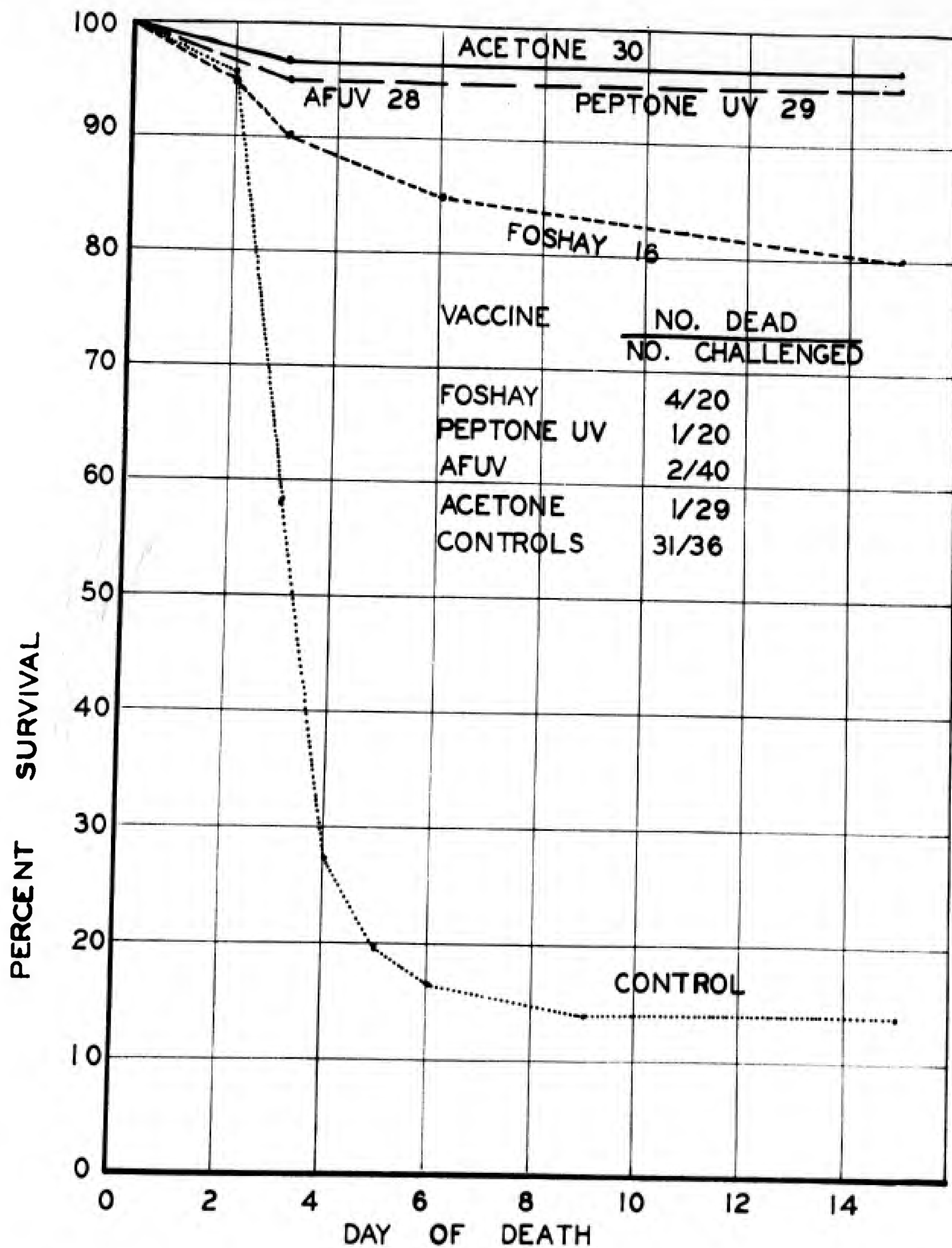

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Table XXXIX

Immunization of Rats with Vaccines Prepared from Peptone Broth Cultures

All Animals Challenged with 1 ml of Decimal dilutions of a T40 Suspension of Strain S

Vaccine #	Mode of Immunization	Route of Challenge	No. of Rats	Challenge Dilution 10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	% Survival*
27 Young broth culture-Ch	0.5 ml x3 subcut.	Subcut.	42		3/15**		1/15		1/12	80,93,92
Control	None	Subcut.	46		11/15		6/15		1/16	27,60,94
29 72 hr. broth culture UV ½	0.5 ml x3 subcut.	Subcut.	20	1/20						95
Controls	None	Subcut.	18	18/18						0
35 24 hr. broth culture	0.5 ml x3 subcut.	I.P.	16			1/8		0/8		87.5,100
Controls	None	I.P.	22			11/13		[illegible]/9		15,11
32 Alum vaccine	0.5 ml x3 subcut.	Subcut.	6	0/6						100
32 Alum Control	0.5 ml x3 subcut.	Subcut.	6	1/6						83
Controls	None	Subcut	8	4/8						50

* % Survival figures are given in order of ascending challenge dilution.

** Number [illegible] over number challenged.

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TABLE XL

Immunization of Rats with Acetone Extracted Peptone Broth Culture
All Animals Challenged with 1 ml of Decimal Dilutions of a T40 Suspension of Strain S.

Vaccine #	Mode of Immunization	No. of Rats	Route of Challenge	Challenge Dilutions 10^{-1}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	% Survival
30	0.5 ml x3 subcut.	6	Subcut.	0/6*							100
30	0.5 ml x3 subcut.	6	"	2/6							67
Controls	None	8	"	4/8							50
30	0.5 ml x3 subcut.	27	"	3/27							87
Controls	None	24	"	21/24							12
30	0.5 ml x3 subcut.	19	"	1/19							95
Controls	None	16	"	13/16							19
30	0.5 ml x3 subcut.	10	"	1/10							100
	0.25 ml x3 subcut.	10	"	1/10							90
Controls	None	18	"	18/18							0
30	0.5 ml x3 subcut.	35	I.P.	5/35							86
Controls	None	35	"	18/35							49
30	0.5 ml x3 subcut.	60	"					16/60			73
Controls	None	20	"					13/20			35
31	0.5 ml x3 subcut.	9	Subcut.				1/9				89
31	0.5 ml x3 subcut.	8	I. P.				1/8				88
Controls	None	10	"		6/6	6/6	4/10	6/10	9/10	4/6	60
31	0.5 ml x3 I.P.	8	"			1/8					87.5
	0.5 ml x3 subcut.	9	"			1/9					89
Controls	None	10	"			4/10					60
31	0.5 ml x 3 subcut.	20	"					10/20			50
Controls	None	20	"					13/20			35
31	0.5 ml x3 subcut.	[illegible]	"			9/79					89
Controls	None	10	"			9/10					10

* Number dead over number challenged.

allantoic fluid vaccine contained one-tenth this amount judged on the basis of bacterial count before killing. From the standpoint of total amount of antigen injected, the allantoic fluid vaccine appeared to be best, however, subsequent vaccination with ten fold dilutions of the acetone extracted vaccine showed no significant decrease in protection. In summary, egg vaccines and Foshay's vaccine were not superior to simple peptone broth vaccines and were more difficult to prepare and reproduce. Therefore, fractionation studies were carried out on peptone broth cultures. (See section H)

(1). Pathology and Pathogenesis of "UL"

(a) The Pathology of "UL" in the Rat

Rats challenged with "UL" usually appear quite ill within 24 hours. They sit in the corner of the cage, the hair is rough and they refuse to eat. The animals become progressively more lethargic, respiration is labored, and terminally they are prostrate. They expire quietly with the belly flat on the floor of the cage and the head extended. The majority of deaths occur between 2 and 5 days after challenge. Animals which survive for 5 or 6 days frequently show a marked symptomatic improvement and go on to eventual recovery.

Microscopic Pathology

At autopsy the lymph nodes draining the challenge site are slightly injected. The spleen is 3-4 times enlarged, dark bluish purple, and friable. The cut surface is firm and no focal lesions are visible. The liver, adrenals, kidneys and lungs appear normal to gross examination. The same gross picture is seen in mice except that the liver is frequently distinctly yellowish in color and the liver and spleen are usually covered with minute yellowish foci.

Microscopically the liver shows multiple small areas of acute focal necrotizing hepatitis infiltrated with polymorphonuclear and mononuclear cells which show marked fragmentation. There is no sharp border between normal and necrotic tissue. The remaining liver cells show moderate to marked fatty change with no particular distribution in the lobule. There are numerous small areas of polymorphonuclear and mononuclear infiltration without necrosis of liver cells. Kupfer cells are prominent. Rounded mononuclear cells are frequently scattered through the sinusoids or gathered in small clumps with necrosis of individual cells in the clump. There are few lymphocytes on the periportal spaces.

The spleen shows extensive coagulation necrosis which involves the Malpighian bodies less than the red pulp. Fragmented polymorphonuclear-leukocytes and large reticulo endothelial cells are seen throughout the red pulp and in necrotic areas. Where the necrosis is limited to focal areas in the red pulp there is no cellular border around the foci. The Malpighian bodies are depleted of lymphocytes and some contain infiltrating polymorphonuclear-leukocytes. There are no distinct germinal centers. Many blood vessels contain thrombi.

Lymph nodes have no distinct germinal centers. There are focal accumulations of large pale reticulo endothelial cells in the nodes and in the sinuses with central necrosis in some. A few large basophilic cells which resemble plasma cells are seen in some germinal centers.

The adrenals show loss of cortical lipoid and less often diffuse infiltration with polymorphonuclear and mononuclear leukocytes. Occasional clumps of large mononuclear cells show central necrosis and occasional cortical cells are necrotic. No unusual findings are seen in the medulla.

The lungs frequently show some edema and depletion of cells in the lymphoid follicles. Occasionally the alveolar walls are thickened and infiltrated with mononuclear and polymorphonuclear cells.

The kidneys, thymus, and pancreas show no consistant changes.

(b) Comparison of the Rat and Mouse

The microscopic pathology in vaccinated animals was compared with that in normal animals in the following experiment carried out by Ensign Spencer (39), (40). Normal and immune rats were challenged intracutaneously with 0.1 ml of a 10^{-1} dilution of a T500 suspension of Strain SM-16. Normal and vaccinated mice were challenged intracutaneously with 0.1 ml of a 10^{-5} dilution of the same bacterial suspension. Two animals from each group were sacrificed at the following intervals after challenge: immediately, ½, 1, 2, 4, 8, 24, 48, 72, and 96 hours. Tissues were prepared for histopathological study and stained sections were examined by Lt. Comdr. Paul E. Steiner. Pooled weighed blocks of fresh liver and spleen from the rats were ground aseptically and serial dilutions were plated on DCBA to determine how soon and in what quantity the organisms appeared in these organs.

Microscopic pathology

Normal Rats

Skin-Injection site. The injected fluid disappeared between 2 and 4 hours after challenge. A slight increase in polymorphonuclear leukocytes and lymphocytes appeared after 1 hour. At 8 hours the inflammatory reaction was intense. It consisted of a large area of polymorphonuclear infiltration with extensive fragmentation of leukocytes. At 24 hours central necrosis was present and showed a progressive increase thereafter. Tiny blue coccoid bodies appeared at 4 hours and increased in numbers at 8, 24, and 48 hours. They were both intracellular and extracellular.

Liver- The first liver changes were seen at 24 hours and consisted of small areas of acute interstitial hepatitis with polymorphonuclear cells predominating. At 48 hours numerous larger areas of acute focal necrotizing hepatitis were seen and the remaining liver cells showed extensive fatty change. At 72 and 96 hours the necrotic areas were larger and very few intact leukocytes were seen.

Spleen-Focal infiltration of polymorphonuclear leukocytes in the red pulp was first seen at 24 hours. These areas were larger and necrotic at 48 hours. By 72 hours there was a massive necrosis of the spleen involving the red pulp and distorting or obliterating the Malpignian bodies.

Vaccinated Rats

Injection site-The sequence of events at the site of injection resembled that seen in the normal rats with two notable exceptions: (1) The inflammatory response came on quicker, having already begun at 30 minutes. (2) There was no visible massive growth of organisms as seen in the normal rats. Fragmentation of leukocytes was almost entirely absent. At 48 hours many microphages were being engulfed by macrophages and at 72 hours the inflammation was subsiding.

Liver and spleen-The liver remained normal throughout this series except for occasional small foci of hepatitis. In the spleen there were no areas of spleitis and necrosis as in the normal rats. Small groups of mononuclear macrophages were present, scattered throughout the red pulp.

Normal Mice

Injection site-The injected fluid caused distension and separation of the tissues. It was nearly absorbed at 2 hours and completely absorbed at 4 hours. A slight inflammatory response, consisting of an infiltration of polymorphonuclear leukocytes, was visible at ½ hour and it increased steadily thereafter. At 48 hours the infiltration of polymorphonuclear leukocytes was intense and some necrosis had begun. There was now some inflammatory edema. By 96 hours the inflammatory reaction was still more intense and the overlying epidermis was necrotic. Great numbers of tiny coccoid, blue bodies were present in sections stained with Giemsa. A few macrophages distended with these bodies were seen.

Liver-The livers of the animals sacrificed up to 72 hours were rich in glycogen. At 96 hours a sever fatty change had developed. Between 24 and 48 hours numerous acute focal lesions appeared. Some of them were characterized by coagulation necrosis of the preexisting liver cells followed by leukocytic infiltration. At 72 hours there was a generalized increase in Kupfer cells and in some areas their cytoplasm contained masses of blue coccoid granules, presumed to be"UL". It is not certain whether or not these cystic cells may also be formed from liver cells. These cysts became enormous by 96 hours. Extracellular blue coccoid granules were sometimes seen in the areas of acute coagulation necrosis.

Spleen-The spleens appeared relatively normal up to 24 hours. At 72 hours small nodular groups of macrophages, some with degenerative changes were seen in the red pulp. At 96 hours the necrosis was severe and involved all parts of the spleen, but the centers of the Malpighian bodies were least affected. The blue coccoid granules seen at the site of injection and in the liver were also seen in macrophages in the spleen.

Vaccinated Mice

Injection site-The inflammatory reaction resembled that in normal mice with respect to cellular and fluid response and speed of its development, but it was quantitatively less and there was no necrosis of the overlying epithelium. Fewer blue coccoid granules were seen at the injection site.

Liver-Only a slight degree of focal hepatitis developed and the macrophages filled by blue coccoid bodies did not appear at all. The areas of focal hepatitis remained small and they consisted mostly of large mononuclear macrophages, rather than polymorphonuclear leukocytes. There was no visible necrosis of liver cells.

Spleen-The spleens remained relatively normal, showing only smallnodular areas of large mononuclear macrophages in the red pulp. The acute splenitis with widespread necrosis and overwhelming growth of the blue coccoid granules was not seen in these vaccinated mice.

Summary of histopathology in Mice

There appeared to be inhibition of the growth of the organisms at the site of injection with resultant sparing of the viscers. Since there was less inflammatory reaction at the site of injection in the vaccinated mice than in the normal animals, phagocytosis must have been more effective. That the local defense was not fully effective is shown by the hematogenous dissemination which occurred; however, some degree of resistance was induced in spleen and liver by the vaccination because there was effective inhibition of the organisms at this point.

Quantiative Culture of liver and spleen at various intervals after challenge

The results of quantitative culture of spleen and liver tissue at various hours after challenge are shown in Table XLI. In normal mice the organisms had reached the spleen and liver 8 hours after challenge and the number increased steadily until at 96 hours there were 1 and 20 billion "UL" organisms per milligram of tissue.

The first positive spleen and liver cultures were obtained at 13 hours in both normal and immune rats. In the normal rats the number of organisms per mgm decreased slightly at 24 hours and thereafter increased logarithmically until death. In the vaccinated rats organisms were absent or reduced in number at 19 hours and absent after 24 hours.

These findings indicate that in the immune rats the organisms are not held at the site of challenge but multiply and invade at the same rate as in normal rats. Then, sometime between 13 and 19 hours after challenge, the immune rats' defensive mechanisms are mobilized and the organisms are destroyed. The same immune mechanism may have been active in the normal rats,

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TABLE XLI

"UL" Organisms per Milligram of Liver and Spleen at Various Intervals after Intracutaneous Challenge

Animal	Tissue	Time in Hours After Challenge											
		½	1	2	4	6	8	13	19	24	48	72	96
Normal	Liver	0	0	0	0	0	0	-	-	727	814	95,900	1,660,000
Mice	Spleen	0	0	0	0	0	0	-	-	77	16,000	962,000	11,900,000
Normal	Liver	0	0	0	0	0	2	-	-	880	1,000	137,000	20,900,000
Mice	Spleen	0	0	0	0	0	47	-	-	35	3,890	295,000	1,310,000
Normal	Liver	-	-	-	-	0	-	119	1950	117	10,600	249,000	Dead
Rats	Spleen	-	-	-	-	0	-	155	2420	1180	31,220	669,000	Dead
Immune	Liver	-	-	-	-	0	-	52	0	0	0	0	0
Rats	Spleen	-	-	-	-	0	-	19	0.14	0	0	0	0

Mice were challenged with 0.1 ml of 10^{-5} dilution intracutaneously

Rats were challenged with 0.1 ml of 10^{-1} dilution intracutaneously

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however, it was inadequate and served only to reduce the number of organisms temporarily.

(c) Bactericidal Property of Rat Leukocytes

The bactericidal effect of rat leukocytes on "UL" was studied in an effort to clarify the mechanism of resistance and immunity. Leukocytes were collected from normal and from immune rats by injecting them intraperitoneally with saline and washing the leukocytes out of the peritoneal cavity after 15-18 hours. This method produced mainly polymorphonuclear leukocytes. The leukcytes were washed with saline then mixed with peritoneal fluid or serum from normal or vaccinated rats and inoculated with various quantities of living "UL" organisms. These mixtures were incubated at 37°C and DCBA plate counts were made at intervals to determine the number of surviving "UL" organisms.

Peritoneal fluid or serum from immune rats appeared to enhance the ability of leukocytes to reduce the number of organisms. Leukocytes from normal rats were as effective as leukocytes from immune rats. Organisms in saline died rather rapidly whereas organisms in normal or immune serum multiplied slowly. In summary, very moderate bactericidal action was demonstrated when immune serum or peritoneal fluid was mixed with leukocytes. This appears to be an immune opsonin effect.

(d) Bactericidal Property of Immune Rat Serum

No in vitro bacterial activity of immune rat, rabbit, or human serum has been demonstrated at Camp Detrick. It was postulated that at the time the defensive mechanism of an immune rat is mobilized, following a rechallenge, there might be an excess of bactericidal substance in the blood stream. This hypothesis was tested by rechallenging 31 rats one month after they had recovered from a previous challenge. Twenty-four hours after rechallenge 15 rats were sacrificed and the blood serum collected, pooled and stored. This was labelled "24 hour serum". Forty-eight hours after rechallenge the remaining 16 rats were similarly sacrificed and the pooled serum labeled "48 hour serum". The spleens of the 48 hour rats were collected, ground aseptically in a mortar with saline and alundum, and the supernatant cleared by centrifuging. The supernatant was labelled "spleen extract". These two pooled specimens and the spleen extract were inactivated by heating at 56° for 30 minutes and tested in vitro with fresh guinea pig complement for "UL" bactericidal activity against dilutions of living "UL". After 2 hours incubation the tubes were plated on DCBA and injected into mice. No bactericidal activity wa demonstrated by any of these preparations.

(e) Passive Protection

Passive protection of rats with the 24 hour and 48 hour rat sera and the spleen extracts was also attempted. Normal rats were challenged intraperitoneally with 1.0 ml of 10^{-3} dilution followed immediately by 1.0 ml of rat serum or spleen extract. The injection of serum or spleen extract was repeated 24 hours later. The results are shown in Table XLII.

Table XLII

Failure to Passively Protect Rats With Immune Rat Serum or With Spleen Extract of Immune Rats

Serum	Agglutination Titer	Total Serum	#Deaths / #Tested
24 hour serum	1:80	2.0 ml	10/10
48 hour serum	1:60	2.0 ml	9/10
Spleen extract	1:10	2.0 ml	9/10
Controls	-	None	10/10

These tests were repeated with immune sera from the rat, horse, man, goat, and rabbit as shown in Table XLIII with similar lack of protection.

Greatest survival was obtained with serum from normal rats with a negative agglutinin titer, while serum from immune rats protected less well. It is believed that the variation in % survival shown in Table XLIII is merely the variation in natural resistance of individual rats. By the use of a smaller challenge one might be able to show slight protection or prolongation of life, but the authors believe that passive protection of the rat is no more feasible and of no more significance than that previously reported for the rabbit (Francis 41).

It was postulated that incubation of the immune serum with the living "UL" organisms before injection might give the opsonins a better change to act. Fresh immune rat serum with an agglutinin titer of 1:80 was mixed in equal portions with saline containing 10-12 living "UL" organisms per ml, and the mixture incubated at 37°C for 2½ hours. One ml portions were then injected into mice as shown in the following table.

These results indicate that passive transfer of serum from vaccinated or immune animals does not protect mice or rats from fatal infection. "UL" is not killed by in vitro contact with serum from immune animals. Apparently in these laboratory animals immunity to "UL" infection is not a function of circulating antibody.

(f) Antibody Response in Normal and Vaccinated Rats Following Challenge

An observation which may have some bearing on the natural immunity of rats was made while doing agglutination titers on normal rats before and after challenge. Two hundred rats were divided into five groups. One group of 40 rats was retained as controls and each of the other groups were vaccinated with acetone extracted vaccine #31. The vaccine was administered subcutaneously in the amount of 4.0 mgm total solids to one group and 0.4 mgm total solids to another group. For the other two groups the vaccine

Table XLIII

Failure of Passive Protection of Rats

# Rats	Antiserum Source	Antiserum Titer	Antiserum Dose	Challenge Dilution	# Dead / # Tested	% Survival
10	Rat	Neg*	2.5 ml sq	1 ml 10^{-2} sq	3/10	70
10	Rat	1:80	"	"	7/10	30
10	Horse	Neg*	"	"	9/10	10
10	Horse	1:1280	"	"	7/10	30
10	Human	Neg*	"	"	10/10	0
8	Human	1:1280	"	"	7/8	13
10		None		"	9/10	10
10	Goat	1:40,960	1 ml ip	1 ml 10^{-3} ip	10/10	0
10	Goat	Neg*	"	"	8/10	20
10	Rabbit	1:160	"	"	8/10	20
10	Rabbit	Neg*	"	"	10/10	0

* Serum obtained from normal animals

Table XLIV

Failure of Passive Mouse Protection With Rat Immune Serum

	#Mice dead / #Mice injected
Rat immune serum plus "UL"	12/12
Same with an additional 0.5 ml of immune serum 24 hours later	6/6
Rat normal serum plus "UL"	10/10
"UL" only	8/8

was heated at 100°C for one hour and administered in the same manner. Three weeks later all animals were challenged with 100 LD_{50}. Five animals from each group were bled for an agglutinin titer on the day of challenge and on the 3rd, 6th, 15th, 28th, and 35th day after challenge as shown in Figure 11.

The titers of all vaccinated animals were so uniform that they are added together for purposes of graphing. All vaccinated animals had a titer at the time of challenge which dropped three days after challenge and returned to the original or higher titer on the 6th day after challenge. Neither the amount of vaccine administered nor heating the vaccine for 1 hour at 100°C had any appreciable affect on the degree of antibody production Unvaccinated controls had no titer at the time of challenge, but 3 out of 5 had an appreciable titer 3 days after challenge and 6 days after challenge all had titers equal to or higher than the vaccinated animals. While the agglutinin titer is not an accurate index of immunity the rapid production of antibody in normal rats after challenge probably indicates that their defensive mechanism is mobilized very quickly. The time of appearance of circulating agglutinins in normal rats following challenge (five or six days) corresponds very closely with the time they begin to show marked symptomatic improvement. Most deaths, in both vaccinated and normal rats, occur within the first challenge 6 days after challenge.

d. Mice (Mus musculus Albino)

Early attempts at Camp Detrick to protect mice by vaccination failed. This was in line with the previous experience of Foshay and Downs (per. com.) However, it was observed at Camp Detrick that even though vaccinated animals died of "UL" infection when challenged, microscopic pathological changes in the liver and spleen were significantly more localized than in unvaccinated mice. Less extensive necrosis, a greater infiltration of large mononuclear cells and less growth of "UL" organisms were the chief changes noted. These pathological findings suggested that with a better vaccine, longer period of immunization and a smaller challenge it might be possible to demonstrate active immunity in mice by means of vaccination.

FIGURE - 11

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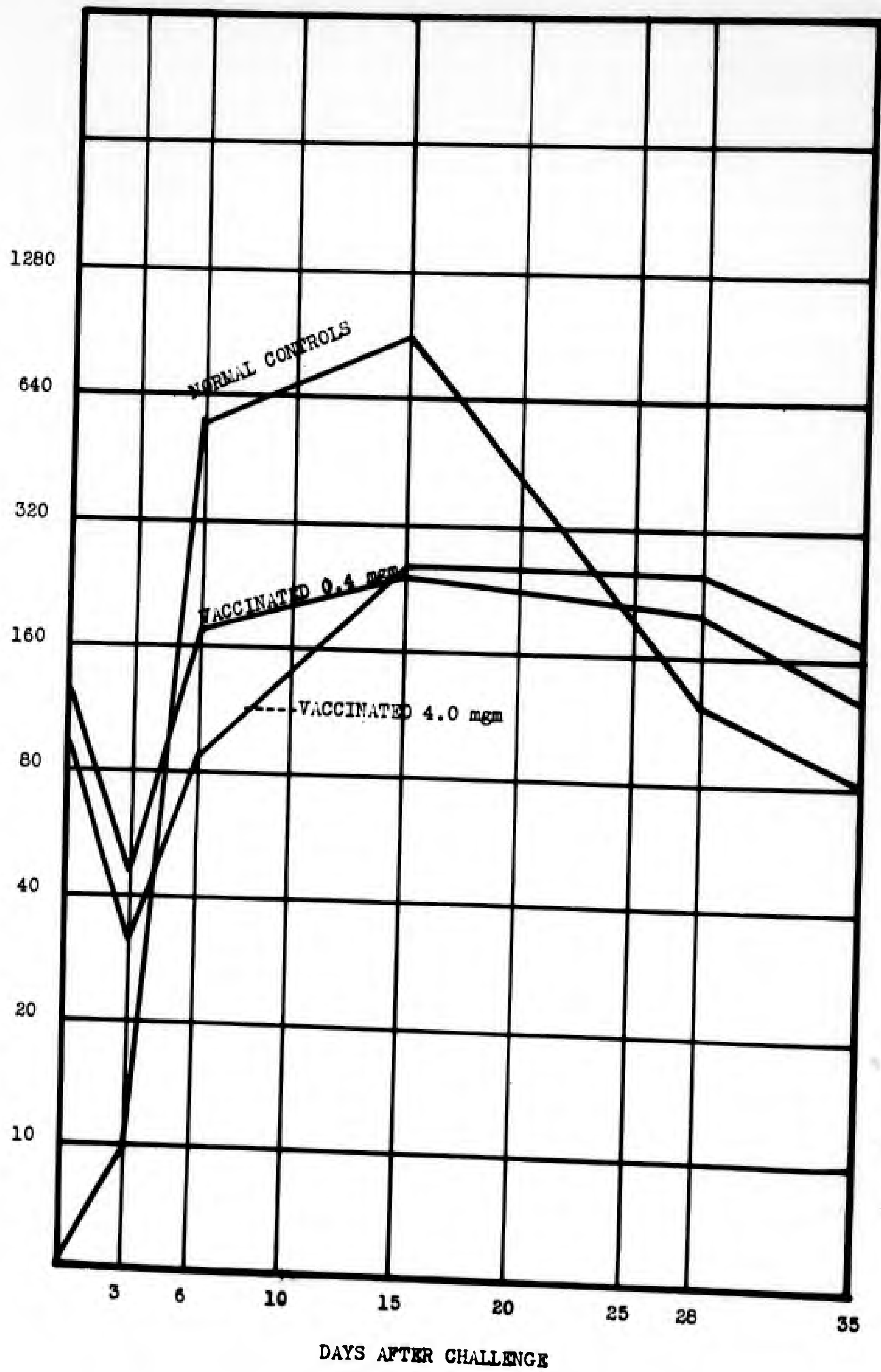
THE AGGLUTININ RESPONSE IN NORMAL AND VACCINATED RATS AFTER CHALLENGE

ACETONE EXTRACTED VACCINE # 31



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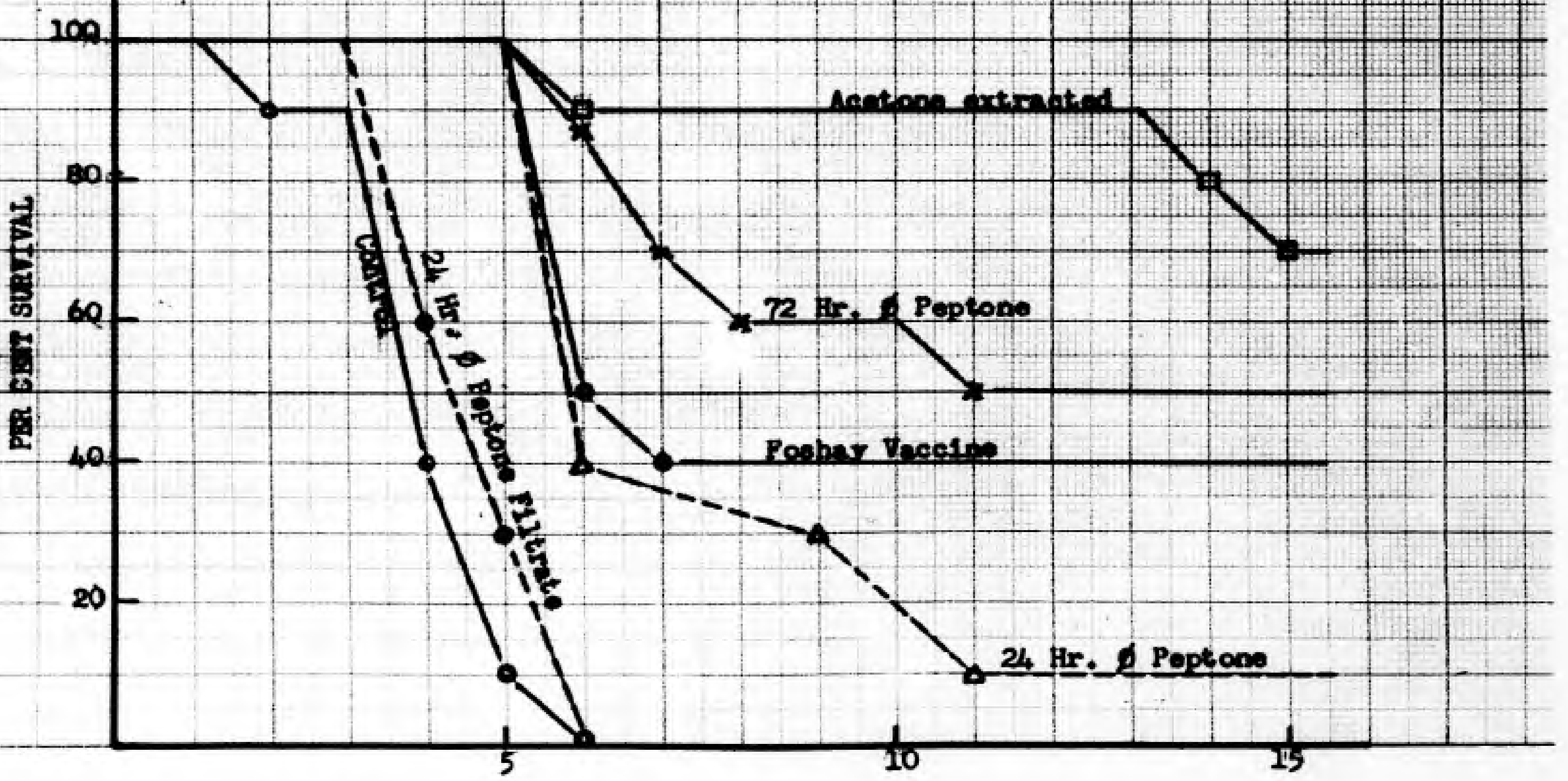
Tables XLV, XLVI, XLVII show the results of four mouse experiments in which vaccinated animals were challenged with graduated doses of living virulent "UL". Table XLV shows the mouse protective effect of 9 ether purified vaccines prepared from infected embryonated eggs when administered over a short period of time and challenged 15 days after immunization. Of these animals, 8.4% were protected against 10 MLD challenge and 0.87% were protected against 100 MLD challenge. Survivors were scattered throughout the groups and no vaccine appeared to be significantly superior to the others. Surviving animals were rechallenged with 100 MLD and all died of "UL" infection.

Table XLVI shows the results of similar mouse immunization with three peptone broth vaccines (numbers 27, 33, and 34), one chloroform extracted yolk sac vaccine (number 24) Foshay's gelatin hydrolysate vaccine (number 16) and acetone extracted vaccine (number 30). The table shows the approximate bacterial count per ml of the vaccines employed. Some protection was conferred by vaccination since 35.4% were protected against 1 MLD, 14.5% were protected against 10 MLD and 4.3% were protected against 100 MLD. With the better vaccines 50 to 100% of animals were protected against 1 MLD and a few animals survived 100 MLD. The degree of protection appeared to be associated with the number of organisms per ml of vaccine rather than with the method of preparation or source of the culture used for vaccine preparation. Surviving animals were rechallenged with 10 MLD and all died of typical "UL" infection.

The above experiment was repeated with a different strain of white mice (Hover). The same vaccines were used except that a new yolk sac preparation (C-5) containing aqueous phase extract from 12 billion organisms per ml was employed, and instead of the 10 hour and 12 hour peptone vaccines, a 24 hour peptone culture containing 6 billion organisms per ml killed with 0.5% phenol and a Seitz filtrate of this same culture were used. Twenty-seven days after vaccination all animals were given a booster dose of vaccine and 12 days after the booster dose they were challenged as before. Results are shown in Table XLVII and Figure 12. Of these animals 40.0% survived 1 MLD and 3.4% survived 10 MLD challenge. The acetone extracted peptone broth culture protected best. Seventy-two hour phenolized peptone vaccine, C-5 vaccine, and Foshay's vaccine gave about equal protection. The 24 hour phenolized peptone broth culture protected slightly but the Seitz filtrate of this vaccine conferred no protection indicating that there was no soluble protective antigen in the broth culture capable of passing through the filter.

The agglutinin antibody production following hyper-immunization of 300 mice with vaccine #30 is shown in Figure 13 and the results of challenge are shown in Table XLVII. These animals were given 0.25 ml of vaccine subcutaneously on 2 alternate days. Twenty-six and fourty-seven days later they were given additional booster doses of vaccine. All were challenged with 1 MLD subcutaneously 7 days after the last booster dose. At intervals throughout the immunization period 10 mice were bled and "UL" agglutination tests were run on each of the ten animals separately. The titers of the ten animals were averaged and the mean titer is shown graphically in Figure 13.



DAYS - AFTER CHALLENGE

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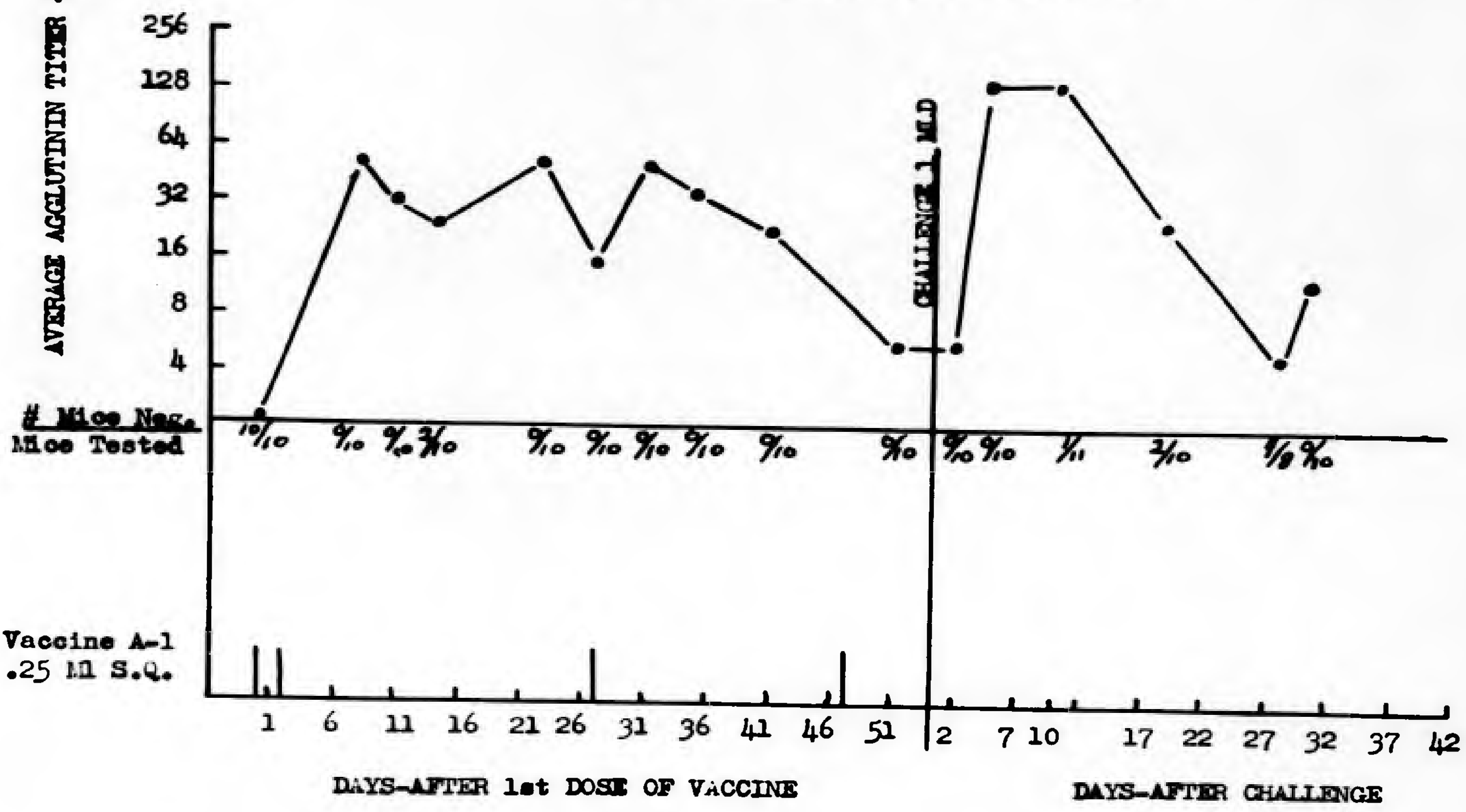
FIGURE 13

AGGLUTININ RESPONSE IN MICE VACCINATED AGAINST AND CHALLENGED WITH "UL"

ACETONE EXTRACTED PEPTONE VACCINE



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The Effect of Gramicidin + Tyrocidine on Various Bacteria
Cora M Downs - J Bact Vol 44, #3, p 392, 1942

Chart 1

SURVIVAL OF "U"

Form of Organism	Agent	Survival	Reference
Culture	Formalin 0.1%	Less than 24 hours	Medicine,1928,7,411
Spleen	1% tricresol	2 minutes	Medicine,1928,7,411
Spleen	Whole glycerin	1 month 20°C	Medicine,1928,7,411
Spleen	Whole glycerin	6 months 10°C	Medicine,1928,7,411
Spleen	Whole glycerin	6 years 14°C	U.S. Public Health Report,1932,47,1287
Spleen	Frozen	3 weeks	U.S. Public Health Report,1932,47,1287
Culture	5% phenol	Less than 10 min.	Downs, personal communication
Culture	70% alcohol	Less than 10 min.	Downs, personal communication
Culture	1.0% formaldehyde	Less than 10 min.	Downs, personal communication
Culture	1-1000 merthiolate	Less than 10 min.	Downs, personal communication
In Water		16 days	American Journal of Hygiene, 1942,36,168
In Mud		31 days	American Journal of Hygiene, 1942,36,168
In dried Rabbit Skin		45 days	Medicine, 1940,19-1
In Milk		20 days	Zeit & Hyg. u Infectkr 1936,119,425
In Frozen Carcass		30 days	Vestnik mikr ep & par 1937,16,308
Bedbug Feces dried		25 days	

Table XLV

Protection of Mice with Purified Embryonated Egg Vaccine

Each mouse given two injections of 0.5 ml of vaccine subcutaneously at 3 day intervals and challenged intraperitoneally 15 days later.

Vaccine # Code	No. of Mice	1	10	100	1000	Deaths Total
9 SYS-M	11		5/6	5/5		10/11
"	19		10/10	9/9		19/19
2 FO38YS#1	14		7/7	7/7		14/14
2 FO38YS#1	11		5/6	5/5		10/11
3 FO38YS#2	22		10/11	11/11		21/22
10 C	11		4/6	5/5		9/11
	20		10/10	10/10		20/20
4 38 Memb inoc YS	21		9/10	11/11		20/21
5 38 Memb inoc Fluid	24		9/12	11/12		20/24
7 S Memb	19		10/10	9/9		19/19
8 S Fluid	20		10/10	10/10		20/20
6 SYS	18		9/9	9/9		18/18
Total # Dead / # Challenged			98/107	102/103		200/210
Per cent survival			8.4	0.87		4.8
Controls	24	6/6	6/6	6/6	6/6	24/24

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Table No. 36

Bone Marrow

Differential Count in Normal and Recovered Monkeys

Animals	#57	#66	#67	#71	Normal
Seg. Neutro.	11.7%	9.7%	23.3%	14.7%	16%
" Eosin.		.3%			1%
" Baso		.3%	2.0%		1.3%
Neut. Band	16%	24.3%	17.3%	17.7%	20.7%
Eo "			.3%	1.0%	0.7%
Baso "					
Neut. Juv.	7.3%	11.7%	7.7%	7.3%	8.3%
Eo. "	.3%	.3%	.7%		
Baso. "					
Neut. Myelo.	7.3%	8.3%	9%	5.7%	9.0%
Eo. "	.7%	1.7%	1%	1.7%	0.7%
Baso. "		.7%	1.3%	0.7%	0.3%
Premyelocytes	3.3%	2.3%	7.3%	7.0%	4.7%
Myeloblasts	8%	3%	7.3%	7.0%	3.0%
Lymphs		0.7%	3%	1.3%	0.7%
Monos.		2.0%			0.7%
Plasma					
Normoblasts	9.7%	23%	4.3%	21.3%	22%
Macroblasts	10%	10%	21.7%	10.7%	8.3%
Megaloblasts	0.7%	1%	3%	3%	2.0%
Megakaryocytes		0.7%		.3%	0.7%
Small cells very little or no cytop. Either nucleated reds or myeloblasts (micro.). Probably normoblasts.	25.3%			.3%	

Table No. 36A

DIFFERENTIAL LEUCOCYTE COUNT ON NORMAL AND

TREATED MONKEY AT TIME OF SACRIFICE

Monkey No.	57	66	67	71	Normal
Monos	0	2	0	0	5
Eosin	2	1	4	2	0
Baso	2	1	0	1	0
Lymph	52	35	48	46	44
Bands	0	3	0	0	1
Segs	44	58	48	51	50

* - These results were obtained at the same time as bone marrow differential of Table .

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TABLE XLVI

Comparison of Mouse Protection by Various "UL" Vaccines

All animals given 0.25 ml of vaccine intraperitoneally on 3 alternate days.

Vaccine # Code	# of Mice	Organisms/ml of vaccine Billions	Interval before Challenge	Challenge Dose-MLD IP 0.1	1	10	100	Survivors 15 days ** #	# Positive Spleen cultures
30 Acetone Extracted	30 6	10	13 days " "		6/10	9/10 5/6*	9/10	6 1	1
24 CHYS Ext	24	0.5	" "		3/4	9/10	10/10	2	0
16 Foshay	28	12	" "		4/8	5/10	10/10	9	0
33 S-72 hr	26	12	" "		0/6	8/10	9/10	2	1
34-B-Conc Peptone	23	3.8	" "		1/3	9/10	9/10	4	1
27 S-10 hr Ch	27	1-2	" "		7/7	10/10	10/10	0	None
Total	# Dead / # Challenged				31/48	65/76	67/70		
	% Survival				35.4	14.5	4.3		
Controls	40	None	- -	0/10	10/10	10/10	10/10	0	None

* Challenged subcutaneously

** All survivors were sacrificed 15 days after challenge and spleen cultures for "UL" made on LCBA

TABLE XLVII

Hyperimmunization and Challenge of Mice

All mice given 0.5 ml of vaccine intraperitoneally in 4 doses over a period of 27 days

Vaccine # Code	# of Mice	Organisms/ml of Vaccine Billions	Interval before Challenge	Challenge Dose-MLD 0.1	1	10	100
30 Acetone Extract	29	10		1/10	3/10	8/9	
25 C-5	28	12	29 Days Challenged Intraperitoneally	0/8	6/10	10/10	
16 Foshay	26	12		1/6	6/10	10/10	
33 S-72 hrs	27	12		2/7	4/10	9/10	
35 S-24 hrs	29	6		2/9	7/10	10/10	
36 S-24 Seitz	29	6 filtrate		1/10	10/10	9/10	
Total Vaccinated		# Dead/# Challenged % Survival		7/50 84	36/60 40.0	56/58 3.4	
Controls	38	None		1/10	10/10	10/10	8/8
30 Acetone Extract	160	.25 ml sq x 4 over 47 days	54 days challenged sq		97/166		
Total Vaccinated		# Dead/# Challenged % Survival			97/166 42		
Controls	67	None		4/12	27/31	12/12	12/12

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Following vaccination all mice developed agglutinin titers which persisted for 3 weeks and started falling after four weeks. A booster dose of vaccine caused a rise in titer after four days which had started to fall again 8 days later. A second booster dose of vaccine 20 days later was not followed by an increase in titer. Two days after challenge the average titer of ten mice was low but four days after challenge the average titer reached a new high. The titer of survivors fell gradually over the next month. Ninety-seven out of 166 vaccinated mice died following 1 MLD challenge (or 42% survival).

A compilation of data for 15 days survival in the four mouse vaccination experiments shows a mean percentage survival of 39.1% when challengeed with 1 MLD; 8.8% survival when challenged with 10 MLD; and 2.6% survival when challenged with 100 MLD.

(1) Latent Infection in Mice

Vaccinated mice which survived challenge were outwardly healthy but an occasional mouse died as long as 30 days after challenge. At autopsy most of these deaths proved to be nonspecific, however "UL" was recovered from some animals and the recovered cultures were shown to be fully virulent for mice. Apparently some surviving mice continued to harbor virulent organisms. To determine the incidence of chronic or latent infection, groups of animals were sacrificed at intervals after challenge. The spleens were removed at autopsy and cultured for "UL" by making impression smears on DCBA plates. A small percentage of positive cultures was obtained by this method but later work showed that a much higher percentage of latent infections could be demonstrated by grinding the mouse spleens with sterile sand and reinjecting the supernatant fluid into mice. Table XLVIII shows the results obtained. Thirty percent of the mice had "UL" in their spleens for as long as thirty days after infection. It is possible that a higher percentage of positive cultures would have been shown if lymph nodes and bone marrow had also been sampled.

(2) Resistance of Surviving Mice to Rechallenge

Previous studies had shown that vaccinated mice which survived a small challenge were not subsequently resistant to a challenge of 10 or 100 MLD. To determine the relative resistance of surviving mice an MLD titration was carried out on a group of mice which had survived for 31 days after a challenge with 1 MLD. The results are shown in Table XLIX.

Table XLIX indicates that immunity induced by vaccination was not enhanced appreciably by recovery from a sublethal challenge. Individual animals . . survived 100 LD_{50} doses but the susceptibility of the group as a whole suggests that those animals which survived the first challange were not rendered immune.

In one experiment surviving animals were dusted with DDT on the 11th day following challenge and 30% of them were found dead the next day. Post mortum spleen cultures showed that many but not all of these animals

Table XLVIII

Latent Infection in Surviving Vaccinated Mice After Challenge

Days After Infection	Spleen Culture	# Positive / # Sampled	% Positive
15	DCBA	3/30	10
19	Mouse injection	5/10	50
20	Mouse injection	10/23	43.5
30	Mouse injection	3/10	30

Table XLIX

MLD Titration on Surviving Mice
Challenge suspension contained 1.05×10^9 organisms per ml

Challenge Dilution	No. of Mice	# Deaths / Total	% Survival	LD_{50}*	Organisms per LD_{50}
Survivors					
10^{-7}	10	5/10	50		
10^{-8}	10	8/10	20	$10^{-8.25}$	4.0
10^{-9}	10	3/10	70		
Normal controls					
10^{-7}	7	7/7	0		
10^{-8}	7	7/7	0	$10^{-9.0}$	0.5
10^{-9}	8	4/8	50		

* Expressed as the dilution of challenge suspension which contained one LD_{50}/ml

harbored "UL" organisms in great numbers. Normal controls of the same age and weight similarly dusted with DDT remained healthy. Apparently DDT was more toxic for animals with latent "UL" infections.

Forty-one days after the original challenge, 11 outwardly healthy surviving mice were placed in the 37°C incubator on a shaking machine which made 70 occillations per minute. Eleven normal controls of equal age and weight were similarly treated. At the end of 6 hours 10 out of 11 of the test animals were dead while all 11 of the normal controls survived. Spleen cultures on all dead animals were negative for "UL". Microscopic sections showed marked acute passive congestion of the spleen and liver, and hemorrhage into the liver tissue. The cytoplasm of liver cells around the central veins

stained pale pink with H and E and the nuclei were pale but still visible. Controls showed nothing unusual in the liver or spleen.

While this was a severe test, the fact that all the normal animals survived indicates a lack of physiological reserve in the recovered vaccinated animals. The negative spleen cultures indicate that these animals did not die of "UL" infection. A prolonged period of convalescence has been observed in man after recovery from "UL" infections. During this period easy fatiguability, weakness and lack of endurance are the chief symptoms and it is possible that in the recovered mouse we have found an experimental animal which can be used to study the mechanism of the prolonged debility following recovery from "UL" infection.

(3) Pathology of "UL" in Convalescent Mice

The pathology of "UL" in normal and vaccinated mice has been described elsewhere in this report. (See section on Immunization of Rats, Pathology and Pathogenesis.) The microscopic findings in vaccinated recovered mice sacrificed 15 days after infection were variable. In a few animals the spleen contained small areas of coagulation necrosis in the red pulp with infiltrating polymorphonuclear and large mononuclear cells. In some of these animals small areas of focal necrosis and mononuclear infiltration were found in the liver. Kupfer cells were prominent. One animal had areas of focal hyperplasia of large mononuclear cells in the lungs, one of which showed central necrosis of the mononuclear cells. These animals had positive spleen cultures at autopsy and may have eventually died of "UL" infection.

The majority of animals sacrificed at 15 days showed only small areas of focal hepatitis infiltrated with mononuclear macrophages. Kupfer cells were prominent throughout the sinusoids. No necrosis of liver cells were seen. The spleens of these animals showed malpighian hyperplasia and an extensive hyperplasia of the reticulum cells of the red pulp.

In general these same variations were seen in animals sacrificed 30 days after challenge. Sections of liver and spleen from a number of mice in this group appeared perfectly normal.

In summary the results of spleen culture, and the microscopic pathology, suggest that vaccination converts "UL" from an acute fulminating disease to a chronic or latent infection in a substantial percentage of mice. The latent or chronic infection finally proves fatal in some animals but others gradually eliminate the organism completely as shown by microscopic pathology and spleen culture. Convalescing mice in which no viable organisms can be demonstrated readily succumb to chemical and physical trauma which is not fatal to normal mice.

c. Monkeys (Macacus rhesus-India)

The occurrence of local skin ulceration and regional lymphadenopathy in monkeys (Section B-2) challenged with "UL" suggested the clinical picture seen in the ulceroglandular form of "UL" infection in man. However, the

mortality rate in monkeys appeared to be greater than in man and therefore a vaccine which would protect the monkey might be very efficacious in man. To obtain more information on the course of "UL" infection in monkeys and to study the effect of vaccination on the course of the disease, two experiments were carried out. In the first experiment young immature monkeys averaging 4½ to 5½ pounds in weight were used. In the second experiment 6 to 10 pound young adult animals were used.

(1) Experiment I

Four young Macacus rhesus monkeys were given 0.5 ml of acetone extracted vaccine #30 subcutaneously on three alternate days in accordance with the standard procedures for human vaccination. One monkey was vaccinated later than the others and was therefore challenged 10 days after vaccination, whereas the other three were challenged 29 days after vaccination. Five normal controls were challenged at the same time. The challenge consisted of 1.0 ml of a 10^{-4} dilution of a 24 hour culture of strain SM21R3 injected subcutaneously on the belly 1 inch to the left and 1 inch anterior to the umbilicus. This challenge contained 217,000 organisms by plate count or between 10,000 and 100,000 monkey MLD on the basis of a previous incomplete monkey titration on animals of the same weight. Daily complete blood counts, clotting time, prothrombin time, and blood cultures were made. Rectal temperatures were taken every 6 hours. Agglutination tests, weights, skin tests, and urinalysis were taken at suitable intervals.

Tabulated results are given in Tables L and LI. All monkeys became ill within 24 hours as shown by an elevation of rectal temperature. The third column in Table L shows that the 5 control monkeys died within 6 days. One vaccinated animal, #51, died after 4 days and the other three lived slightly longer than the controls.

All control monkeys ran a course similar to that previously reported (Section III-B-2 this report). They had a high rectal temperature for several days which fell to subnormal levels 12 hours before death, coincident with a fall in total white blood cell count and extreme prostration. Blood cultures were positive on an average of 3 days prior to death and quantitative counts demonstrated a daily logarithmic increase in number of organisms per ml of blood. Prothrombin and clotting time remained normal in 3 out of the 5 controls and were moderately elevated in 2 animals just prior to death. The outstanding findings in microscopic pathology were fatty degeneration of liver cells and extensive focal hepatitis, massive coagulation necrosis of the spleen, necrosis of the skin without involvement of the muscle layer of the belly wall, and central coagulation necrosis of regional lymph nodes. Congestion, minute hemorrhagic areas, and small areas of focal necrosis were seen in the adrenal cortex together with depletion of cortical lipoid.

Although all the vaccinated animals died, they differed in several respects from the controls. Number 51 which died on the 4th day was found at autopsy to be heavily infested with tapeworms. He had been edematous and had lost weight even before challenge. Monkeys 60 and 64 lived slightly longer than the controls and #58 lived 14 days. During the last three days of life, monkey #58 developed a spiking temperature, accompanied by grunting respiration.

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TABLE L

Summary of Results of "UL" Challenge in Vaccinated Monkeys

Number Indicate the Day of Disease on Which a Finding First Became Positive
All Monkeys Challenged Subcut. With 217,000 Viable Organisms Strain SM21R3

	Animal Number	Day of Death	Local Lesion: Edema	Local Lesion: Hemorrhage	Necrosis	Regional Lymph-adenopathy	Positive Blood Culture	Leukopenia	Generalized Petechial Hemorrhage into skin	Weight lbs. -Oz.	Weight Loss Oz.
Normal Control	56	4	2	3	3	2	2	4	4	4-10	16½
	65	4	2	None	None	4	3	4	4	5-2	14½
	17	5	2	3	4	2	3	5	4.5	8-12	14
	62	5	2	4	5	2	3	4	5	6-3	28½
	54	6	2	3	4	2	4	5	None	4-10	8
Vaccinated	51	4	2	2	3	3	3	4	None	4-9	9
	60	6	2	2	4	2	4	6	None	5-6	14
	*64	7	2	4	6	2	4	6	None	5-5	14½
	58	14	2	2	3	2	7	None	None	4-13	4

* Interval between 1st dose of vaccine and challenge was 29 days, except Monkey #64 which was vaccinated 10 days before challenge.

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In general the vaccinated monkeys developed larger, more extensive local lesions at the site of challenge than did the controls. This is shown in the 5th column in Table L under "hemorrhage into the local lesion". Vaccinated monkeys developed moderate leucopenia before death with nearly normal ratio between segmented and non-segmented cells, whereas the controls showed total white counts of one to two thousand with almost complete absence of polymorphonuclear cells.

Vaccinated animals did not develop generalized petechial hemorrhages in the skin such were seen in 4 of the 5 control animals.

Table LI shows the results of quantitative daily blood cultures. In general the number of organisms per ml of blood was fewer in the vaccinated animals than in the controls at the time of death. Vaccinated monkey #58 had a transient positive blood culture on the 7th and 8th days of illness and again on the 13th and 14th days of illness but the numbers of organisms per ml of blood did not show daily progressive increase.

The agglutinin antibody titer in vaccinated animals was 1:80 in three animals and 1:160 in one animal on the day of challenge. The titers were temporarily elevated two days after challenge in 2 animals and remained the same in two animals. Four days after challenge all titers had dropped but none reached zero before death of the animal.

Urinalysis on Monkey#58 showed albuminuria and hyaline casts.

The gross and microscopic pathology in vaccinated animals was similar to that observed in controls, with the exception of monkey #58. Numerous small semitransparent yellow nodules were present, scattered over the surface and throughout the substance of the lung. No gross evidence of "UL" infection was observed in the liver, spleen, or kidneys. Microscopic sections showed reticulo-endothelial hyperplasia of the spleen, a few small areas of focal mononuclear infiltration in the liver with no necrosis or fatty change in the parenchymal cells. The kidneys were not abnormal. The lungs showed foci where groups of alveoli were filled with necrotic debris infiltrated by polymorphonuclear cells, monocytes and macrophages. No peripheral fibrosis or acute inflammatory reaction was observed around these areas.

Summary of Experiment 1

Vaccination of immature monkeys as carried out in this experiment did not protect them against challenge with 10,000 MLD, however, the disease was sufficiently modified in one monkey to suggest that protection might have been conferred against a smaller challenge.

(2) Experiment II

Twenty-one young adult monkeys were divided into four groups and the animals in each group were injected with a different "UL" vaccine. Each

Table LI

Blood Cultures on DCBA Plates After "UL" Challenge

Numbers Represent the Number of "UL" Colonies on 0.1 ml of Whole Blood

Monkey #	Days After Challenge													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Controls														
56	0	1	186	1980*										
65	0	0	182	2000*										
17	0	0	2	57	1260*									
62	0	0	12	911	4/*									
54	0	0	0	3	29	1780*								
Vaccinated														
51	0	0	31	300*										
60	0	0	0	3	108	676*								
64	0	0	0	8	10	82	840*							
58	0	0	0	0	0	0	1	1	0	0	0	0	1	3*

* Died
4/ Confluent growth

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animal was given 0.5 ml of vaccine subcutaneously on two alternate days followed by a booster dose of 0.5 ml twenty-one days later. All vaccines were standardized to contain 0.035 mgm total nitrogen per 0.5 ml dose. The vaccines used were: #16, Foshay; #31, acetone extracted virulent strain S; #40, acetone extracted avirulent strain 38; and CPE, a purified protein fraction obtained by chloroform extraction of a peptone broth culture (see Fractionation Studies) precipitated with 0.1% alum. Determinations of clotting time and prothrombin time were omitted on this series of monkeys because no significant changes had been observed in the previous group. Sedimentation rates were determined, at two to three day intervals on nine vaccinated animals, two challenged control animals, and one normal animal which was not vaccinated or challenged. Whole blood was added to Heller's and Paul's antiocoagulant (102) (ammonium and potassium oxalate), mixed, and drawn into a 1 ml Exax pipette which was supported on a rubber stopper between two shelves. Readings were made at intervals and reported in millimeters. The same pipette was used for each animal throughout the experiment. The twenty-one vaccinated animals and five normal animals were challenged subcutaneously thirty days after the original vaccination. Part of the animals in each group were challenged with 1.0 ml of 10^{-6} dilution and the remainder with 1.0 ml of 10^{-8} dilution of a standard "UL" suspension of strain S containing 740 million organisms per ml as determined by DCBA count. The 10^{-6} challenge contained approximately 740 "UL" organisms and the 10^{-8} challenge approximately 7.4 "UL" organisms on the basis of the plate count. Judging by the previous MLD titration on immature monkeys the smaller challenge was less than one LD_{50} dose while the larger challenge was in the neighborhood of 100 LD_{50} doses. Table LII shows the results of challenge of the vaccinated and control animals in Experiment II.

It is evident that challenge doses used in this experiment were not sufficient to kill all the control animals. None of the controls were killed by the smaller challenge while two out of three were killed by the larger challenge containing 740 "UL" organisms. This group of animals apparently had more natural resistance to "UL" than those previously tested. The previous titration had been done on young animals weighing 4½ to 5½ pounds, while in the present experiment older monkeys were used. Deaths were not limited to the smaller animals in this group but 4 of 5 monkeys weighing less than 5½ pounds died. It seems very probable that the difference in susceptibility to "UL" in the two groups of monkeys may be partially explained by the difference in age of the two groups.

The data in Table LII also show that as many deaths occurred in the vaccinated groups as in the controls, with one exception. All the animals vaccinated with Foshay's vaccine survived. Previous immunization of rats with this particular lot of acetone extracted vaccine (#31) had shown that it was not as protective as earlier preparations. Rat work had also shown that the chloroform extracted protein fraction was not a very active antigen, but vaccine #40 prepared from avirulent strain NIH 38 had previously protected rats. The number of monkeys on each vaccine was small but apparently Foshay's vaccine was superior to all the others. The temperature chart, weight, agglutinin titer, blood picture, sedimentation rate, and daily blood culture for each monkey are shown graphically in Figures 14 to 40.

Table LII

Monkey Vaccination Experiment II

Vaccine # Code (0.1 mgm)	No. of Monkeys	Challenge 1.0 ml Subcutaneously: 740 organisms Dead/Total	740 organisms % Survival	7.4 organisms Dead/Total	7.4 organisms % Survival
16 Foshay	5	0/3	100	0/2	100
- CPE	6	1/3	67	1/3	67
40-38	5	1/3	67	1/2	50
31-Acetone Extracted	5	2/3	33	1/2	50
None	5	2/3	33	0/2	100
Total	26	6/15	60	3/11	73

At the site of vaccination a subcutaneous indurated nodule 18 by 20 mm in diameter appeared on the day after injection of the alum precipitated chloroform extracted protein fraction, CPE. These nodules became slightly smaller but were still present sixty-two days later when the animals were sacrificed. On cut section at autopsy the nodules contained thick yellow exudate. The other three vaccines caused a less extensive local reaction at the injection site which disappeared completely within a week.

Agglutinin Response

All monkeys developed serum agglutinins to "UL" as a result of vaccination. The animals which received Foshay's vaccine had the highest titers and those that received the acetone extracted vaccine #31 had the lowest titers. The average titer of all monkeys in each group on the day of challenge was as follows:

Vaccine	Average Titer
Foshay	1:160
Acetone extracted, strain 38	1:72
CPE	1:53
Acetone extracted, strain S	1:40

Sera from the six unvaccinated controls did not agglutinate a "UL" suspension.

Monkeys Vaccinated With Foshay's Vaccine and Challenged Subcutaneously With 740 Organisms

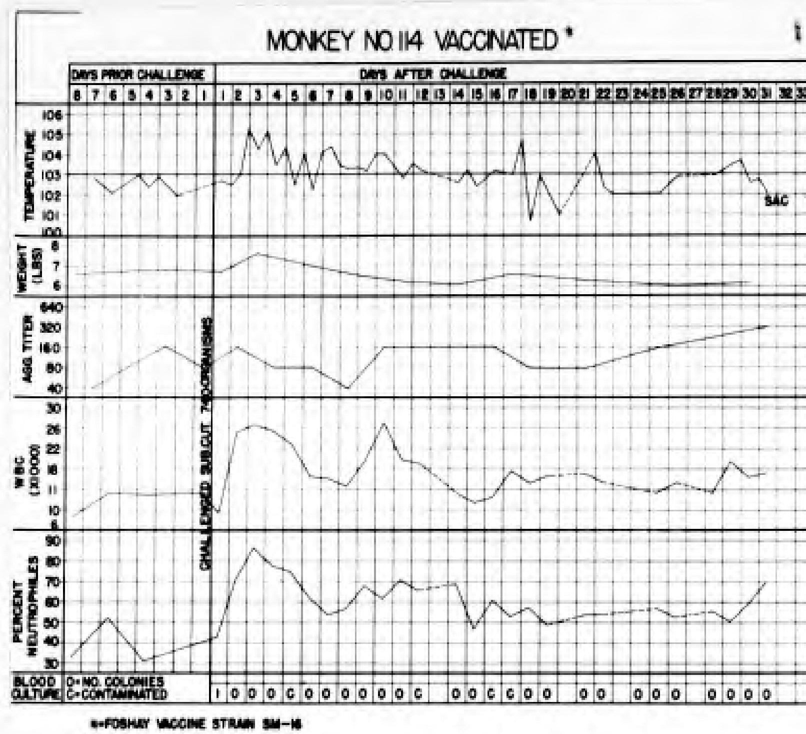

Figure 14

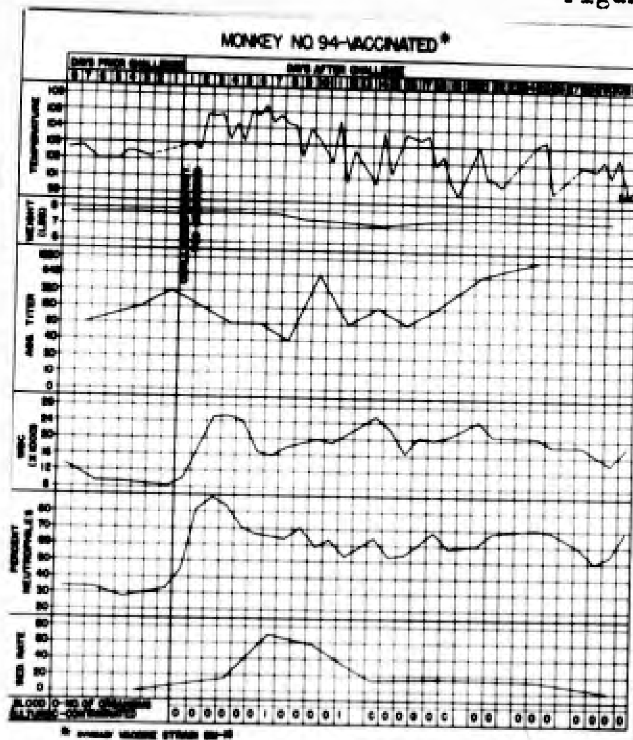

Figure 15

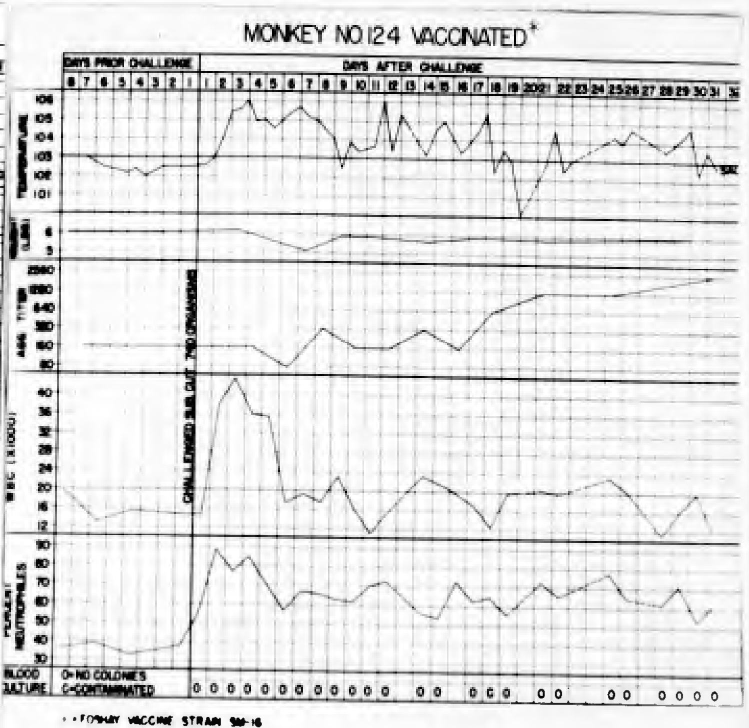

Figure 16

Monkeys Vaccinated with Foshay's Vaccine and Challenged Subcutaneously With 7.4 Organisms

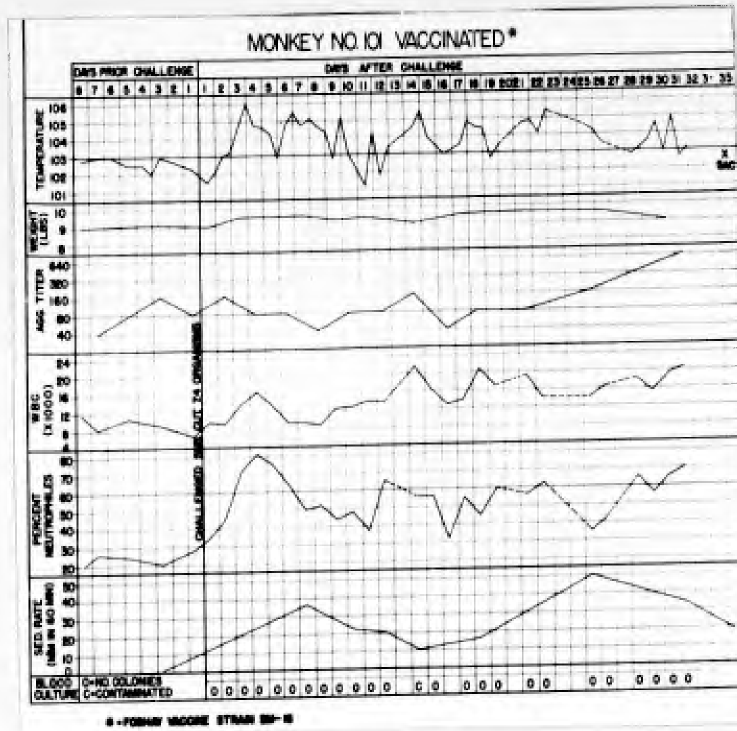

Figure 17

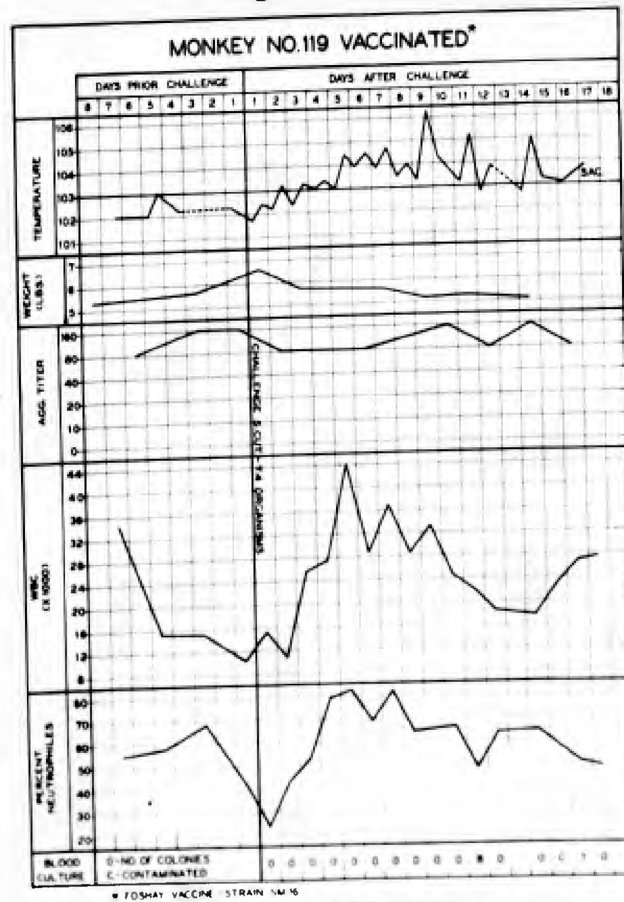

Figure 18

Monkeys Vaccinated With Chloroform Extracted Protein Fraction Vaccine and Challenged Subcutaneously With 740 Organisms

Figure 19

Figure 20

Figure 21

Monkeys Vaccinated With Chloroform Extracted Protein Fraction Vaccine and Challenged Subcutaneously With 7.4 Organisms

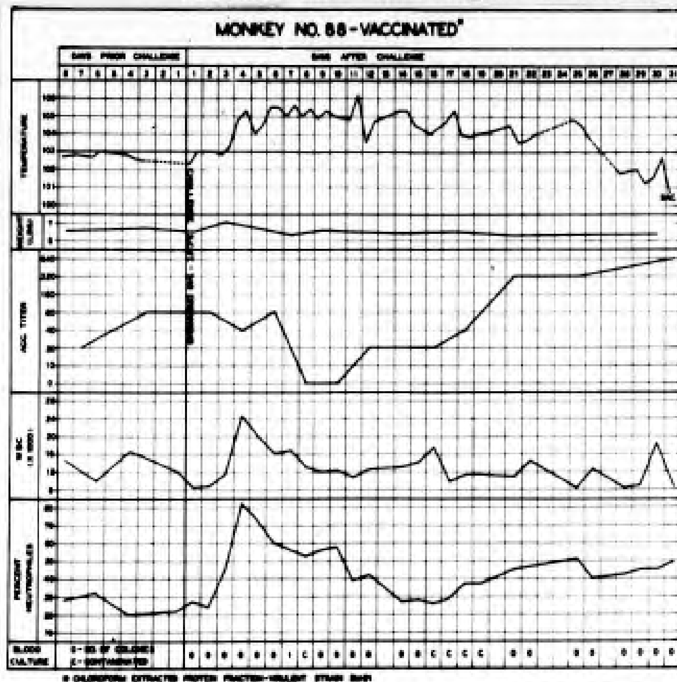

Figure 22

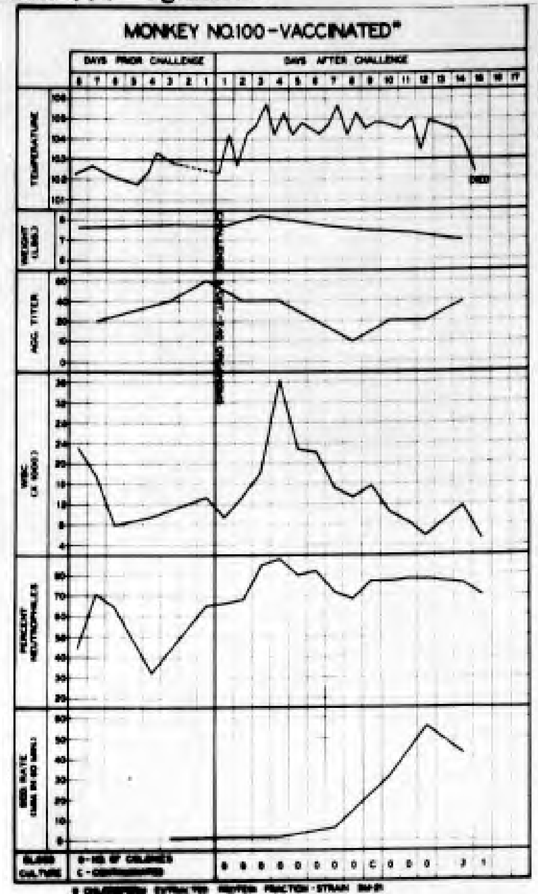

Figure 23

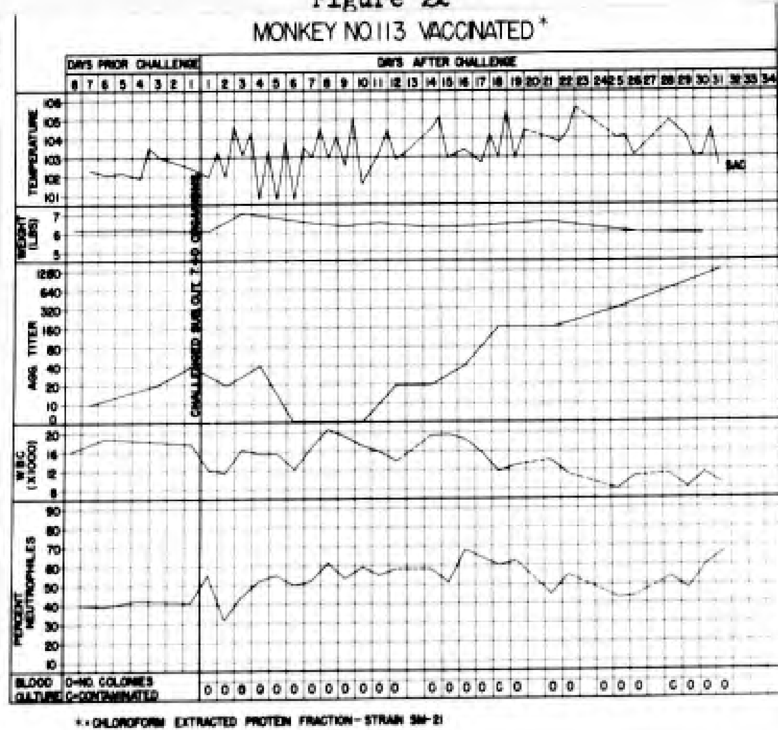

Figure 24

Monkeys Vaccinated With Acetone Extracted Vaccine Avirulent Strain 38 and Challenged Subcutaneously With 740 Organisms

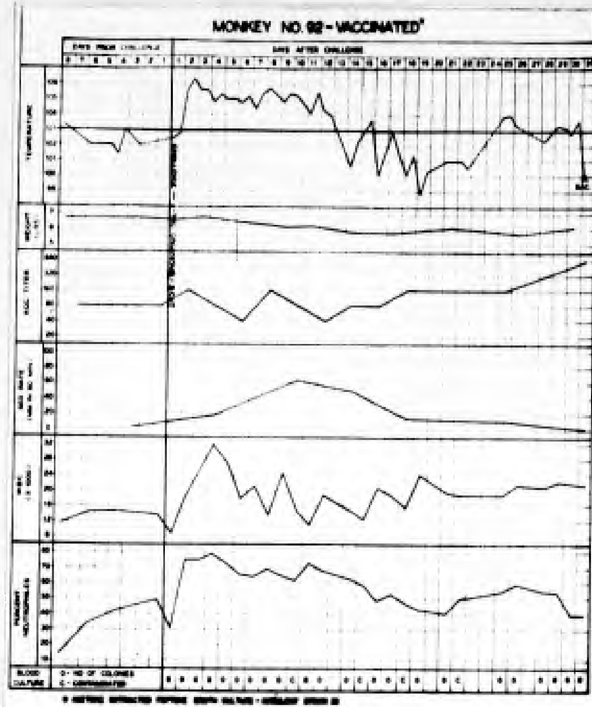

Figure 25

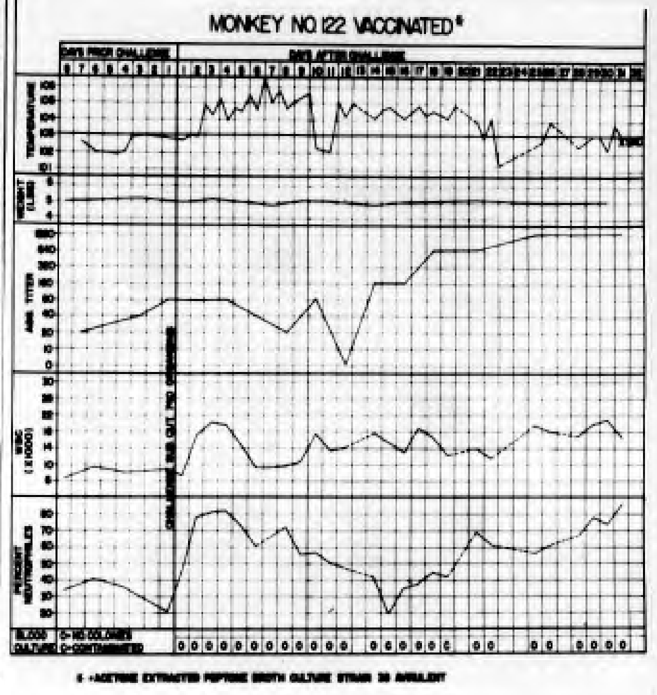

Figure 26

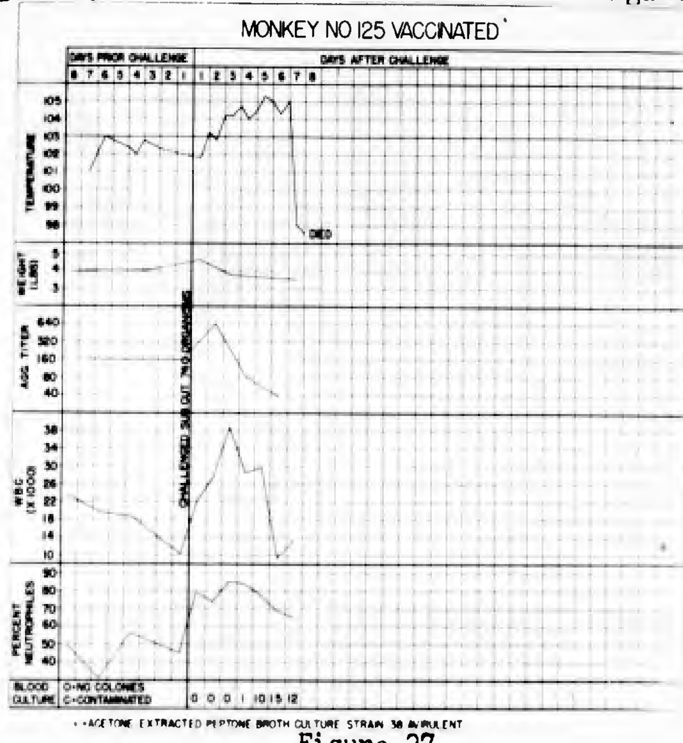

Figure 27

Monkeys Vaccinated With Acetone Extracted Vaccine Avirulent Strain 38 and Challenged Subcutaneously With 7.4 Organisms

Figure 28

Figure 29

Monkeys Vaccinated With Acetone Extracted Vaccine Virulent Strain SM21 and Challenged Subcutaneously With 740 Organisms

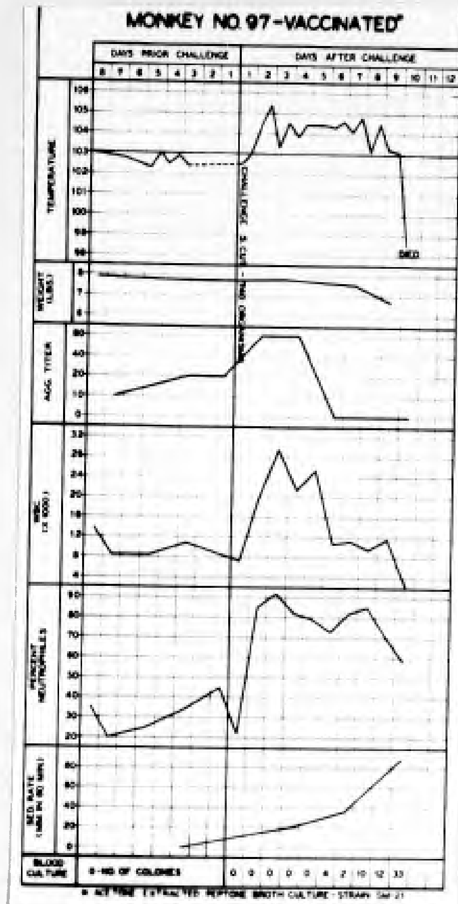

Figure 30

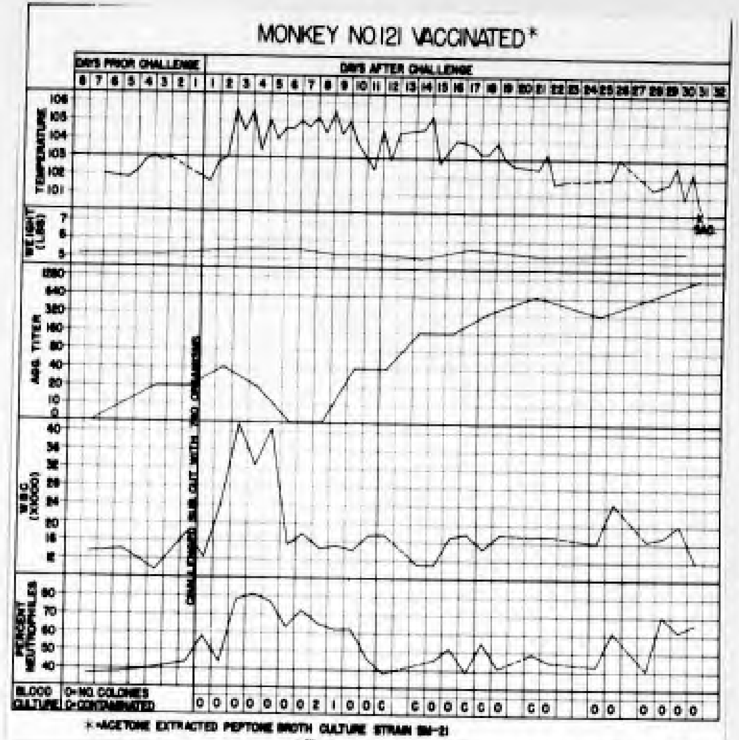

Figure 31

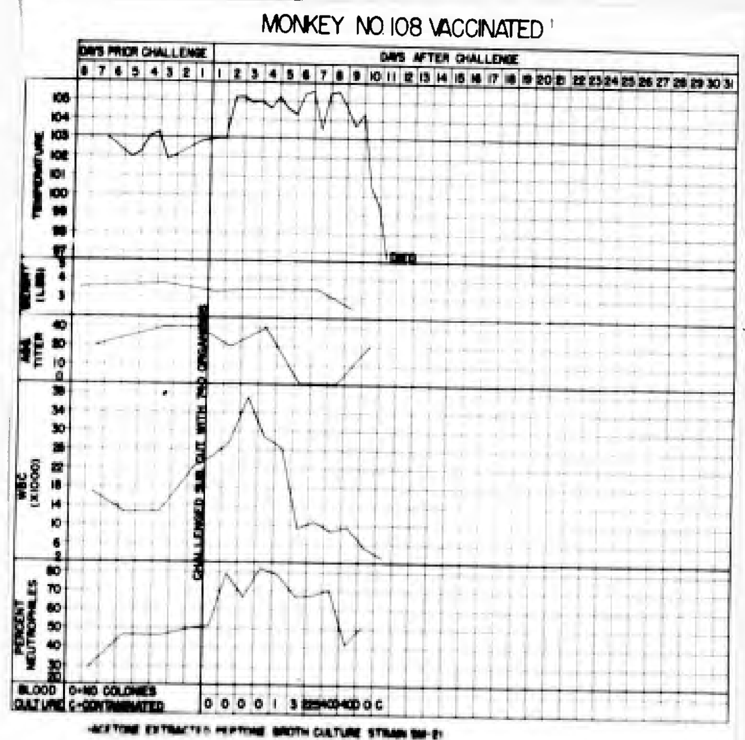

Figure 32

Monkeys Vaccinated With Acetone Extracted Vaccine Virulent Strain SM21 and Challenged With 7.4 Organisms Subcutaneously

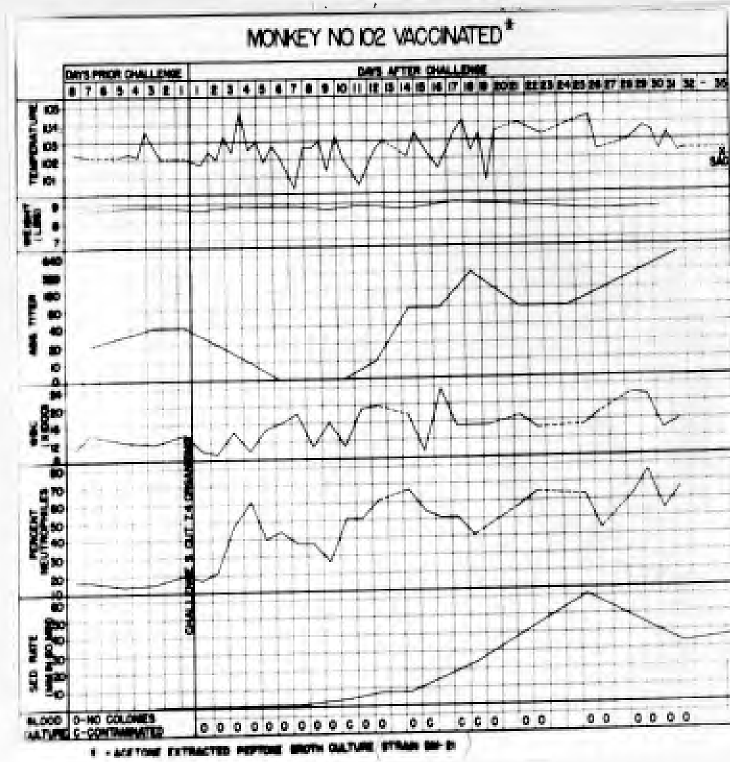

Figure 33

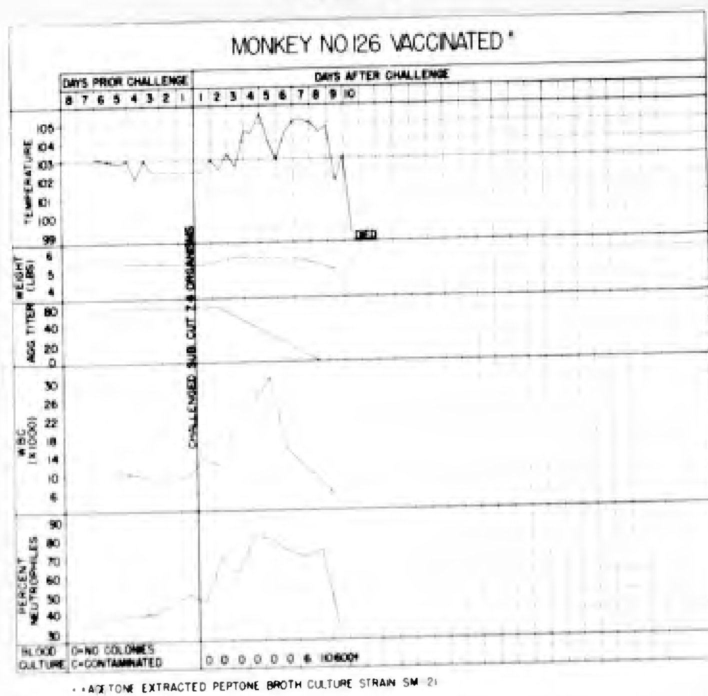

Figure 34

Control Monkeys Challenged Subcutaneously With 740 Organisms

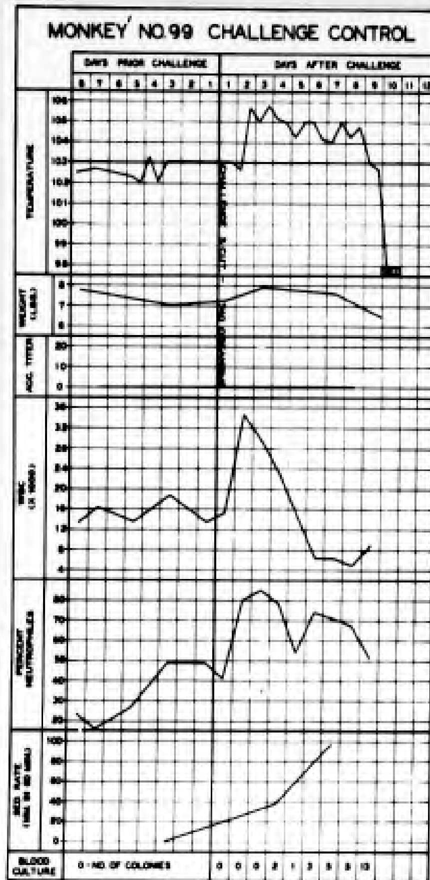

Figure 35

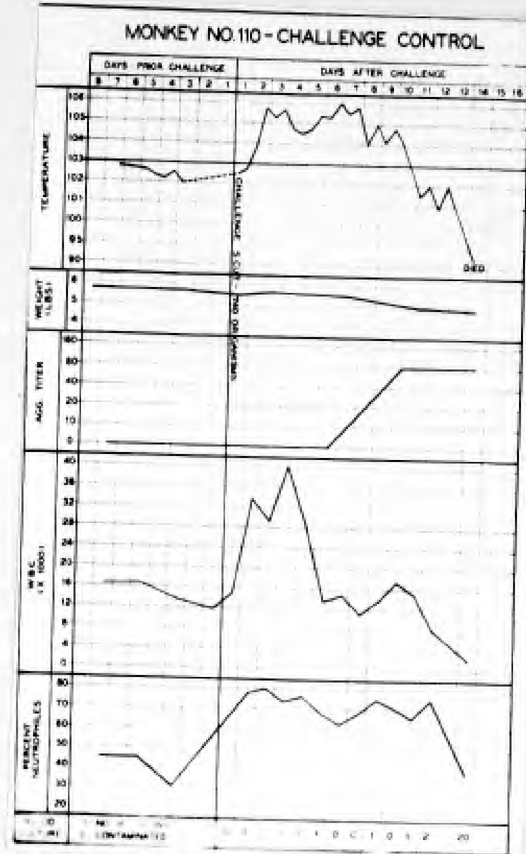

Figure 36

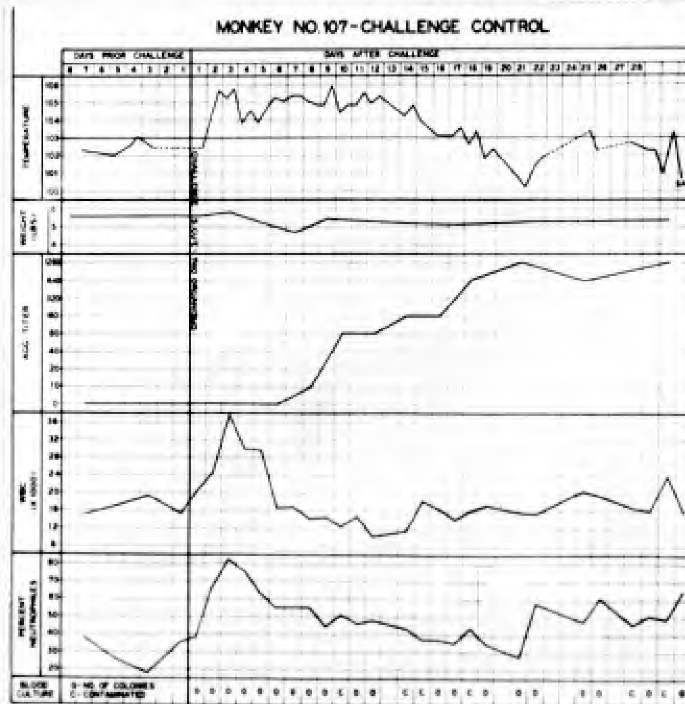

Figure 37

Control Monkeys Challenged Subcutaneously With 7.4 Organisms

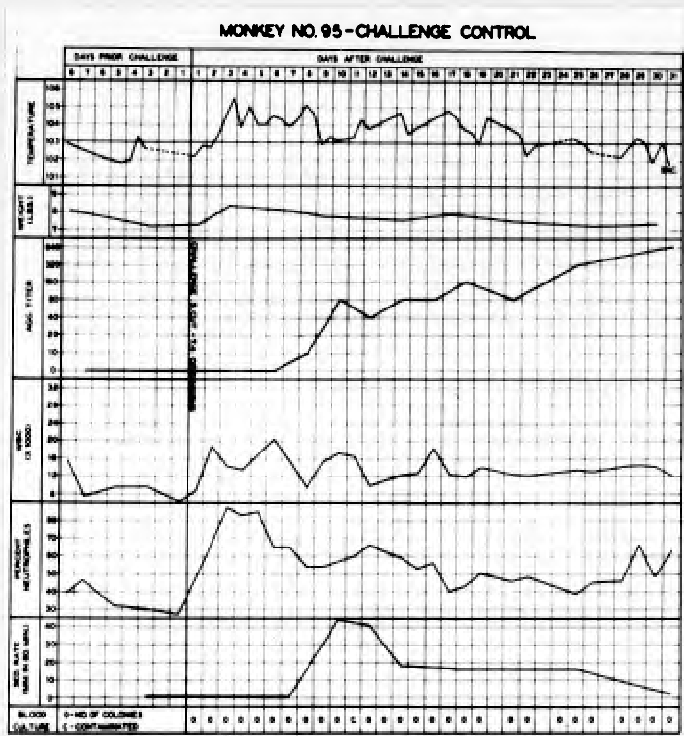

Figure 38

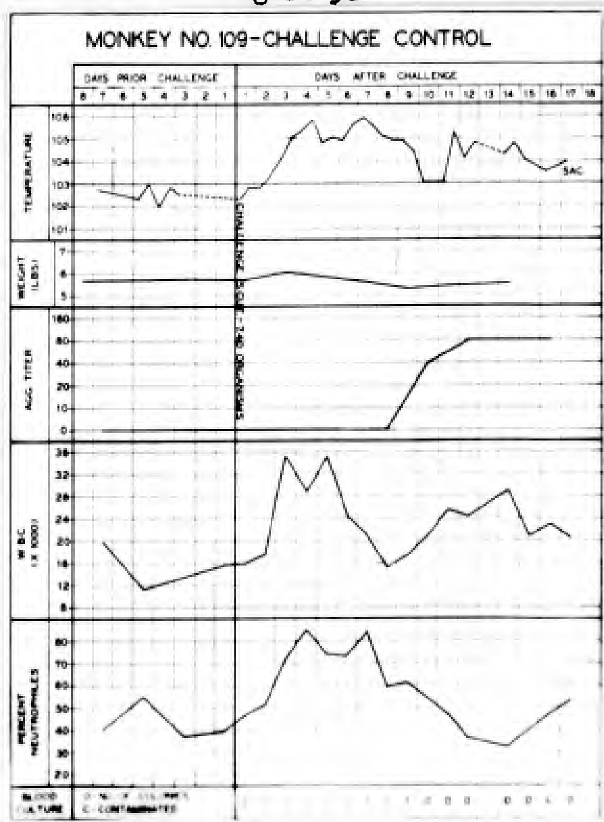

Figure 39

Normal Control Monkey Neither Vaccinated
Nor Challenged

Figure 40

Within two to six days following challenge the agglutinin titer dropped in every vaccinated animal, Figures 14 to 40. In those animals vaccinated with the Foshay vaccine the drop was slight, whereas in the animals vaccinated with other vaccines, twelve out of sixteen showed complete absence of agglutinins in serum dilution of 1:10. During the acute phase the titers remained low in all animals. Eight to twelve days after challenge the titers started to rise and during the succeeding days they gradually increased to reach an average of 1:640 at the end of four weeks.

The drop in titer during the acute phase agrees very well with the findings in human cases occurring in vaccinated personnel. In fact, a drop in titer coincident with the onset of symptoms has been the most consistent early diagnostic sign in the present series of human cases in vaccinated personnel. (See Section F-2 this report).

General Clinical Picture

All monkeys became ill after challenge as shown by an elevation of temperature and loss of weight. On the basis of temperature rise the incubation period was 1-2 days in animals challenged with 740 organisms and 2-5 days in animals challenged with 7.4 organisms. This was similar to the previous observation in mice, Section D and Table XXVI, where the incubation period was also proportional to the size of the challenge dose. The clinical picture was similar to that previously described, namely, high fever, weakness, prostration, weight loss, local ulceration, and regional lymphadenopathy. One animal died on the seventh day, four on the tenth day, one on the eleventh day, one on the fourteenth day, one on the fifteenth day, and one on the sixteenth day. One unvaccinated control and one animal vaccinated with Foshay's vaccine were sacrificed on the seventeenth day to study the course of the infection in the Foshay vaccinated group. The remaining animals were sacrificed between the thirty-first and the thirty-fifth days after challenge when it became necessary to terminate the experiment because of discontinuation of the project. "UL" was recovered from all sacrificed animals at autopsy. It is possible that additional animals would have eventually died had they been observed for a longer period.

Blood Cultures

The day of death and occurrence of positive blood cultures are shown in Table LIII. Sixteen out of the twenty-six animals developed positive blood cultures at some time during the course of the disease. Of the sixteen animals with positive blood cultures, nine eventually died. The other seven animals had only transient positive blood cultures with a small number of organisms per ml of blood.

Animals which died within the first ten days after challenge had a progressively increasing number of organisms per ml of blood, while those that died later had less blood stream invasion. One animal #93 which died on the sixteenth day had a transient positive culture on the seventh and eighth days after challenge and thereafter a negative culture up to and including the day of death. Apparently an increasingly positive blood culture

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Table LIII

Daily Blood Cultures on Monkeys After Challenge With "UL"

0.1 ml Whole Blood Plated on DCBA

Vaccine	Monkey #	Challenge # Organisms	Day after Challenge																	
			1	2	3	4	5	6	7	8	9	10	11	12	14	15	16	17	18	19-31
CPE	111	740.0	0	0	0	0	0	1	0	0	C	0	0	0	C	0	0	C	NC	0
	93	740.0	0	0	0	0	0	C	1	1	0	0	0	0	0	0	0*			
	106	740.0	0	0	0	0	C	0	0	C	0	C	0	0	C	0	0	C	0	0
	113	7.4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	C	0
	100	7.4	0	0	C	0	0	0	0	0	C	0	0	0	3	1C*				
	88	7.4	0	0	0	0	0	0	1	C	0	0	0	0	0	0	C	C	C	0
# 40 Acetone Extracted Strain 38	125	740.0	0	0	0	1	10	15	12*											
	92	740.0	0	0	0	0	0	0	0	0	0	C	0	0	0	C	0	0	C	0
	122	740.0	0	0	0	0	0	0	0	0	0	0	0	0	0	C	0	0	0	0
	116	7.4	0	0	0	0	0	2	5	39	34	132*								
	96	7.4	0	0	0	0	0	0	0	0	0	0	0	0	C	0	0	0	C	0
# 16 Feshay	124	740.0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	NC	0	C	0
	94	740.0	0	0	0	0	0	0	1	0	0	0	0	1	C	0	0	0	0	0
	114	740.0	1	0	0	0	C	0	0	0	0	0	0	C	0	0	C	C	0	0
	119	7.4	0	0	0	0	0	0	0	0	0	0	8	0	0	C	1	0s		
	101	7.4	0	0	0	0	0	0	0	0	0	0	0	0	C	0	NC	0	0	0
# 31 Acetone Extracted Strain S	121	740.0	0	0	0	0	0	0	0	2	1	0	0	C	C	0	0	C	C	0
	97	740.0	0	0	0	0	0	4	2	10	12	33*								
	108	740.0	0	0	0	0	1	3	225	400	400	0	C*							
	126	7.4	0	0	0	0	0	0	6	110	600/	*								
	102	7.4	0	0	0	0	0	0	0	0	0	C	0	0	0	C	NC	C	C	0
Controls	110	740.0	0	0	0	0	0	1	0	C	1	0	1	2	20*					
	99	740.0	0	0	0	2	1	3	5	5	13	*								
	107	740.0	0	0	0	C	0	0	0	0	0	C	0	0	C	C	0	0	C	0
	109	7.4	0	0	0	0	0	0	5	0	1	0	0	0	0	0	C	0s		
	95	7.4	0	0	0	0	0	0	0	0	0	0	C	0	0	0	0	0	0	0

Numbers represent "UL" colonies on plates after 72 hours incubation.
* - Died. 0 = Neg. C = Contaminated. NC = no count made. s = Sacrificed.

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during the acute phase of the disease indicates a probable fatal outcome, whereas in the subacute or chronic disease death may occur in the absence of bacteremia. This was also observed in Monkey Experiment #I.

Blood Picture

The normal red blood cell count for monkeys varied between 5.0 and 6.0 million per cubic centimeter with hemoglobin values of 13-15 grams, Haden-Hausser. The normal total white blood cell count varied between 7,500 and 25,000 with an average of 14,000. The average differential count of white blood cells was: neutrophils 42.6%; lymphocytes 51.8%; monocytes 1.2%; eosinophiles 2.8%; and basophiles 0.3%.

After challenge the blood counts of control and vaccinated animals followed a similar pattern. From 1.5 to 5 days after challenge there was an increase in total count accompanied by an increase in the segmenting and band forms of neutrophilic leukocytes. The day of increase in total white count depended on the size of the challenge which the animal received. Those animals receiving a challenge of 7.4 organisms showed a noticeable increase in either total or differential count in 3.5 days with the peak reached in 4.8 days. Those animals receiving 740 organisms at challenge showed a rise within 1.8 days, the peak being reached in 3.3 days. This increase in total count was accompanied by a marked increase in neutrophils with a slight increase in immature forms. In instances where the total count did not rise above normal the peak was determined by the increase in neutrophils and bands. After the initial peak in total white blood count there was a sharp differentiation into groups:

Group I. Those animals dying within 9-16 days after challenge showed a marked drop in total white blood cells to normal or below with a marked shift to the left. The average height to which the immature forms rose was 26%. One animal in this group showed a neutropenia.

Group II. Those animals living until sacrificed 31-35 days after challenge showed a continued high total or high neutrophile count with little or no shift to the left. The following table shows the distribution of the vaccinated and control monkeys into the two groups.

All animals except one showed a sudden increase in monocytes at the acute invasive stage of the infection. The height of the monocytosis was reached in from 1 to 7 days after challenge, the average being 4.5 days for both groups. This monocytosis continued over a considerable period of time. In Group II 76% of the animals continued to show increased monocytes at the time of sacrifice, and 55% in Group I continued to show an increase at the time of death.

All animals showed an absence or decrease in eosinophils and basophils until just before sacrifice.

All animals which survived showed a slow consistant fall in red count (average fall of 1.5 million per cu. mm.) from the day of challenge until

Table LIV

Distribution of Monkeys Showing
Leukopenia and Leukocytosis

Vaccine	Total No. of Monkeys	Group I Leukopenia (died)*	Group II Leukocytosis (sacrificed)**
CPE	6	2	4
Acetone extracted Strain 38	5	2	3
Acetone extracted Strain S	5	3	2
Foshay	5	0	5
None	5	2	3

15-22 days thereafter. After the third week there was a slow but steady increase in both red cells and hemoglobin despite the fact that the daily bleedings of from 1 to 5 cc were continued.

Monkey 112 was neither vaccin ted nor challenged, but was bled each day to determine the effect of daily bleeding on the blood count. The total white count fluctuated between 8-25,000 with an average of 16,000. The neutrophils varied from 19% to 68% with an average of 45%. There was no correlation between the peaks in total white count abd per cent neutrophils in this normal monkey. The red count and hemoglobin dropped slightly during the first week (approximately 700,000 per cc) but returned to normal by the tenth day after the other animals were challenged. There was a moderate fall during the last days of the experiment probably due to the daily removal of larger amounts of blood for control purposes.

The hematological findings may be summarized as follows:

1. After challenge there was a sharp increase in total white count accompanied by an increase in the percentage of polymorphonuclear leukocytes with a slight shift to the left.

2. The increased total count returned to near normal within two to four days, but the percentage of neutrophils tended to remain elevated in those animals which survived.

3. The nine animals dying showed terminal leukopenia with a marked shift to the left.

4. A moderate monocytosis occurred during the acute invasive stage of the infection in all but one animal, and in 68% it persisted until death or sacrifice.

5. An absolute decrease in eosinophils and basophils was noticed in all animals.

6. A slow progressive drop in red blood cells and hemoglobin occurred during the first two to three weeks after challenge. Thereafter there was a gradual increase.

7. No difference was noted in the blood pictures of the vaccinated and control groups.

Weight:

All animals lost weight during the height of their illness. The amount of weight loss was moderate in most cases. Those animals that died lost from 6 to 23 ounces with an average of 15 ounces. Many animals had almost regained their original weight by the time of sacrifice. Animals with internal necrotic foci did not gain weight.

Urinalysis:

Seven animals showed albuminuria and four showed glycosuria for a brief period during the course of the disease. No urinary casts or hematuria were demonstrated. These clinical findings agree with the microscopic pathology namely, that the kidneys are not severely damaged in "UL" infection.

Sedimentation Rate: Sedimentation rates are expressed as mm of settling when read after standing 60 minutes.

The sedimentation rate before challenge varied from zero to two mm. The sedimentation rate of the unchallenged control monkey #112 was not affected by the repeated daily bleedings and remained below 1 mm throughout the experiment. All eleven challenged monkeys on which successive determinations were made showed a marked increase of the sedimentation rate. Five of these animals died. The maximum sedimentation rates and day of the disease on which it occurred in these five animals are as follows: 96 mm-7th day; 88 mm-10th day; 82 mm-12th day; 56 mm-12th day; and 56 mm-7th day. In five animals the sedimentation rate was elevated and returned to or near normal as the acute phase of the disease subsided. In one animal, #102, the sedimentation rate did not increase significantly until the third week after challenge and remained high at the time of sacrifice. This was consistent with the clinical course of the disease as shown by elevation of temperature. Readings made 31 to 35 days after challenge in additional animals showed normal values in five and elevated values in four. (See Table LV) Of the six animals showing elevated readings at the time of sacrifice five had purulent substernal or rectropleural lymph nodes at

Table LV

Sedimentation Rates on Monkey Bloods Expressed in mm After 60 Minutes*

All Animals Challenged Except #112

Monkey #	Vaccine	Organisms in Challenge	Before Challenge	Days after Challenge 4	7	10	12	14	18	25	31	32	33	35
112	None	None	1		0	1	0	0	0	0	0			0
125	38	740.0			56#									
99	None	740.0	0	38	96#									
97	A-2	740.0	0	20	36	88#								
93	CPE	740.0	0	4	16	73	82	68#						
100	CPE	7.4	1	1.5	6	32	56	42#						
95	None	7.4	1		1	43.7	41	18	16	16	2			2
94	Foshay	740.0	1	16	70	58	34	14	16	14	1			
96	38	7.4					25	14.5	3	1	1			
92	38	740.0	2	18	40	64	56	50	14	11	1	2		
107	None	740.0									0			
121	A-2	740.0										0		
88	CPE	7.4									0		0	
106	CPE	740.0									4	2		
111	CPE	740.0												1
102	A-2	7.40	1		1	4	8	8	19	61	34			38
101	Foshay	7.40	1		36	22	20	10	16	50	34			18
113	CPE	7.4									46			64
122	38	740.0												10
124	Foshay	740.0									8	6		
114	Foshay	740.0									3	6		

A-2 : Acetone Exctracted peptone broth culture - Strain SM21
38 : Acetone extracted peptone broth culture - Strain 38 (avirulent)
CPE : Chloroform extracted protein fraction - Strain SM21
*All sedimentation rates were done on fresh oxylated blood in 1 ml Exax pipettes using the same pipette for each animal throughout
Died

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autopsy and the remaining animal had a purulent punched out ulcer on the right leg.

In summary the sedimentation rate was elevated in all infected animals tested and paralleled the fever chart rather closely. It returned to normal rather promptly in some animals but remained elevated in others and may be the best index of continuing active infection in monkeys.

Hypersenitivity to "UL" Antigen

All monkeys were skin tested with "UL" skin test antigen before vaccination, after vaccination, and twenty-six days after challenge. Because previous skin tests had indicated that monkeys were not readily sensitized to "UL", two skin tests containing ten times and one hundred times the concentration of "UL" antigen usually employed for human tests were conducted on each monkey. In the twenty-nine normal monkeys no skin sensitivity was demonstrated. Vaccination did not sensitize any of the monkeys. Twenty-six days after challenge the surviving animals were mostly in the convalescing stage. Skin tests were positive on 13 of 15 animals. The positive tests consisted of induration and edema measuring 5-20 mm in diameter. Hyperemia was present in about half the animals. The induration reached its peak after 48 hours and thereafter regressed very slowly. None of the tests became vesiculated or necrotic as would be expected in a convalescent human patient given this amount of antigen.

Histopathological examination of the indurated area showed mononuclear infiltration of the cutaneous and subcutaneous tissue with occasional small hemorrhages. Apparently convalescent monkeys became sensitive to "UL" skin test antigen. In this respect monkeys differ from rats and react more like man.

It is unfortunate that the convalescent monkeys could not have been observed for a period of several months to determine whether late deaths would have occurred, how long latent infection persisted, the antibody level, the percentage of recrudescense or relapse, the persistence of the positive skin test, and the eventual level of immunity in completely recovered animals.

Pathology

Local Lesion

Of the twenty-six animals challenged all developed subcutaneous induration and edema near the umbilicus. In twenty-two the superficial skin became hemorrhagic and in twenty of these necrosis and ulceration occurred. Central necrosis of the indurated mass was present in the six animals where ulceration did not occur. Four animals developed ulcers on the extremities in the fourth week of disease from which viable and virulent "UL" was recovered. Three occurred on the hind legs and one on the right wrist. These ulcers appeared to originate as subcutaneous nodules which later developed

into punched out ulcers resembling the primary ulcerative lesion seen in human ulceroglandular "UL" infections. Whether they represent reinfection through skin abrasions or localized foci carried by blood or lymphatic channels could not be determined. It is interesting to note that they all occurred during the fourth week of the disease at which time the animals had developed skin hypersensitivity to "UL" protein. Similar "secondary" "UL" ulcers have not been previously described in man or laboratory animals and it would be of interest to study this problem further. Three animals, all vaccinated with fraction CPE, developed a maculo-pustular rash over the chest during the first week of the disease. Two of these animals survived and one died on the sixteenth day with extensive lobular "UL" pneumonia. Only three animals in this series developed petechial hemorrhage in the skin distant from the local lesion. This finding was very common in previous experiments where monkeys were challenged with an overwhelming dose and died in less than a week. Iritis with a white exudate in the anterior chamber was observed in two monkeys before death.

All animals developed regional lymphadenopathy, and suppuration of the regional nodes occurred in seventeen of the twenty-six animals, or 65.4%. The regional lymph nodes of the other nine animals showed microscopic foci of necrosis. No difference was observed in the speed or manner of development of the local lesion in the control or vaccinated groups.

Gross Pathology

Complete post mortem examinations were carried out on nine animals at the time of death, on two animals sacrificed seventeen days after challenge and on the remaining survivors sacrificed 31 to 35 days after challenge. Cultures on DCBA and guinea pig inoculations were made of the local lesions, lymph nodes, spleen and liver, and tissues were fixed in Zenker's and formalin solutions for histopathological examination.

There was some variation of gross pathological findings in individual monkeys but no distinct difference between the controls and the vaccinated groups.

In two animals in which the local lesion extended into the muscles of the belly wall the overlying skin was not ulcerated. In all others the induration and necrosis was confined to the cutaneous and subcutaneous tissues.

The spleen was enlarged 2 to 3 times normal size and contained numerous small yellow foci 0.5 to 4 mm in diameter in all animals dying before the eleventh day. Animals dying after the eleventh day were similar except the foci were larger and some large foci showed central liquefaction. The cut surface was firm and rubbery in consistency in all animals. The spleens of the control and Foshay vaccinated animals sacrificed on the seventeenth day were only slightly enlarged and contained one to three small yellow foci measuring 2 x 3 mm in diameter. The remaining 15 animals were sacrificed 31 to 35 days after infection. The spleens of these animals were normal in size or very slightly enlarged. Three contained one or two small yellow

foci 2 to 6 mm in diameter which exuded thick yellow pus. In eight the capsule contained irregular plaques of white fibrous tissue and in three the omentum was adherent to these areas.

The kidneys of the animals which died were slightly enlarged, the cut surface bulged on section and the border of the medulla and cortex was indistinct. In the sacrificed animals the kidneys were not abnormal except for faint yellowish striations in the cortex of some animals.

The adrenal glands of all monkeys that died were swollen and in some the capsule was hemorrhagic. The cortex was pale yellow or grey and contained minute petechial hemorrhages. The medulla was not abnormal except in Monkey #125 in which the medulla of the left adrenal was liquid and reddish-black in color. In addition to gross pathological evidence of "UL" infection this animals had a large intussception of the distal ileum with hemorrhage and discoloration of the intestinal wall.

The livers of animals which died were swollen; the cut surface bulged and showed a slight yellowish mottling. Only two contained small yellow foci 1 to 2 mm in diameter. The livers of the sacrificed animals were apparently normal except in two animals in which the livers contained 2 or 3 thick walled abscesses 2 to 8 mm in diameter containing thick yellow pus.

The mesenteric lymph nodes were enlarged in 17 and necrotic in two of the animals. The two necrotic nodes contained viable "UL". The others were not cultured.

The lungs of Monkey #125 were not grossly abnormal. In all animals which died after the 10th day the lungs contained many small raised yellowish-grey nodules surrounded by a narrow band of hemorrhage. These nodules were scattered throughout all lobes. The cut surface of the nodules was yellowish-grey and firm. In the animals which lived longer these nodules were larger, measuring 10 to 12 mm in diameter and some became confluent. Monkeys #100 and 93 which died on the 15th and 16th days had complete consolidation of one or more lobes with extensive edema. Numerous small or confluent yellowish nodules could be seen deep in the semi-transparent tissue of the lobes that were consolidated. Lungs of all sacrificed animals contained yellowish-grey nodules ranging from 2 to 12 mm in diameter throughout all lobes. There were no hemorrhagic areas around the nodules and the intervening lung tissue appeared normal. In many, the lungs were adherent to the parietal pleura by delicate band-like adhesions. The peritracheal nodes were enlarged in all animals and necrotic in several.

Post Mortem Cultures

"UL" was recovered from every animal at autopsy except one, when lymph nodes were ground and injected into guinea pigs. Those animals which died had bacteremia and cultures of all organs were therefore positive. Isolations made on the two animals sacrificed 17 days after challenge and on 13 animals sacrificed 31 to 35 days after infection were positive. The

results are shown in the following table.

Table LVI

Post Mortem Cultures on 17 Sacrificed Monkeys

Method	Tissue	% Cultures Positive
DCBA Culture	Spleen	13.3
Guinea Pig Inoculation	Spleen	41.6
	Liver	50.0
	Lymph Nodes	92.3
	Left inguinal	71.0
	Peritracheal	75.0
	Left axillary	91.0

Infection persisted for as long as 35 days in all but one monkey. The local lesion and lymph nodes gave the highest percentage of positive cultures, indicating that the organism persisted longer in lymph nodes than itdid in the spleen or liver. Animal injection was superior to DCBA cultures in detecting residual or latent infection.

Microscopic Pathology

Spleen: Spleens of animals which died showed marked depletion of the lymphoid tissue of the malpighian follicles. Hemorrhage and thrombi of numerous sinusoids were common. Many small to large areas of focal necrosis were present in the red pulp and in some animals there was diffuse coagulation necrosis of all but the centers of the malpighian bodies. Focal or diffuse areas of rounded reticulo-endothelial cells were seen in some. Polys and mononuclear cells were seen around the focal necrotic areas. General karyorrhexis was marked. The malpighian bodies were hyperplastic and one area of necrosis was sharply circumscribed although there was no cellular wall around the border. The spleens of the control and Foshay vaccinated animal sacrifieed on the 17th day were similar. They showed large hyperplastic malpighian bodies and polys scattered diffusely through the red pulp. Small areas of necrosis were surrounded by a zone of loosely arranged rounded mononuclear cells. Some animals sacrificed 31 to 35 days after infection were similar to the above while others showed only hyperplastic malpighian bodies without necrotic foci.

The livers from fatal cases showed diffuse and focal increase of large mononuclear cells and Kupfer cells in the sinusoids. (Small foci of hemorrhage, fibrin replacement of individual liver cells, and small areas of focal necrosis were seen.) Fatty degeneration of liver cells was not uniformly present, nor was it confined to any portion of the lobule when present. Depletion of glycogen occurred in all fatal cases. Sacrificed

animals showed many small focal lesions composed of large mononuclear cells. Kupfer cells were prominent throughout the sinusoids, and the liver cells appeared normal.

Lymph Nodes: The lymph nodes varied from almost normal appearance to complete coagulation necrosis. In the early stages of the disease lymph nodes throughout the body were depleted of all types of cells. During the second, third and fourth weeks the germinal centers in intact nodes became more and more active and an increasing number of large basophilic staining cells was seen. These resembled plasma cells.

Lung: The progress of the lung lesion as reconstructed from the picture seen in all monkeys was was follows. The alveolar walls became thickened and infiltrated with mononuclear cells arranged singley and in small clumps. There was early necrosis in the center of some small clumps of mononuclear cells. Later small areas of mononuclear exudate and edema fluid filled the alveoli. The center of these areas became necrotic and in the periphery intact polymorphonuclear and mononuclear cells could be seen. These areas extended and became confluent to form larger necrotic lesions. The alveoli surrounding a necrotic area were filled with large mononuclear cells and edema fluid but there was no walling off or limiting zone of cells about the foci. Edema of the surrounding lung tissue was more prominent in some animals than in others. As healing began a few lymphocytes appeared in the alveolar walls about necrotic areas. The mononuclear exudate in the surrounding alveoli later became organized with proliferation of the alveolar lining cells. A few giant cells appeared in this peripheral zone. Organization of the mononuclear exudate progressed slowly. No definite fibrous wall was observed about the central necrosis in animals sacrificed 35 days after challenge. In some animals organization of the exudate and fresh areas of alveolar thickening and necrosis were seen in the same section. The slowness of healing is best shown by a section of the original vaccine site which contained thick pus at autopsy 64 days after injection of alum precipitated vaccine CPE. This showed central necrosis surrounded by a wide zone of rounded mononuclear cells. Outside this zone were polys, lymphocytes, large basophilic staining cells and some connective tissue reaction.

Adrenal Glands: The fatal cases showed loss of cortical lipoid, diffuse infiltration of mononuclear and polymorphonuclear leucocytes with focal hemorrhage and focal necrosis of individual cortical cells. Sacrificed animals showed a normal amount of cortical lipoid and rarely a small focus of mononuclear cells.

Bone Marrow: The bone marrow from fatal cases showed congestion, hemorrhage, small to large areas of focal necrosis, foci of rounded reticulo-endothelial cells and, in most animals, only a small number of young white cells. Animals sacrificed later showed very active bone marrow packed with young red blood cells, immature granulocytes and hyperplastic reticulo-endothelial tissue.

f. Summary of Animal Immunization Experiments

1. For the first time rats and mice have been successfully immunized by vaccination.

2. There was little or no correlation between degree of agglutinin antibody production and immunity in vaccinated guinea pigs, rabbits and rats.

3. Certain data on the pathology, pathogenesis and mechanism of immunity are presented.

4. Foshay's vaccine has been prepared and tested extensively, and Dr. Foshay's finding's have been fully confirmed.

5. In addition, Foshay's vaccine has been shown to protect certain laboratory animals against experimental "UL" infection.

6. Several purified vaccines have been developed. Acetone extracted vaccine prepared in this laboratory protected rats and mice as well as, or better than, Foshay's vaccine, and in addition did not cause as severe a local reaction in hypersensitive humans.

7. Significant immunization of rabbits, guinea pigs and monkeys has not yet been satisfactorily demonstrated although Foshay's vaccine appeared to confer some protection to monkeys.

2. Immunization of Man

In the period from December 1943 to October 1945 a total of 24 persons contracted "UL" infection in the laboratory while doing experimental work in defensive Biological Warfare (BW) studies carried on by the Army and Navy at Camp Detrick, Md. All cases occurred in vaccinated persons and it seems worth while to report the incidence as well as the clinical picture in this series. Five patients were treated with streptomycin and the results are presented to supplement the one published account[46] of streptomycin therapy in "UL" infection of experimental animals.

Numerous reports in the literature describe the epidemiology, diagnosis, clinical course and therapy of "UL" infection in unvaccinated persons.[3,4,47,48,49] The incidence of infection in unvaccinated laboratory workers has been 10 to 100 per cent (3,47,50-55) in the past.

Foshay[5] published the only account of vaccination in a large number of hunters, market men and others exposed to "UL" infection in and around Cincinnati, Ohio. The lower incidence of infection and absence of deaths in the vaccinated group as compared to that in the unvaccinated population at large strongly suggested that vaccination conferred some degree of immunity. However, as Foshay pointed out the degree of exposure in the vaccinated group was impossible to determine and therefore statistical proof from vaccination was difficult to obtain. The occurrence of only three cases out of nine exposed laboratory workers in Foshay's department suggested that vaccination was effective. This vaccine did not protect the mouse, guinea pig or rabbit.

(a) Vaccination Procedures

All personnel who because of their work at Camp Detrick were potentially exposed to infection with "UL" were vaccinated by the Safety Division. A detailed report and statistical analysis of the data obtained from this series of human vaccination is contained in the final report of the Safety Division.[56] Some of the data which have a bearing on our vaccine studies have been extracted from the Safety Division records and are presented below. Two vaccines were used for human vaccination: (1) Foshay's vaccine supplied by Dr. Foshay and later prepared here according to his specifications[30] and (2) acetone extracted peptone broth culture vaccine #30 developed at Camp Detrick. Vaccines were administered in three subcutaneous doses of 0.5 ml at 48 hour intervals.

At the beginning of the work at Camp Detrick active immunization of man by means of vaccination was not generally accepted by the medical profession or by laboratory research workers. The two chief objections to human vaccination were that (1) ordinary formalized suspension vaccines and Foshay's oxidized vaccine[5] produced a considerable number of severe local and systemic reactions, and (2) no vaccine had ever been shown to be effective in protecting laboratory animals. Foshay's newer vaccine[16] gave fewer reactions and was a better antigen than the oxidized vaccine on the basis of its ability to stimulate agglutinin antibody production in the rabbit and in man. Foshay stated that most reactions, and practically all severe ones, could be explained on the basis of hypersensitization acquired through a previous attack of "UL" infection. If these were carefully ruled out by means of an accurate medical history and blood examination for the presence of residual agglutinins, reactions to vaccination, in Dr. Foshay's experience, were no more common than after typhoid vaccination. Two additional precautions were recommended by Dr. Foshay (1) that the first dose of vaccine be small, and (2) that additional doses be given thereafter at 2 day intervals so that the last dose would come not later than 6 days after the first.

After some preliminary variations the routine adopted was to take a careful medical history to rule out previous "UL" infection, obvious laboratory exposure or history of dressing wild rabbits. Blood was withdrawn for an agglutination test and two days later the agglutinin titer was reviewed. Persons giving a history of previous "UL" infection or an agglutinin titer of 1:320 or more were not vaccinated. Those having a low agglutinin titer only were given a small initial dose of vaccine to test for hypersensitivity and if no reaction occurred the series of injections was completed on schedule. The agglutinin response following vaccination was followed at intervals of 1 week, 2 weeks, 2 months and 3 to 6 months. Those persons subject to greatest exposure to laboratory infection were revaccinated at 3 to 6 month intervals upon the recommendation of Dr. Foshay. Before revaccination a skin test was performed and positive reactors were excluded from the series. Reactions to vaccination were classed as mild, moderate, or marked local reaction; and mild, moderate, or marked systemic reaction.

Out of the first 22 persons vaccinated 3 had marked local and systemic reactions accompanied by fever, chills, and prostration, requiring hospitalization. One of these had nine chills over a period of five days with fever up to 104° and was hospitalized 11 days. Two of the three had agglutinin titers of 1:32 or higher. All three had previously worked in the laboratory with "UL" cultures and two had in addition hunted and dressed wild rabbits. The evidence suggests that these three hypersensitive individuals had a previous "UL" infection, probably mild or subclinical, since none of them could recall a previous unexplained illness.

Following this experience more care was exercised in eliciting a careful history of previous illness or exposure to "UL" infection. The agglutinin titer was considered suggestive if positive in dilution of 1:10 or more. These precautions reduced the number of reactions but mishaps still occurred and in an effort to develop a more reliable method of detecting hypersensitive individuals before vaccination an antigen was prepared for use as a skin test.

Since it was not known which antigenic fraction was responsible for the allergic reaction we used the whole organism. Cultures were grown for 24 hours in peptone broth and killed with formalin. Formalin was used because "UL" vaccines killed with formalin are notorious for causing a high percentage of reactions,[5] therefore formalin must preserve or enhance the activity of the antigen. The packed cells were collected by centrifuging and washed twice with saline to remove excess formalin and culture medium. The cells were then diluted in physiological saline containing 1:1000 formalin and standardized to contain 0.03 mgm N/ml. For testing, this stock suspension was diluted 1 to 1000 in saline and 0.1 ml injected intradermally on the forearm. Standardization tests on known hypersensitive and normal persons showed this dose to be satisfactory for detecting hypersensitivity. Positive reactions were of the delayed type. Edema, induration and hyperemia reached a maximum after 48 hours and thereafter faded very slowly. Vesiculation and pustule formation occurred in two persons tested while convalescing from "UL" infection. A positive skin test was one showing 10 mm of hyperemia or any perceptible induration when read at 48 hours. Persons showing a positive skin test were not vaccinated. The skin test antigen after storage in the refrigerator for 1 year showed no signs of deterioration.

Five hundred and ninety-nine persons received a total of 1196 separate courses of Foshay's vaccine and 66 persons received a total of 223 courses of acetone extracted vaccine #30. The percentage of local and systemic reactions was from two to twelve times greater following administration of Foshay's vaccine (Table LVII). The high percentage of marked systemic reactions are of particular concern because these people require hospitalization or bed rest and are lost from duty for several days. Vaccination tended to sensitize as shown by the greater percentage of hypersensitive persons on revaccination. The hypersensitivity induced by vaccination tended to disappear slowly as shown by repeated skin tests at 2 to 3 month intervals.

Table LVII

Per Cent of Local and Systemic Reactions to Foshay's Vaccine and to Acetone Extracted Vaccine #30

Foshay's Vaccine	Course of Vaccine 1st	2nd	3rd	4th	total
# Individuals	599	385	212	None	1196
Type of Reaction Moderate local	6.6	13.0	12.8		Average 10.8
Moderate systemic	4.2	3.5	4.7		4.1
Marked local	1.8	2.3	1.4		1.8
Marked systemic	8.0	14.8	13.3		12.3

Acetone Extracted Vaccine #30	1st	2nd	3rd	4th	total
# Individuals	66	62	59	36	223
Type of Reaction Moderate local	1.5	0	1.7	5.6	Average 2.2
Moderate systemic	1.5	0	1.7	5.6	2.2
Marked local	0	0	0	0	0
Marked systemic	0	3.2	1.7	0	1.2

The use of the skin test prior to vaccination reduced the number of marked reactions and eliminated the severe type of reaction requiring prolonged hospitalization. Table LVIII compares the skin test and the agglutination test as methods of detecting hypersensitivity to vaccination.

Table LVIII

The Efficiency of the Skin Test and the Agglutination Test in Detecting Hypersensitivity

	Skin Test	Agglutination Test
Total persons tested	342	320
Total positive tests	62	75
Total negative tests	280	245
Positive reaction to vaccine in negative group	24	30
Percentage failure	8	12.2

The data on the agglutination test in Table LVIII are from persons to whom no vaccine had been adminstered. The per cent of hypersensitive people who had positive agglutination tests was 87.8. However, only 13.4% of persons with an agglutinin titer were hypersensitive upon vaccination. After one course of vaccine practically all subjects maintained an appreciable agglutinin titer. In this group the titer was of no value as an index of hypersensitivity but the skin test continued to be reliable.

The agglutinin response to Foshay's vaccine in 243 vaccinated persons is shown in Figure 41. All but two cases developed an appreciable titer following vaccination. The highest titer was reached two weeks after vaccination when the average value was 1:482. Similar data on 65 persons vaccinated with acetone extracted vaccine #30 gave an average peak titer of 1:180 reached between 2 and 4 weeks after vaccination.

Of 278 persons on whom both "UL" and "US" agglutinin titers were determined before "UL" vaccination, 71 had a positive "US" titer in a dilution of 1:10 or higher. The average "US" titer was 1:20. Following "UL" vaccination five of the 71 were negative and in 66 the "US" titer increased to an average of 1:40. In the 207 who had a negative "US" titer before vaccination, 48 developed an appreciable "US" titer after vaccination, or 23.2%. These data suggest that Foshay's vaccine contains a common antigen shared by "UL" and "US"; however, the development of "US" agglutinins appears to be conditioned by other unexplained factors.

(b) Report of Cases

Of 625 vaccinated persons 163 actually worked with the organism or frequently entered the laboratories. A total of 24 persons were infected,

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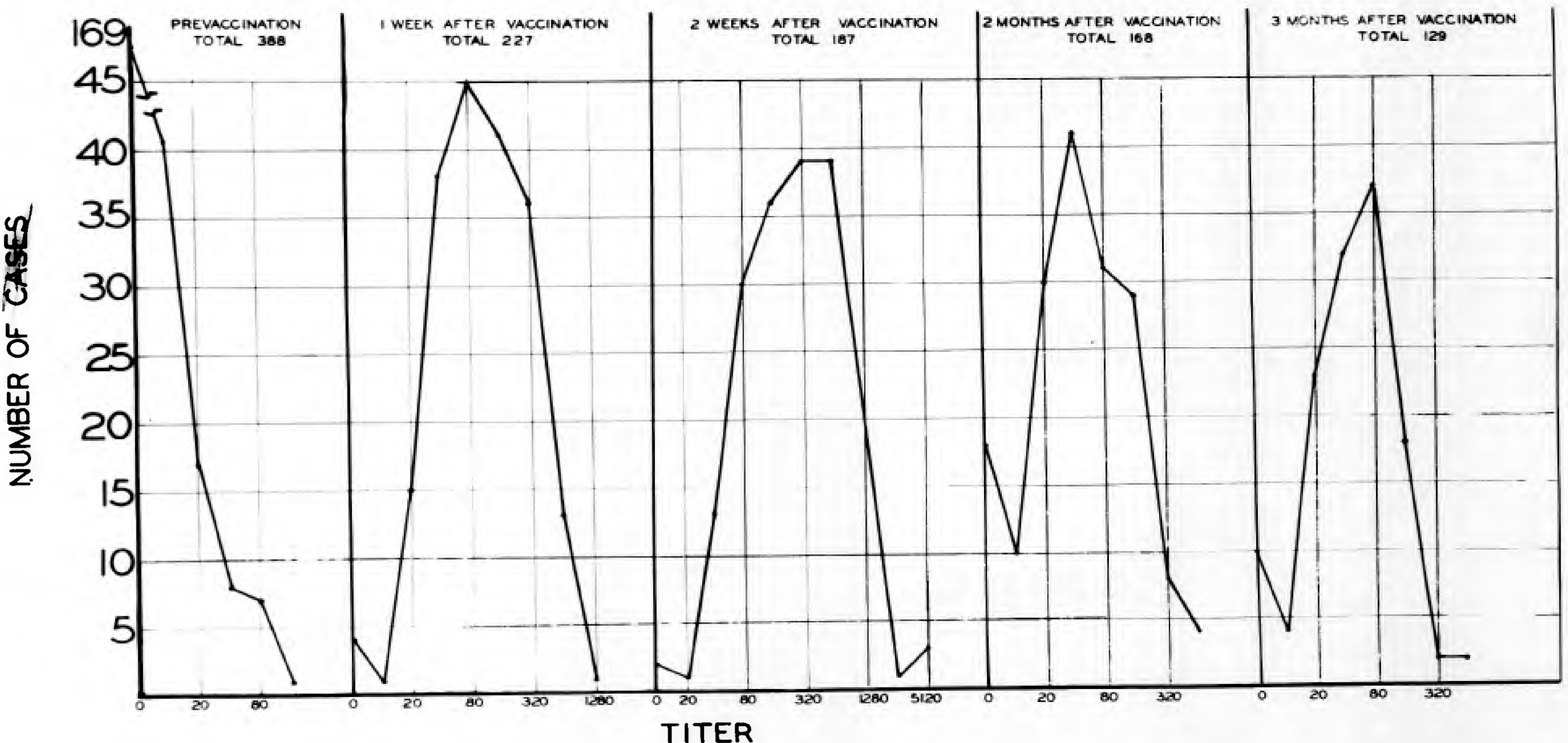
FIGURE 41

AGGLUTININ TITERS FOLLOWING HUMAN VACCINATION
FOSHAY'S VACCINE



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or 14.5 per cent of those possibly subject to exposure. In five reinfections the clinical picture and evidence for diagnosis is presented later in this report. Tables LIX and LIXa show the number of cases according to the type of work performed. The incidence in persons handling cultures and doing post mortem examination of infected animals was 31.9 per cent. Only one case, or 1.1 per cent, occurred in persons who cared for infected animals, washed glassware in the service room or did maintenance repair in the laboratories and equipment.

The overall low infection rate in both the vaccinated laboratory workers and service personnel was much less than in unvaccinated experimental workers. This may be explained in part by the rather rigid safety precautions that were observed. Gowns, rubber gloves, masks and face shields were worn while carrying out hazardous procedures. Five per cent phenol was used freely to disinfect table tops and infected glassware. All glassware and animal cages were autoclaved before they were cleaned. These safety precautions reduced the number of exposures and are probably responsible for the low infection rate in the service personnel. The frequency of exposure in the persons handling cultures and infected tissues was also reduced by these precautions, however, over a period of months of work under crowded conditions numerous known and unobserved accidents occur and it is probable that prophylactic vaccination deserves credit for the low infection rate in this group (31.9 per cent).

The length of time that cases were in contact with "UL" in the laboratory before infection occurred varied from 10 days to 18 months with a mean of 7 months. In the 9 cases with a definite known exposure the incubation period was 1 day in five cases, 2 to 4 days in one, 4 days in one, 6 days in one and 7 days in one. In two additional cases (Table LX) the exact exposure was not known but the incubation period was less than 10 days in one because the person became ill within 10 days after beginning work on the project. In the other case a conjunctivitis followed 3-4 days after an accidental cigarette ash burn in the eye. "UL" was probably carried to the irritated conjunctiva by rubbing with contaminated fingers sometime after the cigarette ash incident.

Source of Infection

Of the 29 cases, 2 were definitely, and 6 were possibly contracted while posting infected animals or handling fresh infected tissues. The other 21 patients or, 72.4 per cent, had no contact with infected animal tissue and were presumably infected while handling cultures. Table LX shows the most probable source of infection based on the type of work performed in the two week period immediately preceding onset.

G Stability of "UL" Vaccines:

1. Storage at Various Temperatures

Since the immunizing potencies of acetone extracted vaccine and Foshay's vaccine were used as standards for assaying new preparations, it

Table LIX

Type of Work and Number of "UL" Infections

	Total Personnel	Infected	% Infected
Maximal Exposure			
Lab and animal work	47	22	46.7
Cloud chamber and lab work			
Chamber A	2	1	50.0
Chamber B	5	0	0
Chamber C	18	0	0
Total Maximal Exposure	72	23	31.9
Slight Exposure			
Animal care (light contact)*	16	0	0
Animal care (heavy contact)**	23	0	0
Maintenance section ***	27	0	0
Service room	25	1	4
Total Slight Exposure	91	1	1.1
Totals	163	24	14.5

* Washed autoclaved cages, fed infected animals on weekends only.
** Cared for infected animals daily over long period of time.
*** Contact with agent was slight. Serviced buildings where agent was handled.

Table LIXa
Type of Exposure

Exposure Known	Number of Cases	Per Cent of cases
Autopsied infected animals	2	6.8
Broken lyophilized tube	2	6.8
Spilled suspension while centrifuging	1	3.5
Aspirated material while pipetting	1	3.5
Cut finger on contaminated tube	1	3.5
Leaking cloud chamber	1	3.5
Percutaneous infection of rats	1	3.5
Exposure Unknown (Type work done in 2 weeks proceding onset)		
Autopsied animals or handled tissues	6	20.7
Pipetting, making dilutions and plate counts	7	24.0
Centrifuging or filtration of mass cultures	5	17.2
Transferred stock cultures	1	3.5
Washed glassware	1	3.5
Total	29	100.0
Total possibly contracted from infected animals	8	27.6
Total contracted from handling cultures	21	72.4

Table LX

Cases of "UL"

Cases	Total Months Contact	Length of contact Before Infection	Incubation Period	Type of Exposure
Known Exposure				
EGC	13	7 months	6 days	Spilled suspension on hands.
LLC	23	18 months	1 day	Injected and autopsied monkey.
RAP	22	13 months	1 day	Broken lyophilized tube.
GBP	21	6 months	7 days	Used new cloud chamber.
GBP	21	15 months	1 day	Cut finger on contaminated test tube.
MAS	2	2 months	1 day	Scratched finger while autopsying infected animal
WMW	12	8 months	1 day	Broken lyophilized tube.
EMS	7	5 months	4 days	Percutaneous infection of rats.
Unknown Exposure				
SSC	14	10 days	10 days	Pipetting, dilutions, plate counts.
FBE	8	4 months	Unknown	Pipetting, dilutions, cloud chamber work.
SFK	21	5 months	"	Pipetting, dilutions, plate counts.
RAP	22	3 months	"	Pipetting, plate counts.
AHK	11	8 months	"	Pipetting, dilutions, plate counts.
TLS	21	12 months	"	Culture work, dilution, plate counts.
PS	2	2 months	"	Pipetting and diluting infected bloods.
WMW	12	4 months	"	Dilutions and plate counts.
MPC	7	6 weeks	"	Centrifuging, harvesting, pipetting.
ALD	2	2 months	"	Centrifuging, harvesting.
OGS	7	4 months	"	Centrifuging, posting animals.
JRS	10	2 months	"	Centrifuging, pipetting concentrated suspensions.
ES	10	1 month	"	Centrifuging, harvesting, posting infected animals.
RTL	22	7 months	"	General lab work, egg and animal work.
BJO	20	16 months	"	Animal work, cultures, serology, antigen preparation.
FAN	8	8 months	"	Transfer of stock cultures.
MB	18	13 months	"	Serology on infected bloods, bleeding infected mice.
MLN	9	9 months	"	Washing glass ware.
HL	11	5 months	"	Mass cultivation and filtration.
REK	11	6 months	3-4 days	Mass cultivation and filtration, cigarette ash in eye.
29 Total	13.4 mean	7 months mean	1-7 days	Known, 8, Unknown, 21.

was of some interest to determine the stability of these vaccines under various conditions of storage. As these vaccines were being prepared in large quantity for possible use on troops in the field, it was also of great interest to know what recommendations should be made regarding conditions of storage, transportation, and probable expiration date. Three lots of Foshay's vaccine #16, one lot of acetone extracted vaccine #30 and one lot of acetone extracted vaccine #31 were stored in the refrigerator, at fluctuating room temperature, and in the 37°C incubator for periods shown in Table XLI. All these vaccines protected 80 to 100% of vaccinated rats against a challenge of 1000 LD_{50} at the beginning of the storage experiment. All were standardized to the same total bacterial nitrogen content (see section on Preparation of Vaccines).

As shown in Table XLI all vaccines deteriorated after storage at 37°C for 8 months. Little loss of potency was observed with the acetone extracted vaccine #30 stored at room temperature and in the refrigerator for 8 months. Acetone extracted vaccine #31 was prepared at 0°C throughout, while #30 was centrifuged at room temperature. It is apparent from the stability of the two vaccines that the procedure used for #30 was preferable.

2. <u>Effect of Heat</u>

The acetone extracted vaccine #30 was subjected to various temperatures for varying lengths of time. The vaccine was then tested in animals in the usual way. Table XLII shows the length of time the vaccine was maintained at the various temperatures, the temperatures used, and the animal results.

It was obvious from these data that no appreciable change occurred in the acetone extracted vaccine when heated as shown in the table. These results with the acetone extracted vaccine have been confirmed at Kansas using vaccines prepared at Camp Detrick. Similar tests on whole culture vaccine have not been carried out but storage of Foshay's vaccine as reported above indicates that the whole vaccine deteriorated faster than the acetone extracted fraction.

3. <u>Effect of pH</u>

Because chemical fractionation was contemplated in the study of "UL" antigens, it was considered important to know their stability at low pH values. Furthermore, it was postulated that acid treatment of organisms might be used for rendering broth cultures non-infective without making the bacterial proteins insoluble as had been observed to occur following the use of acetone, formaldehyde, phenol and heat as killing agents. Sonic disintegration of living organisms had proven to be the best method of rendering "UL" proteins soluble, but, the procedure of centrifuging and handling living organisms was considered so hazardous as to be unsafe for large scale operation.

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Table XLI

EFFECT OF STORAGE AT DIFFERENT TEMPERATURES ON POTENCY OF "UL" VACCINES *

All animals (rats) challenged intraperitoneally

Vaccine	Storage Months	Temperature of Storage					
# Description		Refrigerator 4° C		Room Temperature		37° C	
		Dead/Total	% Survival	Dead/Total	% Survival	Dead/Total	% Survival
30-Acetone Extracted	8	4/20	80	4/21	81	8/19	58
16-Foshay Lot 3	8	5/18	72	6/19	68	9/20	55
16-Foshay Lot 4	8	5/17	71	6/19	68	7/20	65
16-Foshay Lot 5	7	6/19	68				
31-Acetone Extracted	3	10/20	50				
Controls	-	13/20	35				

At the beginning of the storage experiment all vaccines protected 80 to 100% of rats against a challenge of 1000 LD_{50}.
* Summary of two experiments in which the animals were challenged with 10 LD_{50} and 1000 LD_{50} respectively

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Preliminary experiments had shown that the pH must be below 5 in order to kill all the organisms in 24 hours at room temperature. The experiments were performed by adding a predetermined amount of 1 N HCl to flasks containing 24 hour peptone broth cultures of "UL" and allowing them to stand for an hour to overcome the natural buffering action of the media before measuring the pH, Samples were withdrawn after 24 hours and injected into mice to determine whether all organisms had been killed. This procedure was repeated at 4°C with results similar to those obtained when the flasks were stored at room temperature. It was believed that storage at 4°C might prevent excessive autolytic reactions.

A final experiment was conducted to determine the length of time necessary for killing at low pH levels and also the effect of low pH on rat protective (antigenic) power of the organisms so treated. A flask containing a 24 hour broth culture was adjusted to pH 3.9, stored at 4°C and samples removed at intervals for mouse injection and rat immunization. Mice were given 0.5 ml intraperitoneally. Before rats were injected 0.2 per cent formalin was added to kill any surviving "UL" organisms. Rats received two injections of 0.5 ml and one injection of 1.0 ml subcutaneously at 48 hour intervals and were challenged one month later as shown in Table XLIII.

All "UL" organisms were killed by the end of 12 hours as shown by lack of infectivity for the mouse. Rat protection tests showed beginning loss of antigenic potency after only 6 hours exposure to pH 3.9. In summary it appeared that while in the unpurified whole broth culture (1) "UL" was killed in 6-12 hours exposure to pH 3.9, (2) antigenicity was rapidly impaired by this treatment.

Table XLII

The Effect of Heat on Rat Protective Power of Acetone Extracted Vaccine #30

All rats were challenged intraperitoneally with 1000 LD_{50}

Time	Temperature	# Dead / # Challenged	Per Cent Survival
Control	Not Heated	1/10	90
24 hours	Rm Temp Av-26°C	2/9	78
1 week	Rm Temp Ave-27°C	2/10	80
24 hours	37°C	2/10	80
30 min	56°C	1/10	90
3 hours	56°C	0/10	100
15 min	100°C	0/10	100
30 min	100°C	1/10	90
Controls	Not vaccinated	9/10	10

Table XLIII

Effect of Storage at pH 3.9 on Viability and Antigenicity of "UL" Broth Cultures

# Hours at pH 3.9	# Mice Dead / # Mice Injected	Challenge of Vaccinated Rats: 10 LD_{50} # Dead / # Chall.	10 LD_{50} % Survival	1000 LD_{50} # Dead / # Chall.	1000 LD_{50} % Survival
0	4/4	0/8	100	1/8	87
6	4/4	0/9	100	4/9	56
12	0/4	2/9	78	3/8	63
18	0/4	4/9	56	3/9	67
24	0/4	3/9	67	4/9	56
Controls	None	8/9	11	7/9	22

H Comparison of Vaccines Prepared From Virulent and Avirulent Strains

Acetone extracted vaccines were prepared from the fully virulent S strain and from the completely avirulent NIH 38 strain. Cultures of each organism were grown in peptone broth at 37°C and at room temperature (26-28°C), and on solid DCBA media at 37°C making a total of 6 vaccines in all. Each finished vaccine was standardized to contain 0.06 mg nitrogen per ml. The results of vaccination and challenge are shown in Table XLIV and indicate that there was no significant difference in immunizing potency of vaccines prepared from virulent or avirulent strains grown at 37°C or at room temperature. Cultures grown on DCBA appeared to be slightly superior to those grown in peptone broth but there was no difference between virulent and avirulent strains.

These results make it unnecessary to use the virulent strain for vaccine preparation and remove a great hazard from the fractionation studies where it is desirable to work with living organisms during the initial steps of centrifugation, disintegration and filtration. Obviously the virulent and avirulent strains differ in their ability to invade the host and cause death of experimental animals. We have no explanation of this difference but have previously in this report noted that the virulent strain grows better on all artificial media and in embryonated eggs. It survives better on storage and is less susceptible to deleterious agents than the avirulent strain. Electrophoretic analysis of the two strains shows identical components and this is now confirmed by animal protection tests. Apparently the differences noted when the two strains are grown on artificial media are not associated with a difference in antigenic structure. All evidence indicates that the avirulent strain has become more fastidious in its growth requirements, and has become less adaptable to changes in its environment, perhaps through loss of some enzyme system or regulating mechanism. We have frequently observed that the avirulent strain dies out quickly when injected into mice or embryonated eggs. Doubt is now thrown upon our original assumption that the virulent strain produced a haptene,

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Table XLIV

COMPARISON OF RAT PROTECTION BY ACETONE EXTRACTED VACCINES PREPARED FROM VIRULENT AND AVIRULENT "UL" STRAINS GROWN ON SOLID AND IN LIQUID MEDIA AT 37° C AND AT ROOM TEMPERATURE (26 to 28° C)

All animals were challenged 21 days after vaccination with 1 ml of 10^{-6} dilution of strain S (2250 organisms)

Vaccine #	Mode of Immunization	No. of Rats	Route of Challenge	# Deaths / # Challenged	% Survival
37 Strain S-37° broth	0.5 ml x 3 subcut.	20	I.P.	4/20	80
38 Strain S-26-28° broth	0.5 ml x 3	20	I.P.	5/20	75
39 Strain S-37° DCBA	0.5 ml x 3 subcut.	20	I.P.	1/20	95
40 Strain NIH-38 37° broth	0.5 ml x 3 subcut.	20	I.P.	5/20	75
41 Strain NIH-38 26-28° broth	0.5 ml x 3 subcut.	20	I.P.	4/20	80
42 Strain NIH-38 37° DCBA	0.5 ml x 3 subcut.	20	I.P.	1/20	95
Controls	None	20	I.P.	18/20	10

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or protein or capsular material which distinguished it from the avirulent strain. On the basis of the information now available it appears that there is no structural difference between virulent and avirulent strains when grown on the present artifical media.

5. Summary

"UL" vaccines deteriorate when stored at 37°C for 8 months but keep for the same length of time if stored at ice-box temperatures. Acetone extracted vaccine keeps better than Foshay's vaccine. Heating at 100°C for 30 minutes does not impair the protective power of acetone extracted vaccine. Adjusting the pH of a "UL" broth culture to 5 or below causes rapid death of the organisms accompanied by a destruction of protective antigen. Vaccines prepared from completely avirulent strain 38 grown on Snyder's peptone medium or on DCBA protected rats as well as vaccines similarly prepared from virulent strain S1.

I Fractionation Studies of "UL" Antigens

This study was undertaken in an attempt to produce a better antigen by purifying the active material which is known to be present in the vaccines now in use. The Foshay vaccine and the acetone-extracted vaccine showed about equal protective properties in rats, however, there was an antigenic difference between the vaccines since the diluted Foshay vaccine gave very stron skin test reactions with sensitive persons while the acetone-extracted vaccine did not. The Foshay vaccine stimulated greater agglutinin antibody production in man and rats but there was no correspondingly greater protection to experimental challenge in rats. One possible explanation for these observed differences between the whole culture Foshay vaccine and the partially purified acetone extracted vaccine was that the Foshay vaccine contained a number of antigenic components with different immunological activities. Comparative skin tests with the two vaccines indicated that the component responsible for the allergic skin reaction was largely removed by acetone extraction without impairing the protective power of the acetone insoluble fraction. Extraction and purification of the antigenic component responsible for protection might by removing the sensitizing antigen, eliminate or materially reduce the number of vaccination reactions which have been observed in the past with crude vaccines. The purified allergenic component would be of value as a skin test antigen for detecting hypersensitivity or for diagnostic purposes. In the beginning it was assumed that the "UL" antigens under investigation were labile and the techniques and methods used all involved working at low temperatures in order to prevent denaturation of the active material.

1. Disintegration Methods

The first problem in the antigen fractionation study was to find the proper method of disintegrating the organisms in order to get as much of the antigenic material into solution as possible. The first method used was that of freezing and thawing the organisms several times. Even with

ten or more freezing and thawing processes the amount of antigen in solution was not greatly increased. The next method studied was that of mechanically grinding the organisms. The method of Wiggert et al [73] for grinding bacteria with powdered glass in a mortar and pestle was deemed dangerous and was not used with this organism. The conical grinder described by Kalnitsky et al [65] was not available at the time this work was in progress nor were the low temperature ball mills of Mudd [70] or of Booth and Green [57]. In attempting to grind "UL" organisms a number of devices were made and tested. The grinder which was finally developed was built of equipment available in this laboratory and has the advantages of being inexpensive and easily constructed. It did a satisfactory grinding job but had the disadvantage of being rather slow. The operation of the machine is quite simple. (Figure 42)

The organisms, mixed with water and ground glass, were fed into the grinding mechanism by the syringe may be any size Yale Lok syringe, and if a small syringe is used it can be easily removed, when empty, and replaced by a loaded one. The feeder tube was of rubber or plastic, cemented to the bases of two hypodermic needles from which the needles had been removed, and which had been drilled out to permit an easy flow of material to be ground. This tube was connected with a 100 ml Yale Lok syringe (G) in which the grinding took place. The piston of this syringe had been machined in a special way (see insert). Six grooves about ¼ inch wide were cut down the length of the piston; the grooves were cut just deep enough to permit water to pass through the syringe. Very fine scratches connected one groove with the next. These scratches were cut at an angle so that material feeding down through the groove would be caught and sent back into the next groove. In this manner the mixture traveled a maximum distance before finally emerging from the syringe. It was in these scratches that most of the grinding took place. Care had been taken that the scratches were in the proper direction with regard to the turning of the piston so that the material being ground was sent back through the scratches and not forward.

The piston was driven by an electric motor (A) connected by a suitable belt and pulley system (B) to a speed reducing gear (C). The speed of turning was thus reduced to approximately 120 RPM. The speed reducing gear was connected to the glass piston by a large piece of Saran tubing (E). This tube was cemented to the piston and fastened to the shaft of the gear by a rubber stopper. The Saran tube was cut in the center and the two pieces held together by a rubber tube (F) which had been inserted to add flexibility. Holes had been cut through the rubber and Saran tubes and copper wire used to fasten them together. The grinder was mounted on a wooden base and could be easily carried into the cold room for grinding operations.

In order to use the grinder effectively very fine ground glass must be used. This glass was prepared by grinding broken pyrex glassware in a ball mill for 24 hours. The powdered glass was then sieved to remove the larger particles and the fine glass suspended in water. Usually about one

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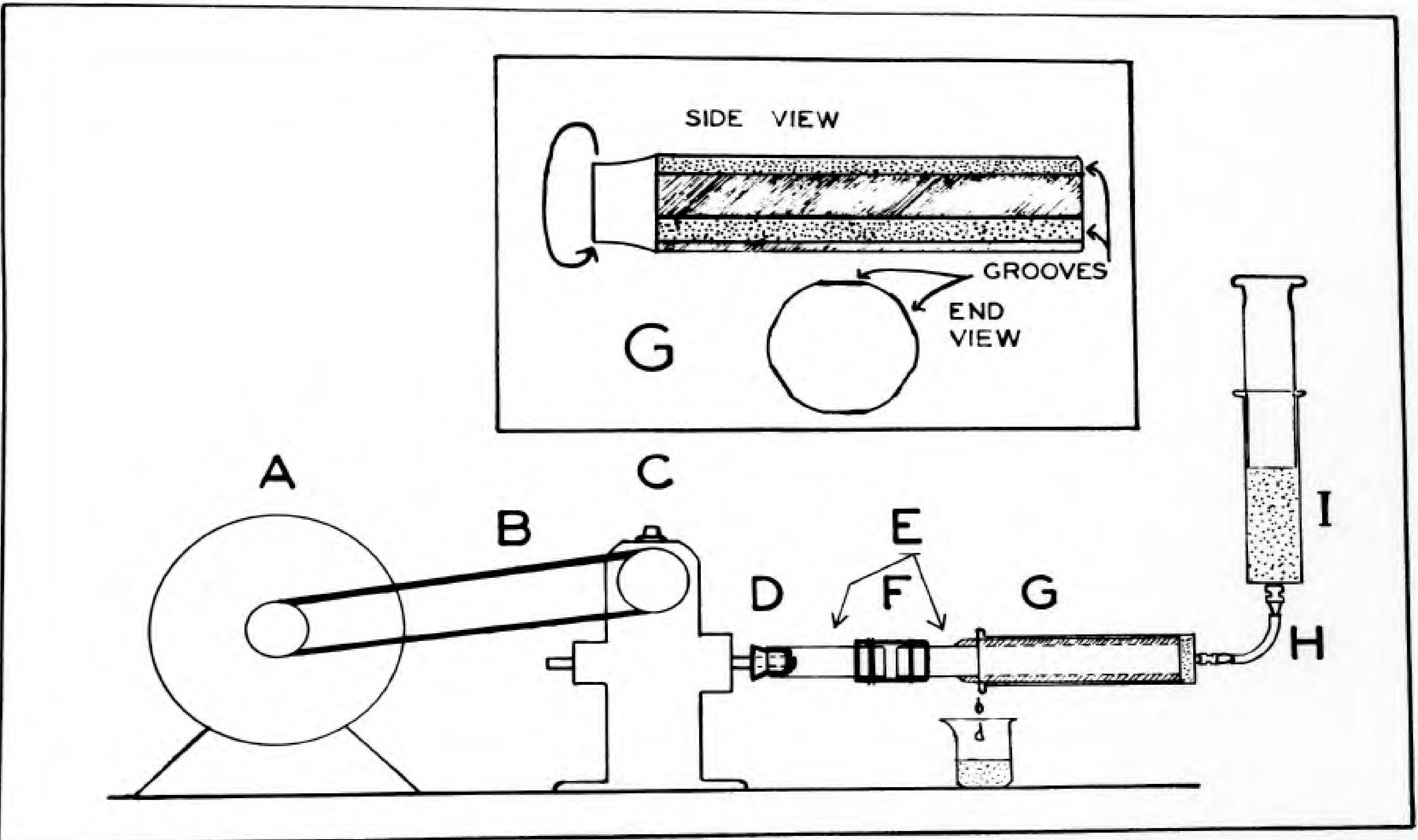
FIGURE 42
BACTERIA GRINDER



pound of glass was suspended in five gallons of water. After the glass had been allowed to settle for an hour the supernatant fluid was drawn off and the glass still in suspension centrifuged out. This procedure yielded glass of a size not exceeding 2 microns and averaging about 1 micron in length. By using this glass* in the grinder maximum efficiency was obtained and the grinding proved to be quite satisfactory.

In an effort to process a larger amount of organisms an attempt was made to grind "UL" in an ordinary porcelain jar ball mill using quartz balls and powdered glass for the abrasive. It was found that several days of grinding in the cold was necessary for reasonably good results. This method was used to prepare a large amount of material for fractionation.

The last and most successful method tried for disintegrating the organisms was the sonic vibrator of Chambers and Florsdorf.[58,59,60] This apparatus was made available to this project through the courtesy of Dr. Leslie A. Chambers of the Johnson Foundation, University of Pennsylvania, Philadelphia, Pa. The first "UL" preparations run in this apparatus were the previously prepared acetone extracted vaccine (ULB) and two fractions of the acetone-extracted vaccine precipitated with ammonium sulfate (SML and SMH). They were exposed for one hour in the ossilator at a frequency of 9,000 cycles. All three preparations were known to contain an active antigen as shown by rat immunization experiments. Following sonic vibration no appreciable decrease in turbidity was observed and, in the case of ULB only a slight increase in soluble total nitrogen was obtained as shown in Table LXV.

The most striking effect of sonic treatment was observed when fresh living "UL" organisms were employed. Twenty-four hour peptone broth cultures were centrifuged to concentrate the organisms and remove the excess of culture medium. The packed sediment was resuspended in physiological saline and colony counts on DCBA plates were made before and after one hour in the sonic apparatus. Turbidity readings were made on the Coleman spectrophotometer.

The results of the turbidity readings showed a 70 fold decrease of opacity measured on the spectrophotometer. Table LXVI shows the effect on the viable count in four separate samples.

This apparatus solved the problem of disintegration of the organisms very well, however, some work[60] had shown that sonic treatment could be harmful to certain proteins and other biological materials.

A sample of sonic disintegrated virulent strain S (ULLSF-3) was filtered through a Selas 03 filter and tested at the same time as sonic treated samples of ULB (acetone extracted vaccine), SMH and SML (ammonium sulfate fractions). Rats were immunized with an equivalent amount of each antigen on the basis of total nitrogen content and challenged as shown in Table LXVII.

* It is important that pyrex glass be used for grinding as soda-glass raised the pH of the suspension to about 9.

Table LXV

Total N in mgm/ml Before and After Sonic Vibration

Preparation	Before Sonic Total N	Treatment Soluble N	After Sonic Total N	Treatment Soluble N
ULB	2.68	.015	2.68	0.22
SMH	0.50	---	0.44	0.14
SML	0.50	---	0.52	0.38

SMH and SML fractions were quite soluble after sonic treatment.

Table LXVI

Effect of 1 Hour Sonic Vibration of Living "UL"

Sample	DCBA Plate Count Before	After	Per Cent Killed
1	311 x 10^9/ml	.162 x 10^9/ml	99.5
2	356 x 10^9/ml	.032 x 10^9/ml	99.9
3	220 x 10^9/ml	.027 x 10^9/ml	99.9
4	159 x 10^9/ml	.055 x 10^9/ml	99.7

Table LXVII

Rat Immunization with Sonic Treated Antigens

Vaccine	Number of Animals	Day of Death 1	2	3	4	5	6	7	8	9	Per Cent Survival
ULBV	7	0	0	0	0	0	0	1	0	0	86
ULLSF3	9	0	0	1	0	0	0	0	0	0	89
SMLV	10	0	0	0	0	0	0	0	0	1	90
SMHV	9	0	0	0	0	0	0	0	0	0	100
Acetone Controls	9	0	0	0	0	0	1	0	0	0	89
Controls	10	0	0	2	0	6	0	0	1	0	10

Judging by the information which this experiment yielded, the sonic method is a most satisfactory way of disintegrating the organisms or making a stable suspension of precipitated fractions without harming the antigen.

2. Fractionation Methods

Before the sonic disintegrator was available a number of experiments had been carried out with material prepared in one of the grinders. All operations were conducted in the cold and all chemicals and reagents were cooled before being used.

(a) Ammonium Sulfate Fractions

The organisms were grown in Snyders peptone media, killed by adding acetone equal to 25% of the volume of broth, and allowed to stand overnight in the cold. The organisms were then collected in a Sharples centrifuge. When a sufficient quantity had collected they were mixed with two parts powdered glass and enough water to form a thin paste, and then ground in the syringe grinder. After grinding, the glass and bacterial debris were removed by centrifuging in the cold at 4000 RPM for 90 minutes. The solution was saturated with ammonium sulfate and allowed to stand in a cylinder for three days. At the end of this time the material had precipitated in a peculiar way. It had separated into three distinct layers. The top layer, a white, flocculant precipitate (SML); a clear middle layer of ammonium sulfate solution, and a third, bottom layer that settled into a heavy, grey-white flocculent mass (SMH). The two principal layers SML and SMH were siphoned off and dialyzed free of ammonium sulfate. They were then lyophilized for storage. Samples of each were diluted to a total nitrogen content of 0.05 mg.N/ml and tested on twenty rats each. The figure 0.05 mg.N/ml is the amount of nitrogen per milliliter which is contained in the acetone-extracted vaccine used as a standard for testing the antigenic potency of these fractions. The results are shown in Figure 43. According to these figures SMH is a much better protective antigen than SML. The antigen present in fraction SMH was not destroyed by ammonium sulfate treatment as shown by rat protection tests.

Relationship of pH to the Turbidity of SML Suspensions

It was shown that the addition of bases in sufficient quantity to raise the pH to about 9 would clear a turbid suspension of SML or SMH. Acid treatment did not decrease the turbidity but rather seemed to increase it. In an effort to investigate the solubility of this material at nearly neutral pH the following experiment was performed.

The SML was diluted to a concentration of 0.05 mg.N/ml and samples of this material were dialyzed against 0.1 M phospate buffers in the pH range from 6.0 to 8.0 in steps of 0.1 pH unit. Upon completion of dialysis there appeared to be very little difference in the turbidity of the samples. When the samples were read in the spectrophotometer the percentage light transmission varied with the pH. These values are plotted graphically in Figure 44.

The minimum point, pH 7.3, of transmission might very well be the isoelectric point of the principal protein and does in fact agree

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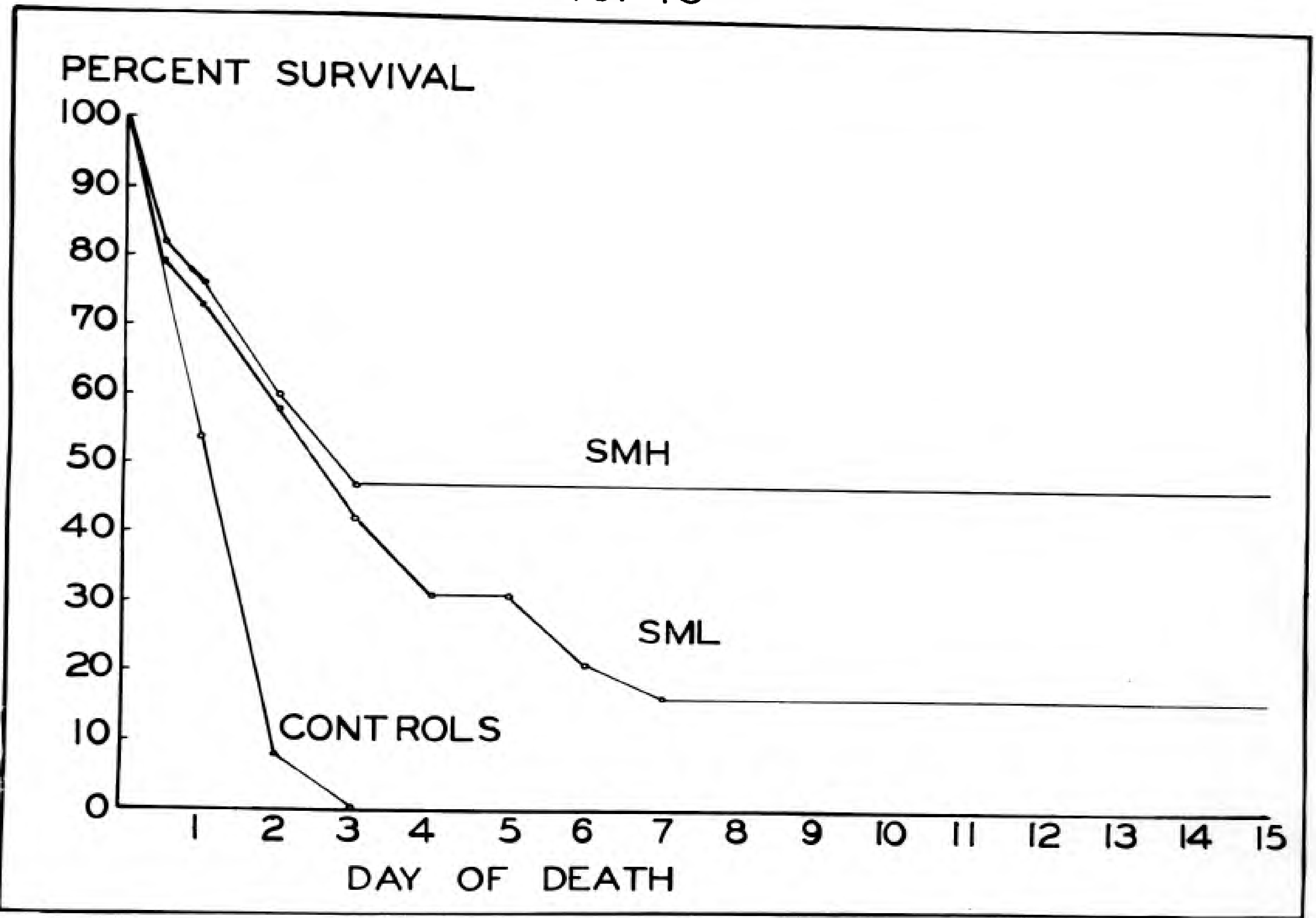
FIG. 43



CHALLENGE OF RATS VACCINATED WITH SML AND SMH

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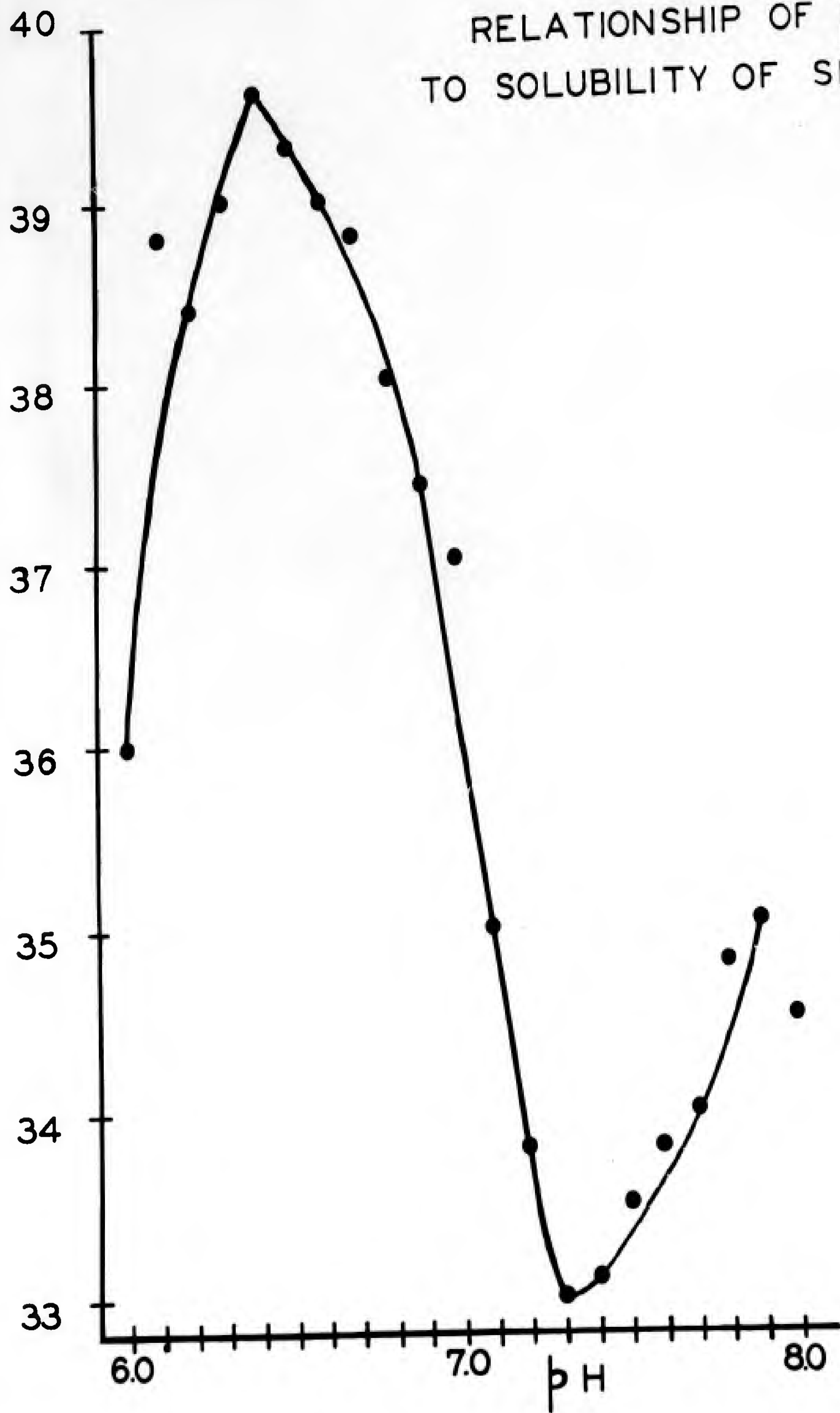

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reasonably well with the isoelectric point value found later by the electrophoresis method.

(b) Fractionation Studies Using Strain NIH-38

It had been demonstrated [62] that a vaccine could be made from the avirulent strain NIH-38. This seemed to offer a solution to the problem of obtaining native material unchanged by contact with acetone, formalin, phenol or any of the other similar agents generally used to kill the organism. Quqntities of NIH-38 were grown, collected in the Sharples centrifuge, disintegrated by the sonic treatment, dialyzed free of soluble material, and then lyophilized for storage. This material was used for nearly all of the fractionation studies hereafter.

(c) Chloroform Gel Method

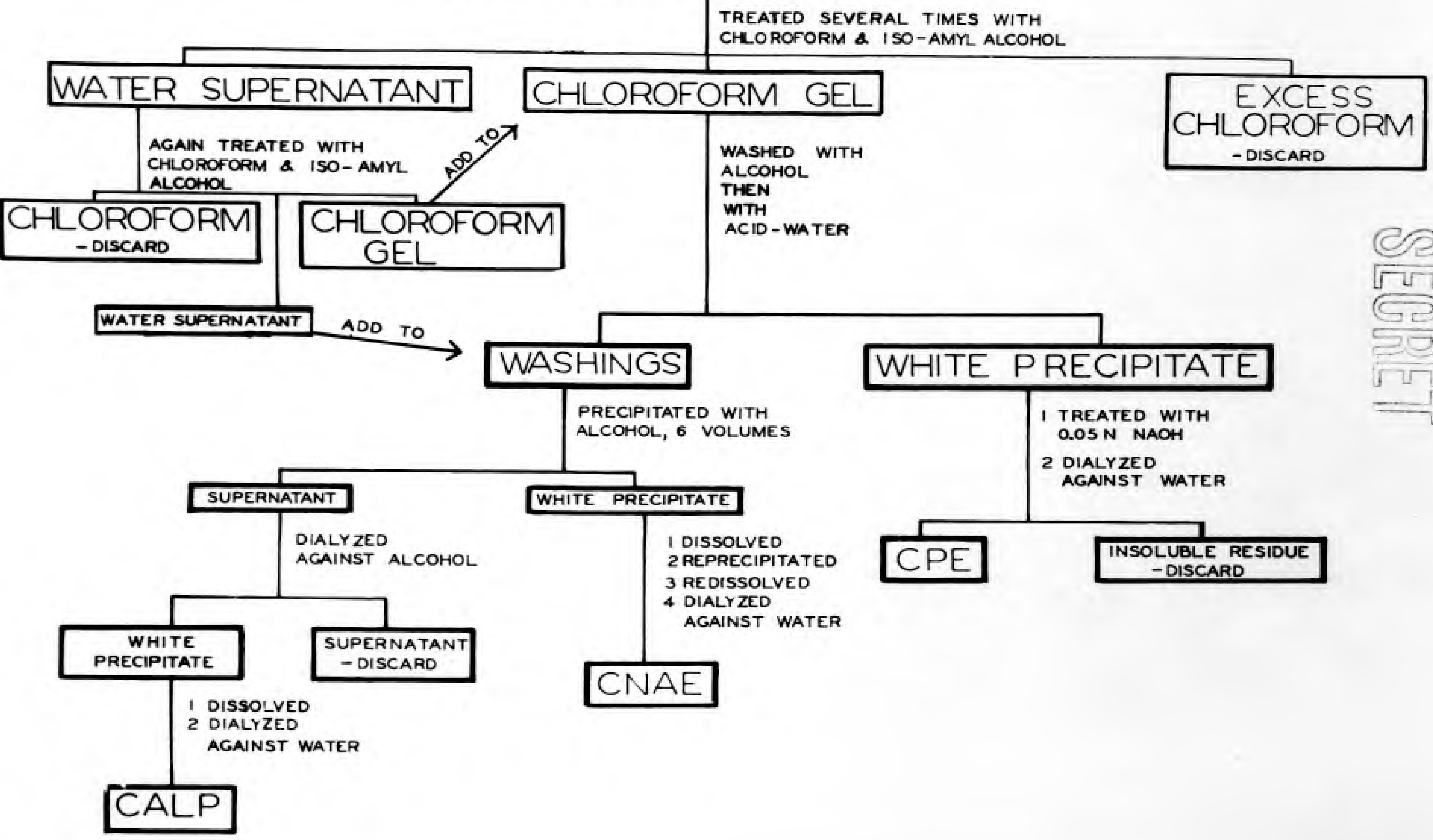
Following the procedure developed by Sevag, Lackman and Smolens [71] a chloroform extraction method was used to extract antigen fractions. All work was done at 1° C. The material used was 12 ml of a suspension of organism which had been treated by the sonic method and then filtered through an 03 Selas filter. To this was added 5 ml of chloroform and 0.5 ml of iso-amyl alcohol. The mixture was shaken well and then centrifuged until a good separation was obtained. After centrifuging the tube contained three distinct layers; the top water layer, a solid chloroform-protein gel, and a bottom layer of excess chloroform. The water layer was pipetted off and treated several times with chloroform until no appreciable amount of gel formed. At this point there were three separate components, the water supernatant, the chloroform gel, and the excess chloroform (Figure 45).

The gel was washed first with alcohol and then with water adjusted to pH 5.0 with acetic acid. The washings were saved for further precipitation. The protein material left upon solution of the gel was suspended in cold 0.01 N NaOH for several hours until it want into solution and was then dialyzed against water to remove the excess NaOH. A slight precipitate formed which was centrifuged off and discarded. The final solution (CPE) measured 21 ml in volume.

The water supernatant from the original chloroform treatment, plus the washings from the chloroform gel were treated with 6 volumes of 95% alcohol and allowed to stand overnight at 1° C. A white precipitate formed which was filtered off. The precipitate was then dissolved in water, filtered, and reprecipitated with alcohol. The precipitate (CNAE) was again dissolved in water and dialyzed free of alcohol. The final solution (CNAE) measured 20 ml in volume.

In order to be sure that no more material remained in solution in the alcohol from the CNAE precipitation this solution was dialyzed against 95% alcohol. At the end of 48 hours of precipitate (CALP) had developed. This was centrifuged off, redissolved in water, dialyzed against water, and

Figure 45



then, as there was very little material present, the entire sample, 5 ml, was sent to the chemistry branch for analysis. The results are shown in Table LXVIII.

Table LXVIII

Chemical Comparison of Chloroform Fractions

Sample	Total N in Sample	Total P in Sample	Total Carbohydrate as Glucose
CPE	6.51 mg	0.105 mg	1.701 mg
CNAE	1.20	0.100	13.80
CALP	0.15	0.005	---

	Biuret	Molisch
CPE	+	-
CNAE	-	-

The two most important fractions, CPE and CNAE, had quite definite characteristics as shown by this table. The CPE seemed to be a protein as indicated by the positive Biuret test. The CNAE seemed to be some sort of carbohydrate material containing nitrogen and phosphorous. The high phosphorous to nitrogen ration suggested a nucleic acid complex. An ultraviolet absorption spectrum was run on the Coleman Spectrophotometer.

The values obtained were plotted against similar data for ribonucleic acid as shown in Figure 46. The points of the first high peaks are identical for the two curves while the minimum in the CNAE appears to be partially masked by impurities.

The carbohydrate was determined by the carbazole method of Dische[61] as developed by Gurin and Hood[64]. The color and curve of the carbazole of this carbohydrate was quite unlike that of any sugar available at this laboratory. The curve was read as glucose as it more closely fitted the glucose curve than any other. However, the curve indicated that the final hydrolysis product was no simple hexose or pentose but seemed to be a sugar of perhaps twelve carbon atoms in size.

An electrophoresis study was made of the protein fraction, CPE, and this showed a single peak, indicating that no other components were present. The material was run at pH 6.98 in cacodylate buffer and had a mobility of -3.5. There was about 10% of inert material at the bottom of the peak, which probably indicated that some decomposition had taken place. A more extensive discussion of electrophoretic data appears near the end of this report.

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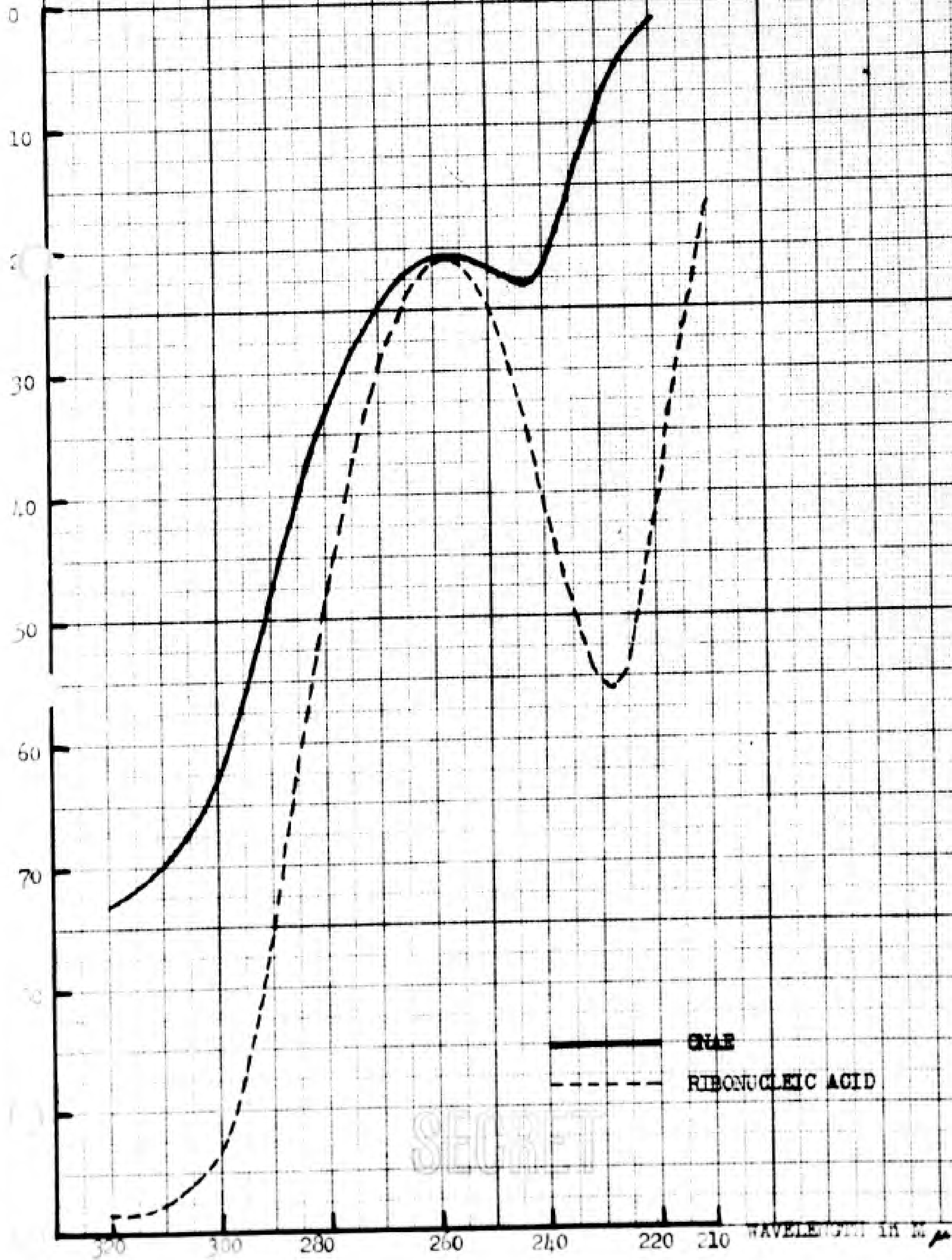
FIGURE 46 ULTRAVIOLET ABSORPTION CURVES OF FRACTION CNAE AND RIBONUCLEIC ACID

PERCENT TRANSMISSION



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Animal tests (Table LXIX) were made on CNAE and CPE, there was too little of the CALP material for anything but a few simple chemical tests.

The antigens were given on the basis of 0.05 mgN/ml of vaccine and were injected 0.5 ml subcutaneously on the first day and two days later with 0.7 ml subcutaneously. The rats were challenged one month later with 100 MLD injected in one ml intraperitoneally.

Table LXIX

Immunization with Chloroform Fractions

Vaccine	Number of Animals	Day of Death												D/T	PerCent Survival
		1	2	3	4	5	6	7	8	9	10	11	12		
CPE	5	0	0	0	0	1	1	0	0	0	1	0	0	3/5	40
CNAE	5	0	0	3	0	0	0	1	0	0	0	0	0	4/5	20
Controls	7	0	0	6	0	0	0	0	0	0	0	0	0	6/7	13

There was a noticeable difference in the way the animals reacted to challenge. The rats vaccinated with fraction CPE did not appear ill on the third day after challenge when the majority of deaths occurred in the controls and in animals vaccinated with fraction CNAE. Delayed deaths occurred in the CPE group on the fifth, sixth and eleventh days after challenge. 40% of the CPE rats survived against 20% for CNAE and 13% for the controls. As the number of rats used in this experiment was limited the data could not be considered statistically significant but indicated that fraction CPE contained the antigen. The poor protection afforded by fraction CPE in comparison with a whole vaccine indicated that it was partially inactivated or destroyed by the manipulation of extraction and purification. This fraction, precipitated with 1 per cent alum, also failed to protect monkeys against challenge with virulent organisms (see Immunization of Monkeys, Section F-c this report). As some decomposition of the antigen was also indicated by the electrophoresis picture this method of extraction was not continued further.

(d) Ultrafiltration Method

In an effort to get away from harsh chemical fractionation methods the physical method of ultrafiltration was used. This consisted of filtering the sonic treated material through nitrocellulose membranes[63] of various porosities in order to separate fractions of grossly different molecular sizes.

The filters were made by impregnating Whatman #1 filter paper with various concentrations of nitrocellulose dissolved in glacial acetic acid. The papers used by Elford[63] were not available. The concentrations of nitrocellulose in acetic acid were 1.0%, 1.5%, 2.0%, 2.5%, 3.0%, 3.5%.

The filters were made by placing the filter paper in small jars containing the nitrocellulose solutions. These jars were than placed in a vacuum dessicator and evacuated. A vacuum was maintained until no more air bubbles were given off by the filter paper. The vacuum was then released and the paper removed. Upon removal from the nitrocellulose solutions the papers were drawn between two glass rods to remove the excess nitrocellulose. They were then placed in water and washed free of acetic acid. The membrane filter was mounted in a Seitz filter with another piece of filter paper placed against the screen for reinforcement. A amall stirrer was used to prevent clogging of the filter. The pressure at which the filter was run was that which was most efficient as determined by plotting the flow-rate against various pressures and then finding the point at which an increase in pressure did not produce an appreciable increase in flow rate. The curves were quite reproducible and were used as a test to select the best filter paper to use.

The first material to be filtered was prepared from sonic treated NIH-38 organisms, sample ULA-3. This material was first treated with 3 ml of 0.05 N NaOH per 100 ml of suspended organisms to clear the solution. The solution was then dialyzed against water until free of base and centrifuged at 4000 RPM for 90 minutes. This solution was diluted with water.

A clear filtrate was obtained by filtering through a 1.5% membrane filter at a negative pressure of 4 cm of Hg. This clear filtrate (ULF-1.5) did not yield any residue even when filtered through the 3.5% membrane. It filtered quite easily and did not change in appearance on filtration.

The ULF-1.5 was lyophilized and yielded 1.05 gm of material. On solution the ULF-1.5 gave a negative Biuret and positive Molisch test and showed turbidity with trichloracetic acid. A sample containing 0.219 gm in 16 ml of solution was run in the electrophoresis machine. Chemical tests and animal protection tests were also carried out.

The material retained by the filter (ULS-1.5) was very turbid and was centrifuged in an effort to remove any precipitated material that might be present. However, even after 30 minutes of centrifuging at 18,000 RPM only a very small amount of ULS-1.5 settled out. Upon completion of centrifugation the ULS-1.5 solution amounted to 30 ml. This was diluted to 100 ml and filtered through another 1.5% filter to wash the solution free of ULF-1.5. Several small amounts of water were later added to complete the washing. There was 50 ml of solution present. Samples were sent to chemistry for analytical work and rats were also vaccinated with this material. The sample gave a faintly positive Biuret test and a negative Molisch test.

The comparison of chemical results is shown in Table LXX.

Table LXX

Chemical Ultrafilter Fractions

Sample	Mg N/ml	Total carbohydrate as glucose	N calculated as protein	Protein to Carbohydrate ratio
ULF	0.06	0.083 mg/ml	0.375 mg/ml	4.52-1
ULS	0.27	0.198 mg/ml	1.69 mg/ml	8.53-1

The ULF-1.5 appeared to be a carbohydrate complex of some sort while the ULS-1.5 was a protein. The protein-carbohydrate separation was exactly the separation hoped for in using the ultrafilter method.

A weighed sample of ULF-1.5 was dissolved in water and dialyzed against water for several days, and then lyophilized. There was very little loss of weight upon dialysis showing that the ULF-1.5 complex is too large a molecule to pass through the cellophane membrane. Another sample of ULF-1.5 was dissolved in water and precipitated with trichloracetic acid. The precipitation took place overnight at -7° C. The precipitate was centrifuged out and the supernatant dialyzed against water. When free of acid the supernatant was frozen and lyophilized. Nothing remained after completion of lyophilization. Evidently all of the material present was removed by trichloracetic acid. The ultraviolet absorption curve of ULF-1.5 (Figure 47) showed a distinct similarity between the ULF-1.5 and ribonucleic acid while the ULS-1.5 was quite differant.

A comparison of the antigenic properties of the fractions is shown in Figure 48. Using acetone-extracted vaccine as a control, animals were immunized with solutions containing 0.04 mg of solids per ml in each case. The rats were immunized with three intraperitoneal injections of 0.5 ml at 48 hour intervals. The acetone control was the best antigen while the ULS-1.5 was considerably better than ULF-1.5 or the whole material, ULA3.

Ten per cent of the unvaccinated controls survived. Since the death curve of controls fell between that for ULA3 and ULF-1.5 it was omitted from Figure 48 for convenience in plotting. These latter two antigens did not confer any protection while ULS-1.5 and the acetone extracted vaccine did protect a significant number of animals. The low percentage survival with ULS-1.5 and the acetone extracted vaccines is explained by the massive challenge used in this experiment (50,000 rat LD_{50}).

The original material used as a starting point for fractionation (ULA3) was a good protective antigen when given in a dose of 4 mg of solids. It protected 22% of the animals when challenged with 50,000 LD_{50}. This suggests that the filtration concentrated the protective antigen or possibly removed some substances which interferred with immunization.

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FIGURE 47

ULTRAVIOLET ABSORBTION CURVES OF FRACTIONS SEPARATED BY ULTRAFILTRATION

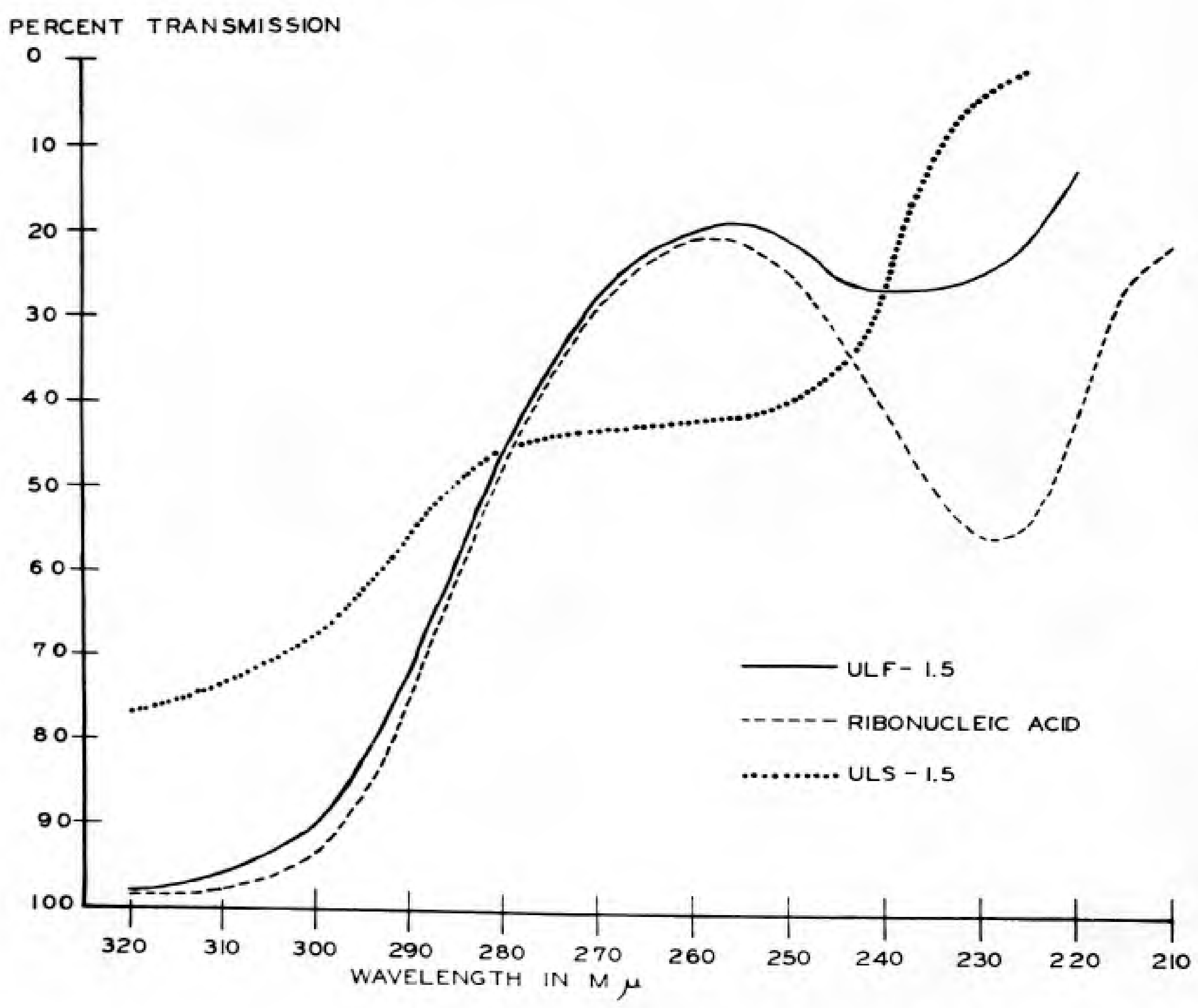

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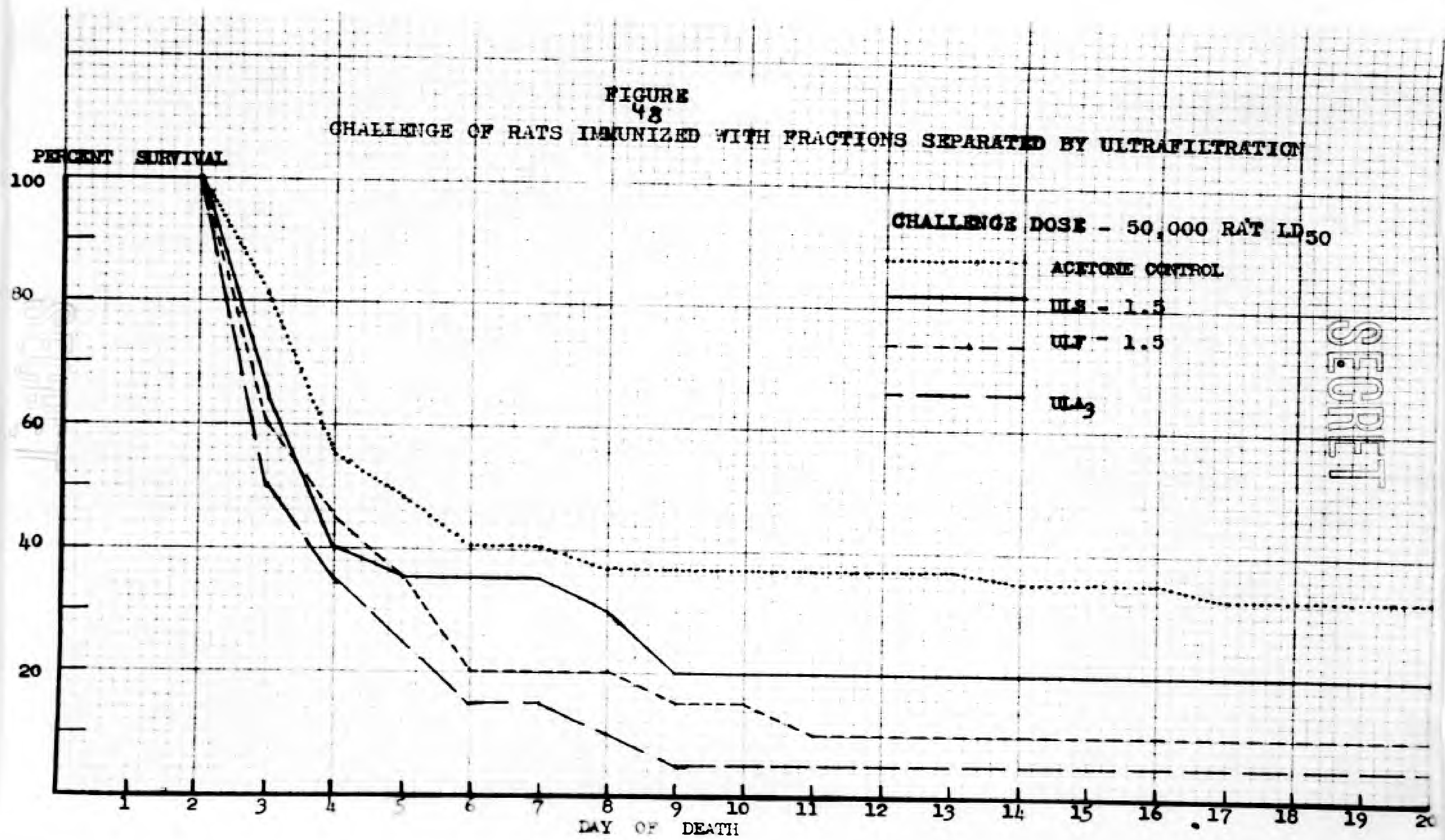
FIGURE 48

CHALLENGE OF RATS IMMUNIZED WITH FRACTIONS SEPARATED BY ULTRAFILTRATION



The electrophoretic data are summarized in the following Table LXXI.

Table LXXI

Electrophoretic Data of Ultrafilter Fractions

Mobility $U_D \times 10^5$

Sample	pH	P_1	P_2	N	Antigenic Properties	Remarks
ULA3	7.02	-1.9	-0.8	-13.1	good	chiefly p_1
ULF-1.5	7.02	-1.7	----	-12.5	poor	chiefly p_1
ULS-1.5	7.02	-1.9	-0.6	-----	good	chiefly p_1 similar to ULA3

Three principal components were demonstrated by electrophoretic analysis, p_1,p_2, and N (See Figures 49, 50, 51). The N component has a very high mobility suggesting nucleic acid. The elimination of it in sample ULS-1.5 shows that this component is not the active antigen. There is no p_2 component in ULF-1.5, which is a poor antigen. It seems then thatthe p_2 or possibly a combination of p_1 and p_2 is necessary for good antigenic qualities.

Summary

These are the facts known and conculusions suggested by the various data obtained in this experiment.

ULF-1.5:

1. Is a poor antigen.

2. Precipitates completely with trichloracetic acid.

3. Decomposes at 240° C-- perhaps similar to glycogen which also decomposes at 240° C.

4. Gives a negative Biuret test when tested in 1.0 per cent solution--not a protein material.

5. Gives a positive Molisch test--the test is weak and develops slowly, suggesting a very complex carbohydrate.

6. Gives an ultraviolet absorption curve similar to ribonucleic acid--nucleic acid may be a part of the complex.

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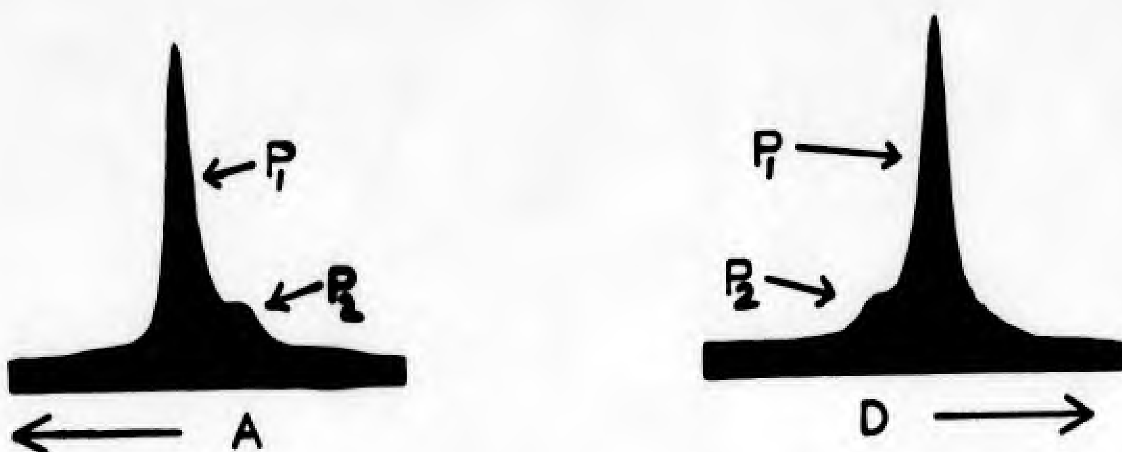

FIG. 49 ELECTROPHORETIC PATTERNS OF ULA-3, CACODYLATE BUFFER, pH 7.02
PATTERNS WERE OBTAINED AFTER 11,813 SECONDS AT 2.80 VOLTS/CM.

FIG. 50 ELECTROPHORETIC PATTERNS OF FRACTION ULF-15 CACODYLATE BUFFER, pH 7.02
PATTERNS WERE OBTAINED AFTER 2109 SECONDS AT 2.49 VOLTS/CM.

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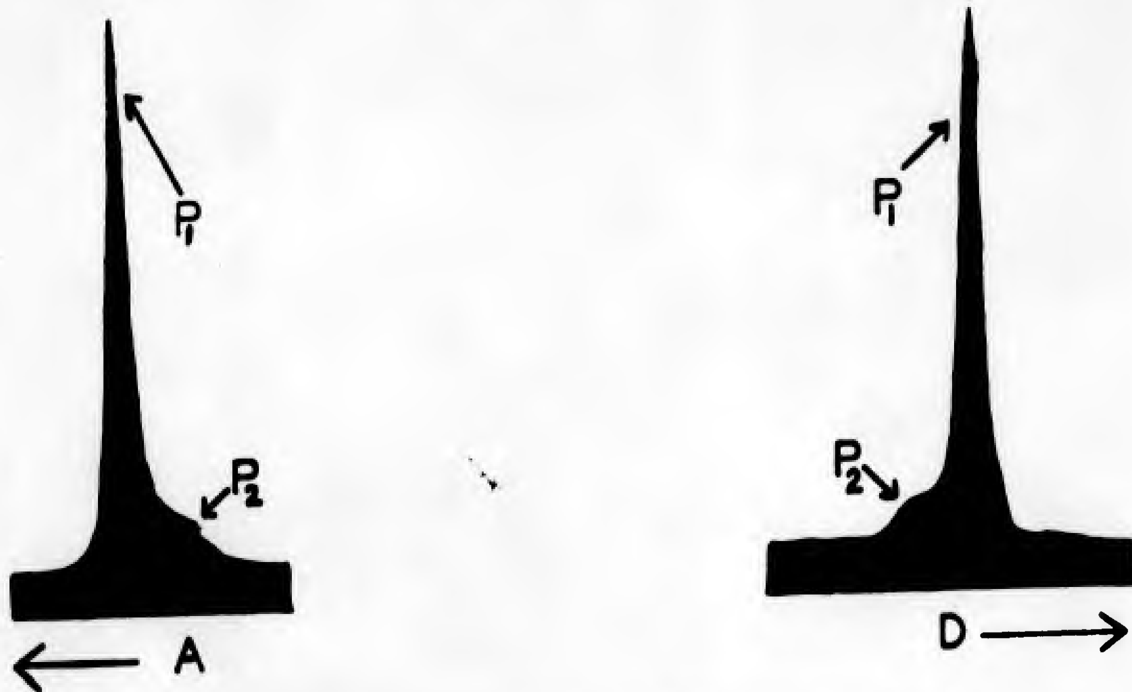


FIG. 51 ELECTROPHORETIC PATTERNS OF FRACTION ULS-1.5
CACODYLATE BUFFER pH 7.02
PATTERNS WERE OBTAINED AFTER 13,185 SECONDS
AT 2.65 VOLTS/CM.

7. Contains a high proportion of carbohydrate---as would be expected by the Molisch results.

8. The carbohydrate as determined by the carbazol method gives a curve unlike the usual pentose and hexose curves even after hydrolysis in 80% sulfuric acid---suggests a basic unit which is more complex than the usual hexose or pentose.

9. Forms osazones which precipitate in 5½ minutes---glucose forms an osazone in 4 to 5 minutes.

10. Gives no color reaction with iodine---as do starch and glycogen.

11. As shown by electrophoresis studies contains two principle components; p_1 and N, with mobilities of -1.7 and -12.5 respectively.

ULS-1.5:

1. Is a good antigen.

2. Precipitates with trichloracetic acid--as would be expected of protein material.

3. Gives a positive Biuret test---the presence of protein is again indicated.

4. Gives a negative Molisch test--no appreciable amount of carbohydrate is indicated.

5. Gives an ultraviolet absorption curve unlike nucleic acid but similar to many other proteins.

6. Has a high protein to carbohydrate ratio compared with ULF-1.5---mostly protein material present.

7. Will not pass through a 1.5% nitrocellulose membrane at pH 7---large molecules are present.

8. As shown by electrophoresis studies ULS-1.5 contains two principle components; p_1 and p_2 with mobilities of -1.9 and -0.6 respectively--this eliminates component N as a part of the antigen necessary for protection.

Conclusions

By filtering sonic killed NIH-38 organisms through a 1.5% nitrocellulose ultrafilter a fractionation of the antigenic material could be accomplished. The filtrate was a poor antigen while the residue was a good antigen. By comparing the two fractions chemically and electrophoretically several conclusions are indicated. The antigen seems to be a protein which is too large a molecule to pass through the membrane. It differs from the

filtrate ULF-1.5 in that it contains no electrophoretic component N. This filtrate is a carbohydrate material which contains the unnecessary component N but does not contain component p_2 which is present in both the ULS-1.5 and the original material ULA3. A study of the electrophoresis pictures shows that the p_2 fraction composes only about 10% of the material present in ULS-1.5 and ULA3, so that if p_2 is the antigen very little is necessary to afford protection. This may account for the slight protection shown by ULF-1.5 as a small amount of p_2 could have been masked by the p_1 component. The antigen then is a protein represented by either component p_2 or by a combination of p_1 and p_2. The unnecessary material is a nucleic acid complex, probably with a carbohydrate.

3. Summary of Electrophoresis Studies

A number of different types of material, in addition to the fractions mentioned, were studied by means of the Tiselius electrophoretic apparatus (71,66,67,68,69). When the data on all of these different samples were compiled certain conclusions were suggested. The samples are listed in the following paragraphs with information concerning their preparation and relative antigenicity.

ULB--- acetone killed and washed cells broken up by the sonic disintergrator....a good antigen.

SMLV--- acetone killed organisms ground in the mechanical grinder, waste cells and cell debris centrifuged off, remaining material precipitated with ammonium sulfate; this top layer which separated on standing was put into solution further by sonic treatment..... a poor antigen.

ULLSF3--- living virulent organisms killed and disintergrated by sonic treatment; filtered through a Selas 03 filter until clear....a good antigen.

ULLSF3-ppt--- ULLSF3 which was precipitated at pH 6.8 with 80% ammonium sulfate at 1° C; redissolved in saline...a good antigen.

ULA-3--- Avirulent strain NIH-38 organisms killed by sonic treatment; the extraneous material centrifuged out, and the suspension dialyzed free of soluble low molecular weight substances.....a good antigen.

ULF-1.5---- The filtrate of ULA-3 through a 1.5% nitrocellulose ultrafilter....a poor antigen.

ULS-1.5---- The residue from ULA-3 filtered through a 1.5% nitrocellulose ultrafilter....a good antigen.

The electrophoretic properties of these antigens are summarised in the following table.

Table LXXII

Summary of All Electrophoretic Data

Sample	pH	Mobility $U_D \times 10^5$ p_1	p_2	N	added	Antigenic Properties	Remarks
ULB	6.82	-1.6	-1.0	-15	---	Good	
SMLV	6.82	-1.8	---	-19	---	Poor	
ULLSF3	6.82	---	-0.4	---	-5.2	Good	
ULLSF3ppt	6.98	-1.3	-0.8	---	---	Good	
ULA-3	7.02	-1.9	-0.8	-13.1	---	Good	Chiefly p_1
ULA-3	8.50*	-3.2	poorly resolved	-13.0	---	Good	
ULF-1.5	7.02	-1.7	---	-12.5	---	Poor	Chiefly p_1
ULS-1.5	7.02	-1.9	-0.6		---	Good	Chiefly p_1 similar to ULA-3

* Mobility was greater at the higher pH.

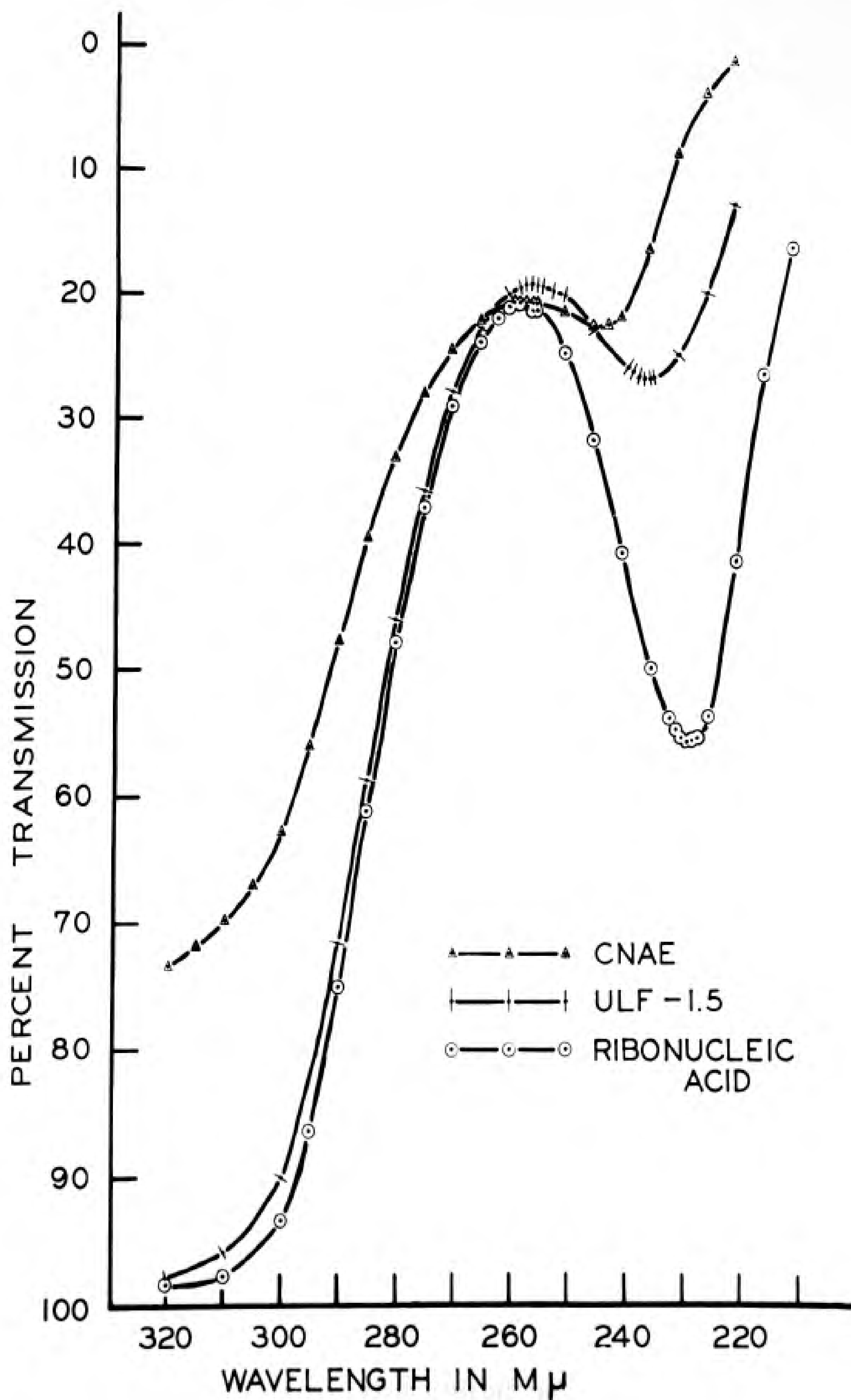
There appear to be three principal components, N, p_1, and p_2. The N component is a fast moving component with a mobility of about -13 and pH 7.02. It is not antigenic as is shown by the fact that it is absent from ULLSF3, ULSF3-ppt, and ULS-1.5 all of which are good protective antigens. The p_1 component is a slower moving component with a mobility of -1.8 at pH 7.02. It is present in all samples, both good and bad, except ULSF3, which is a good protective antigen. The third component, p_2 is a slow moving component with a mobility of -0.6 at pH 7.02. It is present in each sample that showed protective properties and absent from those which did not. The protection which SMLV gave, may be due to occluded p_2 which was present in too small an amount to be detected in the electrophoresis pattern.

4. Summary of Ultraviolet Absorption Studies

When ultraviolet absorption data on various fractions, were plotted, two types of curves were observed. The curves in Figure 52 are of CNAE and ULF-1.5 compared with that of ribonucleic acid. They are quite similar and except for some masking at the lower wave lengths due to impurities they tend to follow the curve of ribonucleic acid. These are all poor protective antigens.

Good protective antigens compared in the same manner yielded

FIG. 52 ULTRAVIOLET ABSORBTION CURVES OF POOR PROTECTIVE ANTIGENS



distinct curves ad demonstrated in Figure 53. In this figure three typical antigens, ULB, ULLSF3-ppt, and ULS-1.5 are shown. The general shape of the curve is specific for the protective antigen from this organism. It also seems to be quantitative, as higher concentrations of protective antigen move the curve up and slightly to the left. These curves are similar to the ultraviolet absorption curves of a large number of proteins. The ULS-1.5 curve in the figure is an example of this.

In summary, two types of ultraviolet absorption curves are obtained from fractions of "UL" antigens. The curve for the poor antigenic fraction is similar to that of ribonucleic acid. The curve of the protective antigen fractions is quite different and may be specific for the protective antigen of this organism.

5. General Summary

It is possible to fractionate the material which "UL" yields upon disintegration into three fractions, N, a nonprotective substance, seems to be a nucleic acid-carbohydrate complex. A large amount of chemical data has been compiled concerning this fraction. The other two fractions, p_1 and p_2, differ in mobilities and probably molecular size as p_1 will pass a 1.5% nitrocellulose membrane ultrafilter while p_2 willnot. The p_2 fraction seems to be the protective antigen. It was possible to differentiate the protective antigen and the nucleic acid-carbohydrate complex by ultraviolet absorption data, by chemical analysis, by rat protection data and by the electrophoresis method. Production of these fractions on a larger scale is indicated to permit a more exhaustive study of their chemical and antigenic properties. It has recently been shown that monkeys develop skin hypersensitivity to "UL" following a sublethal infection. The use of this animal for studying the allergenic potency of various fractions will expedite the accumulation of data since the use of convalescent personnel for this purpose frequently leads to unpleasant hypersensitive reactions.

J. Chemotherapy

An effective therapeutic agent for treatment of human "UL" infection was not available at the beginning of this work. Recognized treatment consisted of good nursing care, bed rest during the febrile stage, and symptomatic treatment of complications. In the average case the febrile stage lasted two to three weeks, followed by two to six months of weakness and easy fatiguability. Obviously a specific agent which would cure the disease in a few days was desirable as protection for laboratory workers as well as for friendly troops in case of an enemy attack with this agent.

A review of the literature (80-85) on Chemotherapy of "UL" infection showed results so conflicting that it was practically impossible to draw any conclusions from the reports. Most reports dealt with a few clinical cases, and usually several types of therapy had been tried on the same cases with no real controls. Ransmeier (86,87,88) in a study of the Chemotherapy of "UL" infection in chick embryos could find no beneficial effect when

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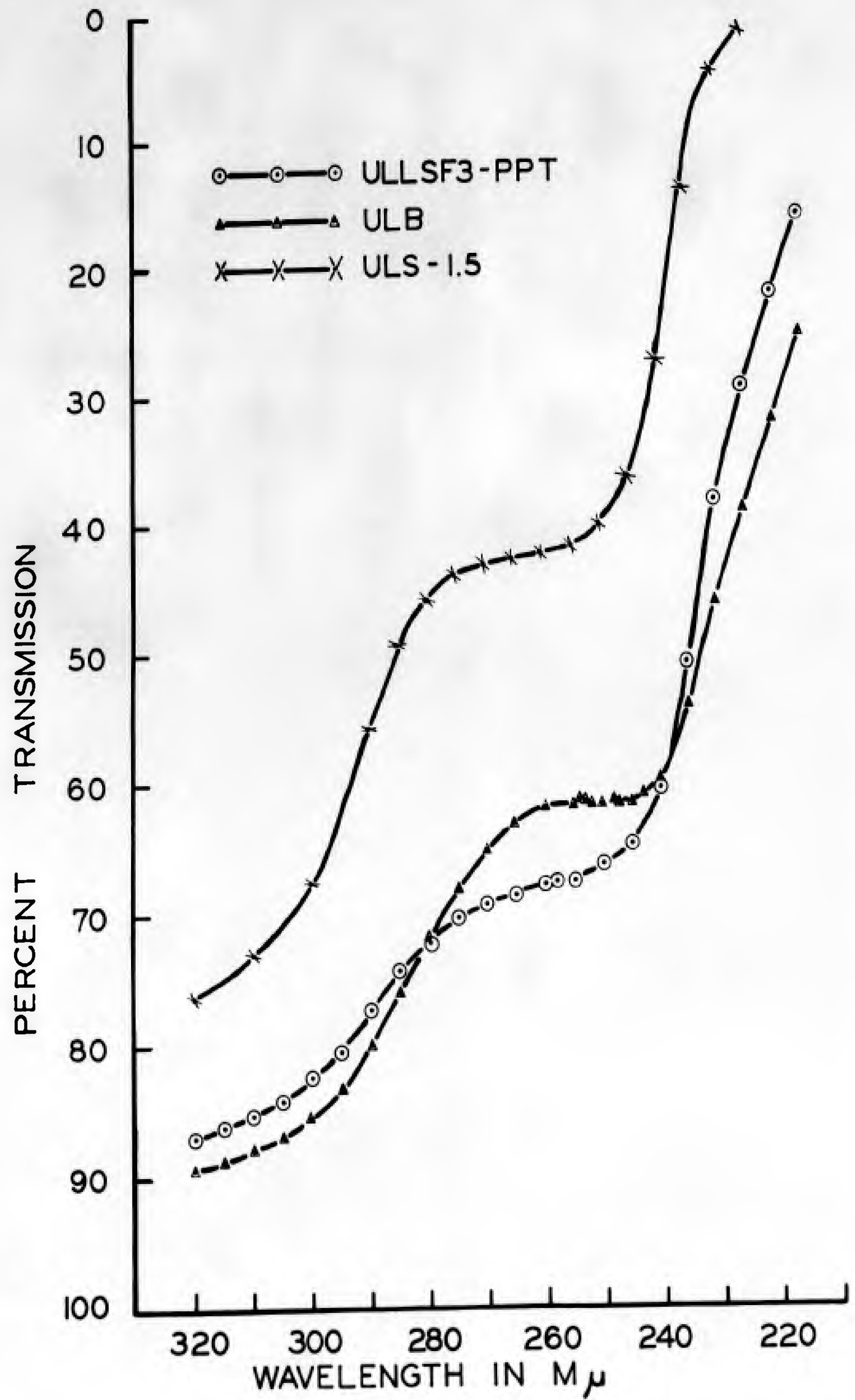
FIG. 53 ULTRAVIOLET ABSORBTION CURVES OF GOOD PROTECTIVE ANTIGENS



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using sulfadiazine. Steinhaus[103] reported that the lives of guinea pigs could not be saved by the administration of a variety of sulfa drugs. It was quite obvious that a careful laboratory study of the problem was needed, and our research program was planned to obtain sound experimental data upon which a statistical evaluation of drugs could be made.

A total of 36 chemotherapeutic and antibiotic agents was tested for anti "UL" activity in vitro and in vivo. The in vitro testing was done in Snyder's peptone broth. The inoculum used was 1 ml of 10^{-2} or 10^{-3} dilution of T500 suspension in 10 ml of medium. In vivo blood levels of 10 to 13 mgm % of sulfonamides were maintained in mice and rats by feeding the drug during the time of challenge. Other drugs were injected into animals at suitable intervals. Dosages were determined by the toxicity of the drug for animals or from its known therapeutic level per kilo for other diseases in man.

Seven sulfonamides, penicillin, sulfarsphenamine, neoarsphenamine, bismuth tartrate, toluidine blue, atabrin, and eleven compounds showing some activity as antimalarials all proved to be ineffective in treating "UL" infected mice. Streptomycin and streptothricin were the only drugs tested which showed any therapeutic efficiency. In mice the toxic dose of streptothricin was only about double the chemotherapeutic dose so further studies were not carried out. Streptomycin was very effective in vitro and in vivo and the toxic dose for mice was in the neighborhood of forty times the therapeutic dose. Details of the experimental work with streptomycin in this laboratory have been included in a special report [89]. Heilemen[46] and Foshay[96] have carried out independent laboratory studies with streptomycin as a chemotheropeutic agent for "UL" infection and their results were similar to those obtained at Camp Detrick. Results of laboratory experiments were so encouraging that streptomycin was given a clinical trial in human "UL" infection by Foshay[per com.] and also at Camp Detrick. (Section F-2 this Report)

In summarizing all evidence available it appears that streptomycin is a safe and effective specific antibiotic for treating human "UL" infection. All the chemotherapeutic agents tested were ineffective or too toxic for human use.

K. The Agglutination Test in "UL"

The agglutination test has long been recognized as a useful laboratory test in the diagnosis of disease, in the recognition of previous contact with a specific disease, and as a measure of immunity following either natural infection or artifical immunization. In the case of "UL" infection it has been the only diagnostic test which could be performed routinely in the average laboratory with safety to the workers involved. It was natural, therefore, that it should be adopted at the outset of this project as a routine procedure for the examination of the sera of human personnel as well as those of experimental animals.

Inasmuch as this procedure was to be employed as one method of measuring the response to "UL" vaccination in man and experimental animals it was essential that a sensitive and accurate test be developed. Comparative studies of antigens and technics of setting up the test were therefore darried out.

(1) Choice of Antigen

The antigen employed routinely throughout the United States in the agglutination test for "UL" is a washed formalized culture prepared according to the method of Francis (90) from a culture known as Strain 38, which was isolated by him in Utah in 1920. A culture of Strain 38 was received from the National Institute of Health, Bethesda, Maryland, and will be subsequently referred to as NIH-38. This strain is completely avirulent and an antigen may therefore be prepared from it without danger to the worker.

In order to ascertain whether or not strain NIH-38 was the most satisfactory for use as an antigen in the performance of the agglutination test a comparative study of antigens were made. The following antigens were employed.

FO 38	prepared by Foshay from Strain 38
MF	prepared by Foshay from strains C and S
NIH-38	prepared at Camp Detrick from Strain NIH-38
C & S	prepared at Camp Detrick from strains C and S

All antigens were made according to NIH specifications from cultures grown on solid media, killed with formalin, washed and resuspended in formalized saline. These antigens were tested against 22 human sera from convalescent or vaccinated persons and the results are shown in Table LXXIII. In 13 instances both NIH-38 and FO-38 gave higher titers than the mixed antigens. In 3 instances FO-38 was greater than NIH-38. In 2 instances all four antigens gave the same results and in 1 instance all antigens except MF were the same. In three cases only, C&S yielded the highest results. On the basis of these findings strain NIH-38 was chosen as the most satisfactory antigen. It was somewhat more sensitive than the antigens prepared from virulent cultures and had the added advantage of being safe to handle.

(2) Technic of the Agglutination Test

The first method employed for the performance of the agglutination test was the standard routine method with incubation at 56°C for 2 hours followed by overnight storage in the ice-box at 4°C. However, in view of the large number of agglutination tests to be performed simplicity and speed were the prime requisites for a satisfactory test. Accordingly the method of Nagle et al (91) was adopted with the following modifications: Serial dilutions (1/10 through 1/2560) of serum in 0.2 ml amounts were placed in a series of nine tubes. To each of these tubes

was added 0.2 ml of NIH-38 antigen diluted to give a transmission of 30 on the Coleman Spectrophotometer. The tests were placed in a Kahn shaker for 5 minutes and then read. It was found that 5 hours incubation at 37°C following shaking, as recommended by Nagle, did not materially alter the readings obtained after shaking alone. Final readings were therefore made after 5 minutes of shaking. These were made with the aid of a mirror and were recorded as 4 + (complete agglutination), 3 + (75% agglutination), 2 + (50% agglutination), 1 + (25% agglutination) and - (no agglutination). The titer reported was the highest dilution showing 1 + or more agglutination.

This method was employed for approximately 6 months and proved very satisfactory for human, rat, and rabbit sera but inconsistent and irregular findings with mouse serum led to its review.

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a. The Agglutination Test for "UL" as Applied to Mouse Serum

When agglutination tests for "UL" were set up using serum from vaccinated mice, very low titers were obtained regardless of the results which might have been expected from the previous treatment of the mice. Normal mouse serum controls were found to give false positive results in relatively high dilutions.

It was thought that the pH of the saline employed in the test (6.5) might be responsible for the occurrence of false positives with mouse serum. Therefore various samples of saline were adjusted with N/5 NaOH and N/5 HCl to give the following pH values: 6.0, 6.4, 6.8, 7.2, 7.6, and 8.0.

Blood was obtained from six normal mice representing two strains, 3 Bagg and 3 Pleasant Valley. The blood of the 3 Bagg mice was pooled. That of 2 of the Pleasant Valley mice was pooled, while that of the 3rd Pleasant Valley mouse was used separately. Agglutination tests were set up in duplicate in serum dilutions from 1/10 through1/2560 using saline at each of the previously adjusted pH values. One set of tests was shaken for 5 minutes and read. At pH values of 6.0 through 7.6 scattered false positives were found to occur in all dilutions. The other set of tests was incubated for 1 hour at 56°C and read and then placed at 4°C overnight and reread. After incubation at 56°C scattered false positives were found in pH values of 6.0 through 7.2 and after ice-box treatment one plus-minus value was found in one 1/10 dilution at pH 7.6.

This experiment indicated that false positives might be expected to occur when the saline employed in the test had a lower pH than 7.6. It also indicated that shaking mouse serum might give false results.

Further tests were then set up using the sera (not pooled) from 11 normal mice and 11 vaccinated mice. The saline used had a pH value of 7.6 and the tests were incubated at 56°C and then placed at 4°C overnight. The normal mice were negative throughout while the vaccinated mice showed positive titers at 1/20 and 1/40. The latter however were not stable until after sitting overnight in the ice-box. Immediately following incubation

Table LXXXIII

Comparison of "UL" Agglutination Antigens

No. of Serum	Antigen	Serum Dilutions 1/20	1/40	1/80	1/160	1/320	1/640	1/1280	1/2560
A	FO 38	-	-	-	-	-	-	-	-
	MF	-	-	-	-	-	-	-	-
B	FO 38	4*	4	4	4	3	1	-	-
	MF	4	-	-	-	-	-	-	-
C	FO 36	4	4	4	4	3	1	-	-
	MF	3	3	3	1	-	-	-	-
D	FO 38	4	4	4	4	2	1	-	-
	MF	4	4	2	-	-	-	-	-
E	FO 38	4	4	4	3	1	-	-	-
	MF	4	3	2	1	-	-	-	-
F	FO 38	4	4	4	4	4	4	2	1
	MF	4	4	4	4	1	-	-	-
G	FO 38	4	4	2	1	-	-	-	-
	MF	3	2	1	-	-	-	-	-
H	FO 38	1	1	-	-	-	-	-	-
	MF	-	-	-	-	-	-	-	-
J	FO 38	4	4	4	4	4	4	1	-
	MF	4	4	4	3	1	-	-	-
K	FO 38	4	4	4	4	2	1	-	-
	MF	4	3	2	1	1	-	-	-
12B	FO 38	4	4	2	1	1	-	-	-
	NIH	4	4	2	1	-	-	-	-
	MF	3	4	1	1	-	-	-	-
	C&S	4	4	3	2	-	-	-	-
27A	FO 38	3	2	1	1	-	-	-	-
	NIH 38	1	1	1	1	-	-	-	-
	MF	3	2	1	1	-	-	-	-
	C&S	3	3	2	1	-	-	-	-
12A	FO 38	4	4	2	1	-	-	-	-
	NIH 38	4	4	1	-	-	-	-	-
	MF	2	1	-	-	-	-	-	-
	C&S	4	4	1	-	-	-	-	-
7A	FO 38	3	3	1	-	-	-	-	-
	NIH 38	3	3	1	-	-	-	-	-
	MF	3	3	1	-	-	-	-	-
	C&S	4	4	3	1	-	-	-	-
23A	FO 38	4	4	4	4	1	1	-	-
	NIH 38	3	4	4	2	1	-	-	-
	MF	3	4	3	2	1	-	-	-
	C&S	4	4	4	4	1	-	-	-

* 4 equal 100% agglutination; 3 equal 75% agglutination; 2 equal 50% agglutination; 1 equal 25% agglutination; - equal no agglutination.

Table LXXIII (cont'd)

Comparison of "UL" Agglutination Antigens

No. of Serum	Antigen	Serum Dilutions 1/20	1/40	1/80	1/160	1/320	1/640	1/1280	1/2560
21A	FO 38	4	3	2	1	-	-	-	-
	NIH 38	4	3	2	1	-	-	-	-
	MF	1	1	-	-	-	-	-	-
	C&S	4	4	2	1	-	-	-	-
1A	FO 38	4	4	4	4	4	2	1	-
	NIH 38	4	4	4	4	3	2	1	-
	MF	2	3	2	1	-	-	-	-
4A	FO 38	4	4	1	-	-	-	-	-
	NIH 38	3	3	2	2	-	-	-	-
	MF	1	1	-	-	-	-	-	-
	C&S	Not tested							
27C	FO 38	2	2	1	-	-	-	-	-
	NIH	1	1	1	-	-	-	-	-
	MF	1	1	-	-	-	-	-	-
	C&S	4	3	3	3	1	-	-	-
28C	FO38	1	1	1	-	-	-	-	-
	NIH 38	2	2	1	-	-	-	-	-
	MF	2	1	-	-	-	-	-	-
	C&S	4	4	3	3	1	-	-	-
29	FO 38	4	4	2	2	1	-	-	-
	NIH 38	4	4	2	1	1	-	-	-
	MF	3	2	1	-	-	-	-	-
	C&S	4	4	3	2	-	-	-	-
30	FO 38	4	4	1	1	1	-	-	-
	NIH 38	4	4	2	1	1	-	-	-
	MF	3	3	1	1	-	-	-	-
	C&S	4	4	1	1	-	-	-	-

Sera # B, C, D, and E are from convalscents; all others are from vaccinated persons.

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Table LXXIV

Final Titers Obtained after Varying Methods of Performing the Agglutination Test on Mouse Sera

Mouse No.	Incubation at 56°C for 1 hr	4°C Overnight Following 56°C Incubation	Incubation at 36°C for 2 hrs.	Incubation at 37°C for 20 hrs.
1 (Neg. Control)	-	-	-	-
2	3 + 1/10	2 + 1/20	1 + 1/32	1 + 1/32
3	3 + 1/10	2 + 1/20	1 + 1/64	1 + 1/128
4	3 + 1/10	1 + 1/20	1 + 1/64	1 + 1/128
5	-	1 + 1/20	1 + 1/32	1 + 1/32
6	-	1 + 1/20	1 + 1/32	1 + 1/32
7	-	1 + 1/20	1 + 1/64	1 + 1/64
8	-	1 + 1/20	1 + 1/64	1 + 1/64
9	1 + 1/20	2 + 1/40	1 + 1/64	1 + 1/128
10	-	1 + 1/20	1 + 1/64	1 + 1/64
11	-	-	-	-
12	-	1 + 1/40	1 + 1/64	1 + 1/64
(B-23) Neg. Controls	-	-	-	-

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at 56°C the agglutination was strongly positive but was dispersed easily when shaken, and disappeared completely when allowed to sit for one hour at room temperature. Following the ice-box treatment the agglutination reappeared and was more stable.

As a result of the inconsistency of the positive findings in this experiment these same sera were retested with incubation at 37°C instead of 56°C and, because of the low titers obtained, with dilutions of 1/2 through 1/512 instead of from 1/10 through 1/2560. The serum from the same series of 11 vaccinated mice and one control when tested in this manner at 37°C showed positive titers as high as 1/64 and 1/128 in 10 cases while 1 was completely negative as was also the negative control. In every positive serum the titer was higher than that obtained by incubation at 56°C. No false positives were observed. See Table LXXIV. In some instances readings were increased by being placed overnight at 4°C. A further series of sera from 17 vaccinated mice and 17 normal mice were tested at 37°C. This time preliminary readings were made at the end of two hours incubation and the tests were then replaced in the 37°C water bath for 18 more hours. All 17 of the sera from the vaccinated mice yielded positive titers after 2 hours while those from the 17 normal mice were negative. After a total of 20 hours all the positive titers had increased in strength, six readings had increased in titer by one dilution and three by two dilutions. After 6 more hours incubation at 37°C there was a noticeable decline in titer indicating that a total of 20 hours incubation gave maximum titers. The sera from normal mice remained negative. These findings have been borne out by subsequent tests carried out on 60 samples of mouse serum.

Table LXXV shows the results obtained on 94 samples of mouse serum by the above method.

Table LXXV

Comparison of Titers Obtained by Reading After 2 Hours Incubation at 37°C with those Obtained by Reading after 20 Hours Incubation on Sera from 94 Mice

No. Negative Tests:	18
No. Tests Showing (1) No increase:	35
(2) Increase of 1 dilution	18
(3) Increase of 2 or more dilutions	23
Increase in Strength of Readings:	100%
Totals: Tests Showing Identical Readings:	53(35 Positive) (18 Negative)
Tests Showing Increase:	41
Total No. Tests Performed:	94

In further experiments agglutination tests were run on 160 vaccinted and challenged mice and consistent results were obtained throughout by means of this method.

These findings suggested that the mouse "UL" agglutinin antibody contains a heat labile fraction which is at least partially destroyed at 56°C and is more stable at 37°C; or it may be interpreted to mean that the whole antigen-antibody complex is unstable at 56°C. This is in keeping with the findings of Heidelberger that heat inactivation may damage antibody in weak human antisera.(92) As has been pointed out mice always yield relatively low titers and consequently agglutination tests on mouse antisera might be expected to be adversely affected by incubation at 56°C.

b. <u>The Agglutination Test for "UL" as Applied to the Sera of Man, Monkey, Rat, and Rabbit</u>

In view of the above findings with mouse serum, the test was investigated in its application to the sera of man, monkeys, rats, and rabbits.

In the case of rats, sera from 24 vaccinated rats were set up in triplicate. One set was shaken for five minutes, read, incubated overnight in the ice-box at 4°C, and reread. The other two were incubated for two hours at 37°C and at 56°C respectively, read, placed at 4°C and reread. The results showed that incubation at 37°C gave lower titers than either shaking or incubation at 56°C even after each of these procedures was followed by treatment overnight in the icebox. Incubation in the icebox was also found to increase the titer over preliminary readings regardless of the procedure followed. Since there was very little difference, if any, between the final titers obtained when the preliminary treatment consisted of either shaking or incubation at 56°C, the shaking method was adopted for future use because of the simplicity and speed of execution.

The results of a similar comparison of methods as applied to human serum are shown in Table LXXVI. A series of 29 sera from non-vaccinated and non-exposed human personnel was tested in triplicate by the same methods. It may be seen that in contrast to the results with rat sera incubation at 56°C gave the poorest results. In 5 instances completely negative results were obtained at 56°C whereas shaking and incubation at 37°C each yielded positive titers. In the majority of instances shaking for 5 minutes gave higher titers than did incubation at either 37° or 56°C. This was again in accord with Heidelberger's findings that heat may partially destroy the antigen-antibody complex. (92,93,94) No false positives were obtained by any of the methods. The shaking method again appeared to be the method of choice.

A comparison was then made of titers obtained by reading immeadiately following shaking for 5 minutes with those made after subsequent overnight storage in the icebox. This was carried out on the serum of man, monkeys, rats, and rabbits and the results may be seen in Table LXXVII.

The occurrence of (1) higher titers, (2) stronger readings, (3) a shift from a final reading of $\pm$ to 1 $+$ and (4) positive findings in some instance where preliminary readings had been negative indicated

Table LXXVI

Comparison of Methods for Performing the Agglutination Test on Human Sera

Serum #	5 Minutes Shaking Reading		37° Incubation 2 hrs Reading		56° Incubation 2 hrs heading	
	Immediate	Over night	Immediate	Overnight	Immediate	O-nite
1*	1/80	1/160	1/160	1/320	1/160	1/160
2	1/20	1/20	1/10	1/10	1/20	1/10
3	1/40	1/80	1/40	1/40	1/40	1/40
4	1/320	1/640	1/640	1/640	1/640	1/320
5	1/640	1/640	1/320	1/640	1/160	1/320
6	1/160	1/320	1/160	1/320	1/160	1/160
7	3/160	1/1280	1/320	1/640	1/320	1/640
8	1/160	1/320	1/160	1/320	2/80	1/160
9	1/80	1/160	1/160	1/160	1/80	1/80
10	1/10	1/20	1/10	1/20	-	1/10
11	1/160	1/320	1/160	2/160	1/40	1/80
12	1/320	1/640	2/320	1/640	1/320	1/320
13	1/20	1/80	1/40	1/40	-	-
14	1/20	1/160	1/40	1/40	1/10	1/10
15	1/320	1/640	1/320	2/160	1/160	1/80
16	1/80	1/160	2/160	1/320	1/80	1/80
17	1/320	1/1280	1/640	1/1280	1/640	1/640
18	3/40	1/80	1/80	1/80	1/40	1/40
19	1/40	1/20	1/20	1/10	-	1/10
20	1/320	1/320	1/320	1/320	1/80	1/160
21	1/320	1/320	1/160	1/160	1/80	1/160
22	1/320	1/640	1/320	1/160	1/320	1/160
23	1/20	1/40	1/20	1/20	1/10	1/20
24	1/20	1/20	1/10	1/10	1/10	1/10
25	2/20	1/40	2/20	1/20	-	-
26	1/20	1/40	1/20	1/10	-	-
27	-	1/10	-	-	-	-
28	1/20	1/40	1/20	1/20	-	-
29	2/40	3/80	1/80	1/160	1/20	1-40
30**	-	-	-	-	-	-
31	-	-	-	-	-	-
32	-	-	-	-	-	-
33	-	-	-	-	-	-
34	-	-	-	-	-	-
35	-	-	-	-	-	-
36	-	-	-	-	-	-

* Sera 1-29 from vaccinated or convalscent human personnel
** Sera 30-36 from non-vaccinated, non-exposed human personnel

that placing the tests overnight in the icebox and reading them following this treatment gave both a more specific and a more sensitive test.

c. Summary

On the basis of the experimental results reported the following recommendations are made for the performance of the agglutination test for "UL".

1. The most satisfactory antigen for routine agglutination tests for "UL" is a formalized saline suspension of washed, whole organisms made from strain NIH-38.

2. The agglutination test with mouse serum will give the most satisfactory results when (a) the pH of the saline employed is adjusted to not lower than 7.6; and (b) the tests are incubated at a temperature of 37°C for a maximum of 20 hours.

3. The agglutination test with the serum of man, monkeys, rats, and rabbits is most satisfactory when the tests are shaken for 5 minutes and incubated overnight in the ice box before final readings are made. It was not necessary to adjust the pH of the saline with these sera.

L. Study of Strain Differences by Serological Methods

The object of this experiment was to determine whether there were antigenic differences in strains of "UL" from various sources and in strains of varying virulence.

Previous work by Downs[95] and Foshay[96] had shown that agglutinin absorption tests did not reveal any strain differences. Nevertheless a part of this work was repeated using a different type of antigen for the production of serum. An attempt was made to determine whether there was any correlation between antigenic differences as revealed by the agglutinin absorption test and the virulence of the strains in terms of mouse LD_{50}. The "US" titers were also determined for the various serums used. In a few experiments high titered human serum from recovered cases was used on the theory that such serum might contain antibodies developed by contact with whole living antigen which would not be present in the rabbit serum prepared by the injection of killed antigens.

Methods

Rabbit antiserum was prepared by the injection of acetone extracted vaccines intravenously in 3 doses, 0.5 ml per dose every other day. The absorbing antigens were heavy suspensions (T2500 turbidity) of acid killed cultures, washed and resuspended in 0.1% formalized saline. The agglutinating antigens were the same but were diluted to T500 turbidity.

After preliminary titration with the antigens to be used for absorption, the absorption test was set up with the serum diluted to a point 3

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Table LXXVII

Comparison of Titers Obtained by Reading Immediately Following Shaking for 5 Minutes with those Made after Subsequent Overnight Storage in Ice Box at 4° C

Number Tests Showing:	Human Serum	Monkey Serum	Rat Serum	Rabbit Serum
No Increase in Titer	38	19	28	3
Increase by 1 Dilution	80	22	45	2
Increase by 2 Dilutions	10	9	28	1
Increase by 3 Dilutions	2	5	12	0
Negative Test Positive after Ice Box	1	4	2	0
Plus-minus in Highest Dilution changed to one plus	15	3	1	1
Decrease in Titer	1	3	0	0
Total Tests:	147	62	96	7
Totals:				
Tests giving Identical Readings	38	19	28	3
Tests showing Change Indicated Above	109	43	68	4

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to four dilutions below the final definite positive agglutination, as for instance a serum giving 2 plus agglutination at a dilution of 1:5120 was diluted to 1:320. This diluted serum was added to an equal volume of heavy suspension of antigen allowed to stand in the ice-box overnight and the supernatant separated by centrifugation. When the supernatant from such an absorbed serum was set up against the antigen for agglutination tests it would have a final dilution of 1:1280 and should give a good agglutination if absorption were incomplete. The sources of the organisms used and the mouse LD_{50} for each strain are listed in Table LXXVIII.

Results

Preliminary agglutination tests did not reveal any strain differences which were consistent. In other words antiserum prepared by the injection of the low virulent Jap strain agglutinated all the other strains used to titer. Conversely a serum prepared by the injection of the virulent SM culture agglutin ted all other strain equally. No significant heterophile titers were obtained either before or after immunization.

Absorption tests using eight antiserums absorbed by seven antigens and set up after absorption against the same seven antigens showed no strain differences. Absorption of agglutinins was complete in every case, whereas control sera diluted an equal amount but not absorbed, agglutinated the respective organisms to titer showing that the lack of agglutination was not caused by a drop in titer from standing overnight in a diluted state.

Agglutination tests using a small series of high titered human and rabbit serums were done to see how much cross agglutination with "US" was present. Table LXXIX shows the results of these tests.

Ten out of twelve human sera contained a significant "US" titer. Eighteen out of eighteen sera from rabbits immunized with eleven different strains of "UL" all developed agglutinins for "US". In summarizing, no antigenic strain differences were demonstrated by the agglutinin absorption tenhnic using sera and antigens prepared from 8 strains of varying virulence isolated in various regions of the world. Eighteen different strains used as antigens in rabbits all stimulated production of "US" agglutinins.

IV-DISCUSSION

The investigations reported herein were undertaken to obtain supplementary information about "UL" which might help to determine its status as a potential BW agent. In the following paragraphs the suitability of "UL" as a BW agent is reviewed taking into account the data which has been accumulated at Camp Detrick, at the University of Kansas and at Cincinnati.

A. Production

Mass production of "UL" is possible on a large laboratory scale in Snyder's peptone broth medium, Foshay's gelatin hydrolysate medium or in embryonated eggs. Yields of 5×10^9 or more can be obtained with ease in all

Table LXXVIII

Source of Strains Used for Production of Agglutinating Serum and as Absorbing Antigens

Strain	Source	Mouse LD_{50}	Antigen Used for Preparation of Antiserum
Ince	Human Downs-Nov. 1944	10^{-9}+	Acetone Extracted
SM	Foshay	10^{-9}+	" "
Camp	"	10^{-9}+	" "
Dieck	"	10^{-9}+	" "
Pier	Human-1938	10^{-1} (Deaths irregular)	" "
26	Human-1921 Utah	10^{-1}+	" "
HD	Human Eye 1935-Austria	10^{-4}+	" "
Max	Russian-1938	10^{-1}+	" "
Russ	Russian-1928	10^{-2}	" "
Jap	Human-1926 Japanese	$10^{-6.5}$	" "
Scherm	Foshay-1944	10^{-9}+	" "
38	Francis	No Virulence	" "
Ohara	Japan-1931	10^{-1}+	" "

Table LXXIX

"US" and "UL" Titers Antisera Obtained from Vaccinated Rabbits or from Convalscent or Vaccinated Personnel

Human Antiserum	"UL"	"US"	Rabbit Antiserum #Antigen Used ≠	"UL"	"US"
1	1280	80	1° Jap	5120	160
2	5120	20	2°° Jap	2560	20
3	640	40	1 Ince	2560	160
4	5120	10	2 Ince	5120	320
5	640	-	1 Hd	2560	40
6	2560	-	2 HD	2560	10
7	640	160	1 Russ	1280	160
8	2560	80	2 Russ	2560	40
9	1280	40	1 Max	2560	80
10	5120	20	1 26	1280	80
11	1280	10	2 26	1280	80
12	2560	20	1 Fier	10,240	320
			2 Fier	2560	160
			1 Camp	5120	160
			1 Dieek	10,240	160
			1 Sch[illegible]	5120	320
			2 Sch[illegible]	320	160
			1 S[illegible]	[illegible]120	160

o #1 Sera from 4 rabbits pooled 9 days after vaccination.
oo #2 Sera from 4 rabbits pooled [illegible] days after vaccination.
≠ Strain used for preparation of acetone [illegible] antigen used to immunize rabbits.

of these culture media. The choice of media for large scale production depends somewhat on how the finished material is to be used. Most work has been done with the simple peptone medium and details of preparation and optimum conditions for growth have been defined by the A Division workers. This medium as presently defined (Final Report, A Division) could be easily adapted to pilot plant production of "UL". The requirements of aeration, size of inoculum, and incubation temperature are all easily obtainable with present pilot plant facilities. As pointed out by Lt. Snyder[16] a cheaper source of nitrogen than Bacto peptone is desirable. Separation and concentration of organisms from peptone broth has been accomplished on a large laboratory scale by the A Division workers[16] and needs only to be adapted to pilot plant equipment. Seventy to one concentration of the organisms in broth has been accomplished[16].

Equally good growth has been obtained in Foshay's gelatin hydrolysate medium but it is not recommended for large scale plant use because procurement of sufficient gelatin and red blood cell extract might prove to be difficult and expensive. Foshay has recently substituted an acid hydrolysate of soy bean meal for the gelatin hydrolysate. He reports that growth in this new medium is as good or better than in the gelatin medium. The saving in expense would be considerable and it is understood that the supply of soy bean meal is ample for production requirements. Another objection to the use of the Foshay medium was raised on purely theoretical grounds by the Safety Division and by the plant engineers at Camp Detrick. The medium contains 0.5 per cent glycerine and it was postulated that it might cause seepage through fittings, valves, and connections in the reactor tanks thus exposing plant personnel to constant danger of infection. It is not known whether this problem was ever resolved or whether the plant personnel carried out any experimental studies. One additional advantage of the peptone medium was that of "UL" would grow in this medium in the presence of tank steel[14] whereas Foshay's medium in tank steel developed a heavy sludge and growth of "UL" was somewhat retarded.

"UL" yields in whole minced embryonated eggs make this medium a definite possibility for mass production. The embryos should be alive at the time of inoculation but death of the embryos from trauma at the time of injection does not diminish multiplication of "UL". Yolk sac inoculation gives maximum yields. These factors would be advantageous in large scale production since yolk sac injection is perhaps the simplest method of inoculating eggs and if the embryos were accidentally pierced and killed during injection no diminution of "UL" growth would ensue. Growth of 15 "UL" strains in embryonated eggs has been studied [illegible] optimal size of inoculum, age of embryos, incubation period, time of harvesting, and conditions of storage have been determined. If a suitable [illegible] were available sufficient laboratory data is now on hand to permit pilot [illegible] production of "UL" in eggs.

One of the primary aims of the B Division [illegible] on media was the development of a technic and medium by means of which [illegible] of viable organisms could be estimated quantitatively. This has been accomplished by means of surface streaked plates of dextrose cystein [illegible] agar (DCBA) using

serial dilutions of the bacterial suspension to be counted. Colony counts were made after incubation and the number of organisms per ml of the original suspension were calculated. This method has been extensively employed in all phases of the studies at Camp Detrick and at Kansas and has added much to the volume and accuracy of the work. The results of many titrations have shown that one organism as determined by DCBA plate count is equivalent to one infections unit as determined by a mouse MLD titration. Lt. Snyder of the A Division has applied this streaking technic and shown that plate counts can also be made on a Bacto peptone agar containing 0.1 per cent cysteine without blood[14]. This medium has been used satisfactorily for colony counts in most of the A Division "UL" studies. A comparative statistical analysis of the two plate count media wa carried out by Lt. Snyder [16] and the following tentative conclusions were made. The peptone agar was simpler to prepare and could be used immediately after preparation. However plates could not be stored and used a later time. DCBA was more complicated to prepare and required incubation overnight before use. DCBA could be stored in the icebox for a least two weeks without loss of growth promoting properties. DCBA was slightly more accurate and gave slightly higher counts than the peptone agar plates although these differences were not great. We have observed that colony growth is more rapid on the DCBA plates.

Determination of "UL" growth curves in Snyder's peptone broth by means of DCBA plate counts of viable cells, mouse LD_{50} titrations of infectious units, and turbidity measurements on the Coleman Spectrophotometer showed a very close correlation. In terms of plant production this means that the stage of growth in the reactor tanks and the time for harvest could be determined quickly and with relative safety by means of simple turbidimetric measurements.

In conclusion it should be stated that repeated serial passage in Snyder's peptone broth, Foshay's gelatin hydrolysate medium, or embryonated eggs did not diminish the virulence of "UL".

B. Infectivity

Titration of the LD_{50} by various routes in nine species of laboratory animals has reemphasized the high infectivity of "UL". Infection was easily accomplished by every route of administration. With the more susceptible species one virulent organism introduced parenterally was sufficient to cause death whereas by percutaneous, intranasal, conjunctival, intravaginal, or respiratory challenge in the cloud chamber a slightly greater inoculum was necessary. If we assume that the more resistant rat approaches man in degree of susceptibility to "UL" infection the routes of challenge in order of efficiency would be as follows: intraperitoneal, respiratory, subcutaneous, intracutaneous, intranasal, and precutaneous. In other words it is anticipated that respiratory challenge might be very effective in man. Two cases of primary "UL" pneumonic infection occurred in laboratory personnel and two reinfections followed accidental breaking of a tube of lyophilized "UL". In these latter two cases infection was presumably by the respiratory route. Foshay (per com) reported a similar accident in his laboratory which was fol-

lowed by "UL" infection. In other human infections at Camp Detrick the portal of entry was probably the conjunctiva, the mucus membrane of the mouth, and the grossly unbroken skin.

Comparative cloud chamber studies of all agents studied at Camp Detrick have shown (Rosebury final report) that "UL" compares favorably with the other agents on the basis of infectivity.

In summary, when all portals of entry are considered, "UL" is probably the most infective agent studied at Camp Detrick. Exact experimental data on the minimum infective dose for man by various portals of entry is not available but using information from human cases, and judging from animal data, conclusions may be drawn which are probably somewhat better than pure opinion. On the basis of practical importance from a BW standpoint the portals of entry of most significance in man are probably (1) respiratory, (2) intracutaneous, by means of scratches and abrasions, (3) conjunctival and intranasal, and (4) percutaneous, via the apparently unbroken skin.

C. <u>Storage</u>

All planning and research for offensive BW at Camp Detrick has been carried on under the assumption that to be effective BW agents must be used in bombs, rockets, artillery shells, or some other form of munition. If used in this way, basic problems of logistics demand that the agent be capable of surviving storage for a period of weeks or months while it is being transported and concentrated at forward areas. No minimum standards of stability have been established but it has been generally assumed that to be practical a BW agent must be capable of storage in the final container for at least two to three months. It should be remembered in this connection that recent and future advances in the use of long range rockets may make it possible to use BW munitions against distant objectives within a period of two or three days after preparation. In a personal communication Lord Stamp recommended that a bomb fill must contain at least 1 x 10^{10} viable organisms per ml at the time of use. This recommendation was based on the results of field trials with agent "US" in the British 4 pound bomb.

The use of BW agents by saboteurs against key personnel or installations has been largely disregarded in British and American planning. Various nebulous intelligence reports have implied that this method has been given serious consideration by the Japanese. If used in this way it is entirely possible that BW agents could be produced on a small scale behind enemy lines and used effectively without a prolonged period of storage. Downs (28) working at Kansas has shown that "UL" will remain infective when stored in cold water for several months. Guinea pigs were infected by eating carrots which had been stored in contaminated soil for 20 days.

"UL" does not survive storage sufficiently well to meet present munition requirements. Greatest stability was observed on storage at low temperature in the moist state. Whole infected eggs stored at -40° C

contained 2 x 10^9 infectious units per ml after 35 days or 33 per cent survival. The infectious units per ml were 7 x 10^7 after 56 days storage and 1 x 10^6 after 23 weeks. Survival after lyophilization was much poorer than when stored wet. Simultaneous colony counts and mouse titrations have shown that virulence is maintained during storage. In fact virulence of "UL" cultures has been remarkably constant under all conditions of handling.

The A division workers have carried out extensive storage experiments using organisms grown in Snyder's peptone broth. Surival of moist cultures was similar to that observed with infected egg material; however, by using concentrated sediments with an initial count of 1 x 10^{12} per ml survival after 2 months was above the minimum requirements recommended by Lord Stamp. Survival after lyophilization has been increased from 0.0002 per cent to roughly 2.0 per cent by the A Division workers. On the basis of 2.0 per cent survival, lyophilization of broth cultures also becomes a practical method of meeting the minimal requirements for storage and potency of the pay load. It is anticipated that if experimental work is permitted to continue, further improvement in storage and stability of "UL" may be expected.

Foshay first called attention to the correlation between the morphological structure of "UL" and ability to survive the lyophilization process. Although we have not been able to attack this problem as such, a morphological study of "UL" under the electron microscope has shown that the majority of "UL" morphological forms have no rigid cell wall of the type described for most bacterial species by Womascher.[79] Organisms rupture very easily and the cytoplasm of individual organisms has a very low electron density. Chromatin material is not distributed throughout the cell but is frequently concentrated in several masses with clear water-like cytoplasm between. It is postulated that the absence of a cell wall and the delicate cellular organization accounts in part for the lability of "UL" when dried, frozen, or subjected to mechanical strain which occurs in cloud chamber nebulization. Future improvement of stability might be accomplished by selective cultivation of the bacillary morphological variants which do appear to have a delicate but definite limiting cell membrane. This subject is entirely unexplored at the present time.

D. Dispersion

1. Cloud Chamber Studies

Of all BW agents studied at Camp Detrick "UL" gave the poorest percentage recoveries when nebulized and subsequently recovered by impinger sampling of the nebulized cloud. Five hundred to 1000 fold improvement in percentage recovery was later obtained by the A Division workers[16] and cloud chamber C workers during the course of their studies. These improvements were brought about by modifications in the conditions under which the organisms were grown, the addition of glycerine to the suspending medium and by changes in the operation of the cloud chamber. Recoveries averaged around 2 to 3 per cent under the best conditions.

Because of the high respiratory infectivity of "UL", recovery on the basis of mouse infectious units per unit of cloud compared very favorably with other agents which on the basis of percentage recovery alone appeared to be much better than "UL". The discrepancy between the number of organisms necessary to cause respiratory infection as determined in chamber A[1], chamber B[11], and chamber C[70] are discussed by Rosebury.[97] In addition to the explanations hereby put forward it is our opinion that another factor is of prime significance. The total lapse of time between nebulization of the cloud and contact with the respiratory membrane or impinger in the three chambers was as follows: chamber A, 10 to 30 seconds; chamber B, 3 seconds and chamber C 10 minutes. Dessication appears to be one of the chief causes of the poor survival of "UL" when nebulized. It seems possible that more organisms would be damaged, to the point where they would not infect animals but still be capable of growing on artifical medium, when nebulized in Chamber C where they would be exposed to drying for 10 minutes before sampling. If true this explanation is but additional evidence of the lability of "UL".

Certainly the danger of retroactivity against our own troops would be minimal with this agent which is not viable in the cloud for more than a few hours.

Additional cloud chamber experiments carried out during the close-out period are not reported in the final reports of the cloud chamber or A division groups. Recoveries of 8 to 25% have been obtained in these tests and the results have been repeated and confirmed with surprising regularity in sharp contrast to the previous experience. The mechanism of this remarkable improvement in stability is being investigated insofar as time and the retention of trained personnel will permit. At present it appears that at least two factors are of importance. First, the strain of UL and/or rapid serial passage in peptone broth at 26°C, and secondly, the operation of the cloud chamber at low temperatures. Under the best conditions 25% recovery appeared to be attainable. This figure is as good as or better than the percentage recovery obtained with other vegetative agents or simulants. If animal infectivity of the clouds is equal to that previously observed then "UL" becomes the most effective agent thus far studied in the cloud chamber. If work is permitted to continue further improvements will very probably be made.

5

2. Field Trials

Field trails in the British four pound bomb with "UL" concentrates prepared by the A Division workers were scheduled to be carried out in September, 1945 thru arrangements made by Lord Stamp with the Canadian BW group. This project was cancelled at the last minute because of the end of hostilities in Japan. Some valuable experience was obtained, however through preparation of the pay loads and comparative studies of cloud sampling methods to be used in the proposed trials. It is impossible to predict what the results of field trials might have been, however laboratory studies have progressed to the point where such trials are possible, and probably indicated as a guide for future offensive studies.

6

E. Biological Protection

The ideal method of protecting friendly troops or civilians against BW attack, with a specific infectious disease agent, is by means of prophylactic vaccination. Vaccination could be carried out in peace time, or in rear areas during mobilization. An efficient vaccine, properly administered, should practically eliminate the agent as a weapon in the hands of the enemy. Furthermore, if the enemy did not have an equally good vaccine, he would be vulnerable to attack, and our own troops could prepare and handle the munition with relative safety.

At the beginning of BW studies at Camp Detrick in November, 1943, a vaccine which would protect laboratory animals against "UL" infection was not available. All authorities on the subject were agreed on this point. Foshay had developed a vaccine (5) for use in man, and a later improved preparation[30], both of which showed some promise in human prophylaxis. Neither of these preparations had been shown to protect the rabbit, guinea pig or mouse, so controlled experimental proof of their efficiency was lacking. Upon recovery from infection, man has a solid immunity, therefore, we were encouraged to study the problem further.

The approach at Camp Detrick was two-fold. First, a survey of all laboratory animals was made in an effort to find one which could be immunized. Secondly, vaccines were prepared from "UL" grown in embryonated chicken eggs on the theory that organisms grown in the presence of living tissue might produce a more complete antigen than cultures grown on the usual <u>in vitro</u> media.

White rats, dogs and chickens all proved to have considerable natural resistance and following recovery from a sublethal infection they were resistant to rechallenge with virulent organisms. Rats were deemed the most suitable animal for our purpose and it was demonstrated that they could be readily protected by vaccination. Because of the individual variation in susceptibility it was necessary to use large numbers of rats on each experiment. Larson[101] apparently working independently, obtained similar results with rats and published his findings a year after the first Camp Detrick monthly reports. It is of interest that Foshay's latest vaccine[30] also protected the rat. This has been confirmed by Dr. Foshay.

Later studies demonstrated that the mouse could also be protected against a small challenge. This animal may offer a more delicate method of comparing vaccines than the rat. Future studies should include more extensive experimentation with this animal.

Several types of vaccines proved to be effective in the rat and the mouse. Preparations from cultures grown in embryonated eggs were not significantly superior to those prepared from DCPA or Peptone Broth cultures[14]. For this reason and for convenience in preparation of materials, peptone broth cultures were employed for further vaccine preparation and purification.

Analysis of the number of exposed personnel and the number of cases at Camp Detrick indicated that Foshay's vaccine also protected man. Protection was not absolute, but the incidence of infection in a large group of laboratory workers was 31.9 per cent, and the severity of the disease in the 24 cases was markedly reduced. Infection of unvaccinated laboratory workers in the past has been 90 to 100 per cent[3,47,50-55]. For human use we recommend the purified acetone extracted vaccine because it protected rats as well as Foshay's vaccine, and gave rise to fewer local and systemic reactions in the vaccinated personnel[98].

By means of a prevaccination skin test developed at Camp Detrick, the number of severe reactions to vaccinations were practically eliminated. This reduced one of the chief objections to human "UL" vaccination.

Preliminary fractionation of the "UL" antigens has been accomplished and if work is permitted to continue this new information can be further developed and applied to human vaccination with the hope of further reduction in the number of vaccine reactions and improvement in the degree of immunity conferred.

F. <u>Specific Therapy</u>

Streptomycin has been shown to be effective in treating established "UL" infection in experimental animals[89]. It is non-toxic in therapeutic levels. This antibiotic is effective in treating clinical "UL" infection in man as shown by Foshay (per com), and by the Camp Detrick station hospital staff. The drug is effective in man at dosages less than one-seventh of the amount which has been safely tried in other human diseases. Streptomycin appears to be a true specific for "UL" infection. Marked symptomatic and clinical improvement has been observed within 12 hours after the beginning of treatment.

G. <u>Detection</u>

With the development of effective vaccines to prevent infection and a specific antibiotic to treat cases the importance of emergency physical protective measures in the field become less important. However, if BW is used against our troops all known protective measures should be employed, especially since "UL" vaccination at present does not confer a solid immunity. Much good work on development of physical and chemical protective measures has been carried out at Camp Detrick[100]. Examples are protective clothing, masks, and ointments. If these measures are to be used most effectively it is imperative that methods of quickly recognizing a BW attack be available. Subsequently it would be of value to identify the specific BW agent being used as rapidly as possible so that prophylactic or chemotherapeutic measures could be instituted before cases of infection developed. This latter problem has been studied in the "UL" laboratories and the results obtained have been confirmed and extended by the Safety Division and the Detection project. At the beginning of this work four to ten days were required for identification of "UL" in unknown samples. By

application of many new technics developed hre, "UL" has been successfully isolated and identified from contaminated simulated field specimens in 24 to 36 hours. Since the average incubation period of "UL" is 3 or 4 days it should be possible to identify the organism and institute specific chemotherapy before the occurrence of widespread morbidity following exposure.

V SUMMARY

A. Production

Mass production is feasible on a large laboratory scale and no more than the ordinary difficulties should be experienced in converting to plant scale operation.

B. Infectivity

"UL" is almost unique among disease organisms in its ability to infect both man and animals via multiple portals of entry. Virulence is maintained without difficulty.

C. Storage

"UL" does not meet present standards for storage required of a potential BW agent. The conditions of storage necessary for survival have only been partially explored and additional investigation would probably lead to further improvement in stability.

D. Dispersion

The percentage recovery of "UL" from nebulized clouds is equal to or better than that obtained with other vegetative BW agents or simulants. Further improvement is probable.

E. Biological Protection

Effective vaccines are available. Protection is not absolute but the incidence and severity of infection in vaccinated personnel are markedly reduced. Precautionary measures have been defined, by means of which untoward hypersensitive reactions to vaccination may be largely avoided.

F. Specific Therapy

A safe, effective, specific, chemotherapeutic agent, streptomycin, is available for treatment of "UL" infection.

G. Detection

Rapid methods of detecting and identifying "UL" in unknown contaminated specimens have been defined.

H. General

With the single exception of stability for prolonged periods of storage "UL" meets the requirements of an ideal BW agent. Past experience indicates that with additional study stability may be improved.

VI APPENDIX

A. Project Specifications

1. Project number and title:

   CD-DB-3, Immunization against Tularemia

2. Date: 1 October 1943

3. Basis of Authority: Directive, Chief, Chemical Warfare Service

4. Project Specification Superseded; None.

5. Object: To develop a means or method for immunization of man against tularemia.

6. Previous Work Done: It is not possible by any method and/ or means now available to immunize any animal against tularemia. Some claims have been made by various workers as to immunization of man, but these claims have been questioned by some observers as resting upon rather weak ground. It is known that man is extremely susceptible to tularemia.

7. Governing Principles:

   a. Use of Item: The primary use of this item would be in the the protection of workers engaged in the development of BW work. The eventual use would depend upon the success of the developmental phases.

   b. Defects in existing Similar Items: ~~No method is~~ now available available for the immunization of animals, ~~and~~ the methods advocated for immunization of man have frequently been criticized as resting upon clinical rather than scientific grounds. It is a general opinion that no completely adequate method for immunization of man against tularemia now exists.

   c. Performance Characteristics: Any method and/or means of immunization of man against tularemia must give a degree of immunity reckoned as completely protective against exposure to the disease if it is encountered in BW, and must be of such nature as not to prejudice the life or health of the individual being immunized.

d. Technical Characteristics:

(1) The method or means of immunization must confer a high degree of immunity.

(2) The method or means of immunization must not prejudice the life or health of the individual, nor may it be of such nature as to affect the normal activity of the individual.

(3) It is desirable that the method and/or means of immunization be of such nature that immunity will be acquired after one or two injections, and that this immunity will develop very rapidly into its full strength.

(4) It is desirable that any vaccine be of such quality as to permit storage for a period as long as one year without impairment of its potential efficiency.

(5) It must be established absolutely that the vaccination does not render the individual more susceptible rather than more resistant.

(6) No vaccine will be admitted as adequate which fails to immunize animals.

(7) No vaccine will be admitted as adequate which cannot safely be administered to animals.

8. Procedure: Considering the amount of work which has already been done on this subject and considering the lack of success which has attended these endeavors, it is impossible to predict that any procedure now envisaged could be regarded as offering success. In general, the approach to this problem involves taking advantage of every available idea and pursuing that idea until it is proven erroneous. All developmental attempts must be carried out with Lower animals before any attempt is made to immunize man. The following lines of experimentation will be followed in the initial development of this subject.

(1) The study of the antigenic behavior of Pasturella tularensis as grown in embryonic tissue.

(2) The determination of whether species vaccines can be developed by growth of the organisms in the serum of the species to be immunized.

(3) The study of the antigenic pattern of the organism to determine whether some fraction might be more effective than the whole organism.

9. Cooperation: None

10. Estimated time: No estimate

11. Estimated Cost:

12. Addenda: It is obvious that the solution of this problem might have a profound significance in the field of public health. As the years pass there seems to be a decided increase in the number of cases of tularemia, particularly among hunters, butchers, and laboratory workers.

SECRET

VI Appendix

B. PERSONNEL AND SPACE ASSIGNED TO THE "UL" PROJECT

Date	Space No. Rooms	"B" Division			"A" Division		
		Officers	Technicians	Service	Officers	Technicians	Service
Nov. '43	1	3	2	0			
Dec. '43	1	3	3	C			
Jan. '44	1	5	3	1	1	1	0
Feb. '44	1	5	4	2	1	1	0
Mar. '44	3	7	4	3	1	1	0
May '44	3	7	5	3	1	1	0
June '44	4	6	6	3	1	1	0
July '44	4	6	6	3	1	1	0
Aug. '44	5	7	6	3	1	1	0
Sept. '44	5	7	9	3	1	2	0
Oct. '44	9	7	9	3	5	3	0
From Oct. to Nov. Dr. Henderson and group gave part time to "UL" studies.*							
Nov. '44	9	8	9	3	5	3	0
Dec. '44	10	10	10	4	2	3	0
Moved to buildings D-25 and L-26							
Jan. '45	22	11	12	5	3	5	5
Feb. '45	22	10	16	4	3	5	5
Mar. '45	22	9	16	4	3	6	6
From Mar. to Sept. Dr. Rosebury and group gave part time to "UL" studies.*							
April '45 **	22	9	15	4	4	7	5
May '45	22	9	15	4	4	5	4
June '45	22	9	13	4	4	5	3
July '45	14	8	12	4	4	5	2
Aug. '45	14	8	12	4	4	5	2
Sept. '45	14	8	9	3	4	4	2

*Cloud chamber studies.
**Capt. Tatlock and group spent two weeks on protective ointment studies.

"B" DIVISION

Officers	Rank	Date Joining	Date Leaving
Coriell, Lewis L.	Capt.	Nov. '43	Oct. '45
Pinchot, Gifford B.	Lt(jg)	Jan. '44	Oct. '45
Owen, Barbara J.	Lt(jg)	Jan. '44	Oct. '45
Klauber, Alice H.	Lt(jg)	Mar. '44	Feb. '45
Chapman, S.S.	Lt(jg)	Mar. '44	July '45
Eigelsbach, Henry T.	Ens.	Aug. '44	Oct. '45
Smith, Joanne R.	Lt(jg)	Nov. '44	Oct. '45
Steinglass, Paul	1st Lt.	Dec. '44	Jan. '45
Hayes, Thomas H.	1st Lt.	Dec. '44	Mar. '45
Wachtel, Lewis W.	Lt(jg)	Jan. '45	Oct. '45
Schadwald, Melvin A.	Lt(jg)	Jan. '45	Mar. '45
Clapp, Mary R.	2nd Lt.	Mar. '45	Oct. '45
King, Elizabeth O.	1st Lt.	April'45	Oct. '45

Technicians	Grade	Date Joining	Date Leaving
Doiron, Ruth T.	T/4	Nov. '43	Oct. '45
Plitt, Karl F.	T/Sgt.	Nov. '43	Oct. '45
Kowal, Stephen F.	M/Sgt.	Dec. '43	Oct. '45
Butler, Margaret K.	PhM3/C	Feb. '44	Oct. '45
Zarraga, Lupe	PhM1/C	May '44	Sept.'45
Carey, Rebecca M.	PhM3/C	June '44	June '45
Chapman, Eleanor G.	PhM3/C	Sept.'44	Oct. '45
Etter, Mary E.	PhM3/C	Sept.'44	July '45
Smith, Claire G.	PhM3/C	Sept.'44	April'45
Clifton, June W.	PhM1/C	Sept.'44	June '45
Nelson, William E.	PhM1/C	Dec. '44	Sept.'45
Nardi, Francis A.	PhM2/C	Jan. '45	Sept.'45
Sable, Ernest M.	PhM2/C	Jan. '45	Oct. '45
O'Dellick, Joseph A.	S/2C	Feb. '45	Sept.'45
Devine, Alice L.	Pvt.	Feb. '45	April'45
Jastrebzski, Leonard T.	H.A.1/C	Feb. '45	Oct. '45
Farley, Douglas M.	PhM3/C	Feb. '45	Oct. '45
Rogers, Mary L.	T/5	April'45	Oct. '45

Civilians			
Downs, Cora M.	Dr.	Nov. '43	Oct. '45
Smadel, Elizabeth M.	Dr.	Nov. '43	June '44

"B" DIVISION CON'T

Service Animal	Grade	Date Joining	Date Leaving
Egan, John F.	Pfc.	Feb. '44	July '44
O'Connor, Frank	Pvt.	March '44	July '44
Craig, Charles J.	Pfc.	July '44	Oct. '45
Mason, W. C.	T/5	July '44	Jan. '45
Boele, Henry J.	S1/C	Jan. '45	Oct. '45
Henson, Kenneth C.	T/5	Jan. '45	Feb. '45
Service Room			
Neusse, Margaret L.	S1/C	Dec. '44	Sept. '45
Pyne, Marilyn A.	PhM2/C	Jan. '44	April '45
Rines, Evelyn L.	PhM3/C	April '45	July '45
Etter, Mary E.	PhM3/C	July '45	Oct. '45

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"A" DIVISION

Officers	Rank	Date Joining	Date Leaving
Snyder, Thomas L.	1st Lt.	Jan. '44	Oct. '45
Klein, Robert E.	Ens.	Oct. '44	Oct. '45
Gerhardt, Phillip	1st Lt.	Oct. '44	Nov. '44
Mizera, J. W.	1st Lt.	Oct. '44	Nov. '44
Spencer, G. R.	Ens.	Oct. '44	Nov. '44
Engley, Frank B.	2nd Lt.	Jan. '45	Oct. '45
O'Kane, Daniel J. Jr.	Major	April'45	Oct. '45

Technicians	Grade	Date Joining	Date Leaving
Penfield, Ruth A.	T/4	Jan. '44	Oct. '45
Waits, W. M. Jr.	PhM2/C	Sept.'44	Sept.'45
Lefkowitz, Harry	T/5	Oct. '44	Oct. '45
Gennert, William	PhM2/C	Jan. '45	Oct. '45
Bennett, Jackson	T/5	Jan. '45	July '45
Pascal, Rinaldo	S2/C	Mar. '45	May '45
Kaleta, Gerald J.	PhM3/C	April'45	May '45
Creasy, John	PhM3/C	July '45	Oct. '45

Service	Grade	Date Joining	Date Leaving
Gaspari, George	S1/C	Jan. '45	April'45
Marhefka, Anthony	Pfc.	Jan. '45	Oct. '45
Morris, Howard	Pvt.	Jan. '45	Oct. '45
Schnell, Ralph	S1/C	Jan. '45	July '45
Quill, Thomas E.	S1/C	Jan. '45	June '45
Zarchy, Joseph	PhM2/C	Mar. '45	May '45

"B" DIVISION CLOUD CHAMBER STUDIES

Officers	Rank	Date Joining	Date Leaving
Johnson, Newell	Capt.	Oct. '44	Nov. '44
Boldt, Martin	1st Lt.	Mar. '45	Oct. '45
Aaron, Jerome	1st Lt.	Mar. '45	Oct. '45
Cournoyer, Norman	1st Lt.	April'45	Oct. '45
Rosenwald, Arnold	Capt.	Aug. '45	Oct. '45

Civilians		Date Joining	Date Leaving
Henderson, David	Dr.	Oct. '44	Nov. '44
Rosebury, Theodore	Dr.	Mar. '45	Oct. '45
Olson, Frank	Dr.	April'45	Oct. '45

Technicians	Grade	Date Joining	Date Leaving
Sherry, Edward S.	PhM1/C	Oct. '44	Nov. '44
Cassell, R. E.	PhM2/C	Oct. '44	Nov. '44
Abrams, Harold	PhM1/C	Oct. '44	Nov. '44
Virgil, William	PhM2/C	Mar. '45	Oct. '45
O'Bryon, Charles E.	Pvt.	Mar. '45	Oct. '45
Franzen, Carl	PhM2/C	Mar. '45	Oct. '45
Bicletto, Allan	S1/C	Mar. '45	Oct. '45
Neff, Everett L. Jr.	Pfc.	April'45	Oct. '45
Palmer, Wm. F.	PhM3/C	April'45	Oct. '45
Kress, Helmut	Pfc.	April'45	Oct. '45
Gupton, Russell C.	Pvt.	May '45	Oct. '45
Drimmer, Gideon	Pvt.	Sept.'45	Oct. '45
Moses, Saul	Pvt.	Sept.'45	Oct. '45

Service Animal	Grade	Date Joining	Date Leaving
Carr, Robert	S1/C	Mar. '45	Oct. '45
Veto, Alexander	S2/C	Mar. '45	Oct. '45
Glucksnis, Albert	Pvt.	June '45	Aug. '45

"PC" DIVISION

Officers	Rank	Date Joining	Date Leaving
Tatlock, Hugh	Capt.	May '45	June '45
Swanson, Beatrice	Lt(jg)	May '45	July '45

Technicians	Grade	Date Joining	Date Leaving
Arey, Barbara	PhM2/C	May '45	June '45
Tripp, Leonard M.	H.A.1/C	May '45	June '45

C. Preparation of Vaccine

Vaccine #1 Formalized 38 NIH, S, and C.

72 hour growth washed from 7 slants of DCBA with 10 ml normal saline. Shaken with glass beads and formalin added q.s. to make 0.25%. Incubated overnight at 37° C and diluted to T500 turbidity with normal saline. Tested for sterility by planting 5.0 ml in 100 ml plain broth, 0.1 ml on DCBA plate and slant. Turbidity, approximately 100 ppm. Final formaldehyde concentration 0.1%.

Vaccine #2 OF038YS I.

9 day embryonated eggs were given 0.2 ml YS injection of 10^{-1} dilution of T500 suspension of strain OF038. Yolk sacs, membranes, and fluid were harvested separately when embryos were dead, usually at 72 hours. They were shaken with glass beads to emulsify the tissue. DCBA plate counts were made and yolk sacs, membranes, and fluid were each made into vaccines by the method described below for yolk sacs. Yolk sacs were frozen overnight, thawed, and 1 volume of 0.85% saline containing 0.5% formalin was added, shaken to break up yolk sac and refrigerated overnight. This was poured into large bottles through a sieve, and the bottles rinsed with 0.85% saline. Enough 0.85% saline was added to bring the egg concentration to 30% and allowed to stand 3 days. An equal volume 0.85% saline containing 1-10,000 merthiolate was added. (15% crude yolk sac). The mixture was adjusted to pH 7.0 with Na_2HPO_4, 200 ml of ether was added to 1500 ml of crude extract and shaken a little by hand. This was frozen in CO_2-ether and stored in the CO_2 box overnight, then thawed in water and 300 ml of ether was added. Shake thoroughly and let stand at room temperature overnight. The aqueous phase was removed by aspiration. 900 ml of saline was added to the ether left in the bottle, shaken and permitted to stand. The aqueous phase was removed and combined with the first aqueous phase. The sediment was washed with 200 ml of ether, shaken, and allowed to stand overnight. The aqueous phase was removed and added to the first and second aqueous phases. These combined washed aqueous phases constituted the vaccine. The finished vaccine contained approximately 1,000,000,000 organisms per ml. This number was based on a DCBA plate count of viable organisms before the addition of preservative and ether extraction.

Vaccine #3 OF038YS#2

Vaccine prepared from membranes by procedure described under Vaccine #2. Count 100,000,000 organism/ml.

Vaccine #4 OF038 Memb-YS.

Vaccine prepared by procedure described under vaccine #2 from yolk sacs of eggs which had been inoculated via the membrane. Count 6,000,000 organisms/ml.

Vaccine #5 OF038 Memb-Fluid

Vaccine prepared by procedure described under vaccine #2 from fluid of eggs which had been inoculated via the membrane.
Count 1,000,000 organisms/ml.

Vaccine #6 S-YS

Prepared from yolk sacs of eggs inoculated via the yolk sac with strain S by procedure described for vaccine #2.
Count. 1,000,000,000 organisms/ml.

Vaccine #7 S-Membrane.

Similar to vaccine #6 but prepared from membranes.
Count 500,000,000 organisms/ml.

Vaccine #8 S-Fluid

Similar to vaccine #6 but prepared from fluids (allantoic fluid, amniotic fluid, amniotic fluid and Yolk).
Count 500,000,000 organisms/ml.

Vaccine #9 S-YSM Were prepared like vaccine #2 from
Vaccine #10 C-YSM pooled membranes and yolk sacs of eggs inoculated with Strain S or strain C. These vaccines contained not over 1 billion organisms per ml.

Vaccine #11 SYS 2nd Supernatant-Crude centrifuged vaccine.

Ten day eggs were inoculated via the yolk sacs as before. After death of the embryo the yolk sacs were harvested, shaken with glass beads, and frozen in CO_2, then melted and refrozen, and remelted. They were then shaken for ½ hour. The material was diluted with an equal volume of saline containing 1.0% formalin to make a final dilution of 0.5% formalin. This was allowed to stand in the refrigerator overnight. It was centrifuged at 2000 rpm for 35 minutes with usual resultant layering. The sediment was resuspended in the liquid middle layer and cantrifuged for fifteen minutes at 1000 rpm. It separated into 2 layers. The supernatant contained large numbers of organisms as shown by smears stained with Giemsa. This was the final vaccine and contained approximately 3 billion "UL" organisms per ml.

Vaccine #12 S Memb F8-IV-44

11 and 12 day embryonated eggs were injected via the yolk sac with 10^{-2} dilution of "UL" strain S and were harvested after death on the 3rd day. Membranes were harvested and shaken with glass beads for 15 minutes. An equal volume of saline containing 0.5% formalin was added. This was stored in the refrigerator overnight. The next day they were

centrifuged at 2700 rpm for 1 hour. They separated into 3 layers. Smears stained with Giemsa showed that the top layer contained few organisms and debris, this was discarded. The fluid middle layer contained numerous organisms and was saved. The sediment contained the largest number of organisms and was suspended in saline, homogenized in the Waring Blendor for 20 minutes, allowed to stand in the ice-box overnight where it again separated into 3 layers. The sediment and top layer were discarded. The middle layer was cantrifuged at 4000 rpm for 1 hour and the supernatant discarded. The sediment was pooled with first supernatant to make the finished vaccine which contained 3.9 billion organisms/ml.

Vaccine #13 SYS-F 8-IV-44

The eggs were injected as for vaccine #12. The yolk sacs were harvested and diluted with saline to 30% and 0.5% formalin was added. These were stored overnight in the ice-box, homogenized for 30 minutes in the Waring Blender. They were then centrifuged at 2650 rpm for 3 hours. The fluid separated into 3 layers. The top layer was discarded. The sediment was saved and the middle fluid layer was centrifuged at 4000 rpm for 1 hour. The sediment from this was saved and pooled with the first sediment. These sediments were diluted with 0.1% formalin in saline to the original volumes.

Count approximately 4,000,000,000 organisms/ml

Vaccine #14 SYS 10-IV-44

Eggs were inoculated via the yolk sac and the yolk sac harvested as for vaccine #12. After being homogenized in the Waring Blendor, they were frozen in dry ice-acetone and stored overnight in the CO_2 box. They were then thawed and centrifuged 1 hour at 2650 rpm. The fluid separated into 3 layers. The sediment contained a very large number of organisms and some debris. It was pooled with the liquid middle layer and saved. The top layer was diluted with 10 volumes of saline and homogenized in the Waring Blender for 10 minutes. It was then centrifuged at 4000 rpm for one hour. There was again separation into 3 layers. The sediment contained a large number of organisms and was pooled with the original sediment and liquid layer mentioned above to make the final vaccine.

The final volume was 360 ml and represented 150 ml of original yolk sac material.

Count Approximately 3.3 billion organisms/ml

Vaccine #15 SYS 30%F 6-IV-44

Ten yolk sacs were harvested from yolk sac inoculated eggs and diluted with 2 volumes of saline. Formalin was added to make 0.5% final dilution and this was then homogenized in the Waring Blender for ½ hour. After appropriate sterility tests this was bottled as the finished vaccine.

Count Approximately 3 billion organisms/ml.

Vaccine #16 Foshay vaccine.

Made at Camp Detrick according to personal communication from Dr. Foshay(30) by adding 0.5% phenol to 72 hour culture of strain S grown in gelatin hydrolysate medium and adjusted to a turbidity of 3,000 on the Fullers earth scale. Count--approximately 10-12 billion organisms/ml. Mg N/ml--0.06 determined on sediment after high speed centrifuging at 21,000 times gravity.

Vaccine #17 AF-13-III-F

Eleven day embryonated eggs were inoculated via the yolk sac with 0.2 ml 10^{-1} dilution of strain SM1 and harvested three days later after overnight storage in the refrigerator. The allantoic fluid was aspirated from 30 eggs, pooled and a plate count made. Formalin was added to make 0.2% concentration.

Count 1,600,000,000 organisms/ml.

Vaccine #18 A-F-13-III-Ch

Same as vaccine #17 except preservative added was 0.75% chloroform.

Count 1,600,000,000 organisms/ml.

Vaccine #19 YS-13-III-F

Yolk sacs from eggs used to prepare vaccine #17 were shaken with glass beads and an equal volume of saline containing 0.2% formalin was added and reshaken. It was allowed to stand in the refrigerator for 1 week then the aqueous layer was aspirated and bottled.

Count 2,200,000,000 organisms/ml

Vaccine #20 YS-14-III-Ch

Similar to vaccine #19 except preservative was 0.75% chloroform.

Count 2,200,000,000 organisms/ml

Vaccine #21 M-13-III-F

Similar to vaccine #19 prepared from membranes.

Count 2,100,000,000 organisms/ml.

Vaccine #22 M-14-III-Ch

Similar to vaccine #19 prepared from membranes and 0.75% chloroform added as a preservative.

Count 2,100,000,000 organisms/ml.

Vaccine #23 MAF-UV

12 day embryonated eggs were inoculated via the membrane and four days later the allantoic fluid of all embryos was aspirated, pooled, and counted on DCBA plates. The fluid was exposed in a thin layer at a distance

of 3 inches to ultraviolet light generated by a Hanovia lamp, then bottled. In subsequent tests this vaccine caused typical "UL" deaths in mice but was avirulent for rats. It was therefore an ultraviolet attenuated vaccine.
Count 400,000,000 organisms/ml.

Vaccine #24 CHYS Ext.

Seven day embryonated eggs were inoculated as uaual into the yolk sac and harvested after 4 days incubation. Yolk sacs were pooled, shaken with beads, and allowed to stand in the refrigerator overnight. An equal volume of saline containing 0.5% formalin was added and shaken before storing overnight in the ice-box. This was diluted to 15% yolk sac and 20% by volume of chloroform added and thoroughly mixed before storage in the CO_2 cabinet. The pH was adjusted to 6.9 and the mixture frozen quickly in a CO_2-acetone bath and stored frozen overnight. It was melted in a water bath and stored in the refrigerator where the mixture separated into a top yolk layer, a middle aqueous layer, a lower sludge layer, and a bottom layer of chloroform. The aqueous layer was aspirated and the excess chloroform removed under a vacuum. 1:10,000 merthiolate was added as a preservative.
Count 500,000,000 organisms/ml.

Vaccine #25 C-5

Same as vaccine #24 but concentrated by lyophilization and resuspended in sufficient sterile water to make a final concentration of 12 billion organisms/ml.

Vaccine #26 MAF-Ch

Similar to vaccine #23 except preservative was 0.75% chloroform.
Count 600,000,000 organisms/ml.

Vaccine #27 Young Broth Culture Ch.

A ten hour broth culture in Snyders peptone medium killed with 0.75% chloroform.
Count 1 to 2 billion organisms/ml

Vaccine #28 AF-UV3/4

Similar to vaccine #23 but this suspension was exposed to ultra violet for 3/4 second in apparatus at Parke-Davis laboratories in Detroit.(37) (Exposure for 1/4 second did not kill all of the "UL".) Count 175,000,000 organisms/ml.

Vaccine #29 Broth Culture-UV½

A 72 hour peptone broth culture was centrifuged at 4000 rpm for 45 minutes and sediment resuspended in broth to match a T 500 turbidity.

This was exposed to ultra violet for ½ second similar to vaccine #28.
Count 3,300,000,000 organisms/ml.

Vaccine #30 Acetone Extracted Vaccine A-1

Twenty-five per cent by volume c.p. acetone added to 24 hour peptone broth culture of strain SM14 and allowed to stand overnight at 4°C. Centrifuged at room temperature and resuspended in 50% acetone in saline. Stored at 4°C overnight. Centrifuged at room temperature and resuspended in 100% acetone and stored overnight at 4°C. Centrifuged at room temperature decanted and dried over vacuum. Resuspended in 0.1% formalized saline to match turbidity T500. Count 10,000,000,000 organisms/ml. MgN/ml--0.06. Total solids--8.0 mgm/ml.

Vaccine #30A

Control for #30. A 24 hour peptone broth culture concentrated by centrifugying to match turbidity of T500.

Vaccine #31 Acetone Extracted Vaccine A-2

Similar to #30 except all procedures , including centrifugying, were carried out at 0° C and with each addition of acetone the sediment was shaken for 45 minutes on a mechanical shaker. Mg N/ml--0.07.

Vaccine #32 Alum Vaccine

11 ml of 10% alum added to 110 ml of 10 day old Snyder broth culture, pH 5.5 produced a floc and change of pH to 4.1. pH adjusted to 7.0 with NaOH (7ml). Allowed to stand in ice box 48 hours and one sample bottled and labeled Alum Vaccine 24VI44. The remainder was centrifuged andthe sediment resuspended in 0.1% formalized saline, pH 6.5. Recentrifuged and taken up in an equal volume 0.1% formalin pH 6.2. Bottled and labeled Alum Vaccine pH 6.2 27VI44.

Vaccine #32A

Ten day Snyder broth culture with 0.1% formalin. No other treatment; to be used as control for vaccine #32.

Vaccine #33 Peptone-Foshay-72

A 72 hour peptone broth culture killed with 0.5% phenol and allowed to stand 24 hours in the cold. Centrifuged at 4000 4pm and packed cells resuspended in a sufficient amount of the supernatant to make a final turbidity of T3000. Count approximately 10-12 billion organisms per ml.

Vaccine #34 B-Conc. peptone

Similar to vaccine #33 but prepared from 12 hour broth culture.
Count 4,000,000,000 organisms/ml.

Vaccine #35 24 hour peptone

A 24 hour culture in peptone broth with 0.5% phenol added as a preservative.

Count 6,000,000,000 organisms/ml.

Vaccine #36 24 hour Seitz Filtrate

Same as vaccine #35 but Seitz filtered and filtrate used as vaccine. Contains soluble material capable of passing a Seitz filter from 24 hour peptone broth culture containing 6,000,000,000 organisms per ml.

Vaccine #37

Virulent "UL" strain SM21R3 was grown in peptone broth with continuous shaking for 24 hours at 37°C. The culture was harvested and acetone extracted vaccine prepared and standardized as for vaccine #31.

Vaccine #38

Similar to #37 but the culture was grown at 26-28° C.

Vaccine #39

Similar to vaccine #37 but the culture was grown on DCBA at 37° C.

Vaccine #40

Similar to vaccine #37 except avirulent "UL" strain NIH 38 was used.

Vaccine #41

Similar to vaccine #38 except avirulent "UL" strain NIH 38 was used.

Vaccine #42

Similar to vaccine #39 except avirulent "UL" strain NIH 38 was used.

Before these vaccines were used safety and sterility tests were carried out as described in Appendix III.

D. Procedures used in Testing "UL" Vaccine During Stages of Preparation and on Completion of the Product (Camp Detrick)

The following outline was adopted after consulting all available publications of the U. S. Public Health Service and conferences with Dr. Workman of NIH and Dr. N. P. Hudson representing the Surgeon General's Office. Dr. Workman had no specific recommendations regarding standardization of "UL" vaccines since the NIH had never been asked to approve such a product.

1. Inoculum and Final "UL" Culture-Identification

a. Identify the organisms to be used for vaccine production both before inoculation and at the end of incubation.

1. Gram stain.

2. Plate on dextrose cysteine blood agar (DCBA) at time of harvesting.

3. Agglutination slide test with specific antiserum.

2. Biologic in Bulk

a. Sterility Test. Prior to bottling, at least 10 ml from each bulk container holding more than 1 liter and from bulk containers of less than a liter capacity at least 3 ml shall be planted in one or more tubes holding sufficient culture medium so that preservative present in the biologic will no longer exert its biologic effect.

1. 2 ml in each of 2 bottles containing 100 ml thioglycollate medium, or:

2. 10 ml in bottle containing 500 ml thioglycollate medium

b. Safety test.

1. Guinea Pigs: Inject each of 2 pigs with 5 ml I.P.

c. Antigenicity Test

1. Using 20 white rats of 150-200 gms, inject each rat subcutaneously with 0.5 ml vaccine every other day for 3 doses. Challenge subcutaneously 14-21 days after the last dose with 1 ml of a 10^{-1} dilution of virulent "UL" culture. 20 control animals should be challenged at the same time. The dilution is made from a 24 hour saline suspension giving 40% transmission on the Coleman Spectrophotometer. The criterion for minimal potency is still in the experimental stage but if the vaccine is to be used for human immunization not less than 80% of the vaccinated animals should survive.

d. Complement Fixation Test- Highest dilution of vaccine which fixes complement in the presence of human convalescent serum. Minimum standards not yet determined.

3. Filling of Final Containers:

As far as practicable, bottles used for bulk storage of a completely processed product shall be of such capacity that the entire contents will be used up at a single filling.

The filling room shall be exclusively used for the intended purpose during the filling process. The room should be provided with a filling

cabinet having a sloping glass front extending approximately one-half way down from the top with an open space below for the operators arms, and, in addition openings for filling apparatus; well lighted and equipped with a bunsen burner.

4. <u>After bottling - Specific Tests on Final Container</u>

a. Sterility tests - Of the final containers selected at random from each filled lot, 3-10 shall be tested in accordance with the following schedule.

Total No. Containers in lot	No. Containers to be tested
100 or less	3
101-150	4
151-200	5
201-250	6
251-300	7
301-350	8
351-400	9
Over 400	10

Procedure:

1. Fluid thioglycollate medium: Add 2 ml of vaccine to 100 ml thioglycollate medium.

2. DCBA slants: Inoculate 0.2 ml on each of 6 DCBA (Dextrose Cysteine Blood Agar) slants. Incubate for 7 days and observe on the 2nd, 4th, and 7th day.

b. Safety Test - Use 1 container from each lot.

1. Guinea pigs: Inject each of 2 pigs with 5 ml I.P.

c. Identification Tests: Use either complement fixation or slide agglutination.

5. <u>Labeling Final Containers</u>

a. Gummed Labels are used with the following information contained thereon:

Proper name of product
Lot No.
Bottled: (date)
Expires: (date if known)
Made by (Name or initials)

6. Storage:

a. A total of 200 ml from each lot to be divided among 3 locations:

1. Ice box
2. Room temperature
3. Incubator

b. The remaining quantity of each lot shall be stored in the ice box.

7. References:

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E.

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RESPONSIBILITY

The experimental studies on various problems recorded in this report were so integrated that almost everyone assigned to the project did some work on each problem. In assigning responsibility for problems it should be understood that only the major assignments of each person are mentioned. These were as follows:

Media Studies; Dr. C. M. Downs; Capt. L. Coriell; Lt. (j.g.) B. J. Owen; Ens H. Eigelsbach; T/4 Ruth Doiron.

Growth Curves; Ens. H. Eigelsbach; Lt. (j.g.) Alice Klauber.

Studies with Embryonated Eggs; Dr. C. M. Downs; Capt. L. Coriell; Lt. (j.g.) Alice Klauber; T/4 Ruth Doiron.

Storage and Lyophilization; Capt. L. Coriell; Dr. C. M. Downs; Mrs. Bette Hamilton.

Electron Microscope Studies; Ens. H. Eigelsbach.

Rapid Detection Studies; Capt. L. Coriell; Dr. C. M. Downs; Lt. G. Pinchot.

Decontamination; Capt. L. Coriell.

Animal Immunization Experiments; Capt. L. Coriell; Dr.C.M.Downs; Lt. G. Pinchot; Ens. H. Eigelsbach; T/4 Ruth Duiron; PhM2/c F. Nardi; Lt. E. O. King.

Vaccine Preparation; Ens. H. Eigelsbach; Lt. (j.g.)Alice Klauber; PhM2/c F. Nardi; PhM3/c Eleanor Chapman; Pfc. Alice Devine.

Chemical Fractionation of Antigens; Capt. L. Coriell; T/Sgt. Karl Plitt

Chemistry; T/Sgt. Karl Plitt; T/5 Mary Rogers; HA1/c L. Jastrebzski.

Chemotherapy; Lt. (j.g.) S.S. Chapman; M/Sgt. Stephen Kowal; PhM1/C Wm. Nelson; PhM2/c Douglas Farley; PhM3/c Rebecca Carey; PhM1/c Lupe Zarraga.

Studies on the Agglutination Test; Lt. Mary Clapp; PhM2/c Margaret Butler; PhM1/C June Clifton.

Serology; Lt. (j.g.) B. J. Owen; Lt. Mary Clapp; PhM1/c June Clifton; PhM2/c Margaret Butler.

Hematology; Lt. Elizabeth King

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