STUDIES ON THE BACTERIOPHAGE OF D'HERELLE.

VI. On the Virulence of the Overgrowth in the Lysed Cultures of Bacillus pestis caviæ (M. T. II).

By J. BRONFENBRENNER, Ph.D., R. S. MUCKENFUSS, M.D., and C. KORB, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, June 15, 1926.)

When susceptible bacteria are grown on broth in the presence of a suitable lytic agent, the initial turbidity of the culture gradually disappears because of the lysis of the bacteria. In exceptional cases the lysis may be complete and permanent, resulting in sterilization of the culture. But more often a few bacteria fail to undergo lysis and multiply in spite of the presence of lytic agent in the medium, thus giving rise to more or less profuse overgrowth of resistants, following a temporary clearing of the culture (1).

In the case of *B. coli*, Bordet and Ciuca (2) found that the overgrowth differs from the original culture, in addition to its resistance to lysis, by its active motility; and Gratia (3) found further that the overgrowth is more highly virulent. The heightened virulence of bacteria resistant to lysis was observed also by other investigators and has been suggested as the source of the failures of bacteriophage to influence favorably the course of infection in certain instances (4–7).

In view of the fact that the virulence of a strain M. T. II of *B. pestis caviæ* was carefully established during several years of study of experimental mouse typhoid at The Rockefeller Institute (8–10), we thought that a lysis-resistant overgrowth of this culture would offer exceptional material for comparison of its virulence with that of the original culture.

Isolation of Resistants.

In order to obtain pure strains of resistant bacteria, the stock culture M. T. II was grown in broth for 24 hours, in the presence of each of the two lytic filtrates

active against this organism. These lytic filtrates were selected from several isolated by us at different times from the stools of mice recovering from experimental mouse typhoid, because they were typical of the variations in the character of lytic activity observed in these filtrates.[1]

The overgrowth which was present in the cultures after 24 hours of incubation at 37°C. was streaked out on agar plates. Single colonies appearing on the plates were repeatedly transferred to fresh agar (each time from a single colony) in order to eliminate all traces of lytic principle. After seven successive transfers, 24 hours apart, each of the cultures was returned to broth. They proved to be free from lytic principle when filtered and tested against the original susceptible stock culture of M. T. II. Broth cultures themselves were tested at the same time for their susceptibility to lysis by each of the lytic filtrates in turn. As the results recorded in Protocol I demonstrate, each culture obtained from the overgrowth was resistant to lysis by the lytic agent used in its production, but only one of the cultures was resistant also to the activity of the second lytic agent.

Protocol I.

Identification of Resistants Isolated from Lysed M. T. II Culture.

Lytic agents used to obtain resistants	Character of growth in broth	Agglutinability with stock M. T. II serum 1:2000	Resistance or susceptibility to lysis	
			Resistant W–Little R_7*	Resistant W–178 R_7*
W–Little.....	Agglutinated	Positive	Resistant	Resistant
W–178.....	Diffuse	Positive	Susceptible	Resistant

* The legend R_7 added to the name of each culture indicates that it represents a resistant (R) variant of the seventh (7) passage on agar.

The identity of these cultures with the parent culture of M. T. II was checked by the agglutination test, in which a stock anti-M. T. II serum diluted 1:2000 was used as agglutinin.

It was observed that in some cases the overgrowth appeared diffuse; in others it was spontaneously agglutinated. Since this difference in the appearance of overgrowth has been stressed in the literature (6, 7) we have carried out all the experiments with Strains W-178 and

[1] We are greatly indebted to Dr. Ida Pritchett for placing at our disposal the greater part of the animals used for isolation of lytic agents. In passing, it should be recorded that the lytic filtrates yielded by the examination of over 300 mice were, for the most part, active only against *B. dysenteriæ*. Next in frequency were those active against *B. enteritidis* Gaertner, and least frequently those active against the infecting organism M. T. II.

W-Little as representing this difference in growth. In order to avoid spontaneous return of susceptibility to lysis, the cultures were grown on agar and transplanted only as often as the experiments required. Whenever resistant bacteria were needed for the test, a subculture from agar was made into broth and used as such after 18 hours of incubation at 37°C.

Virulence of the Resistants by Feeding.

After having carried the resistant strains W-Little R_7 and W-178 R_7 for four more generations on agar (eleven in all) we attempted to determine their virulence as compared with that of the original strain of *B. pestis caviæ*.

Protocol II.

Virulence of Resistants by Feeding.

Bacteria fed		Group A (control) Stock M. T. II	Group B W-Little R_{11}	Group C W-178 R_{11}
No. of bacteria given		3,000,000	3,000,00	3,000,000
No. of mice surviving by days	5th day	25	25	25
	6th "	23	25	25
	7th "	19	25	25
	8th "	15	25	25
	9th "	12	25	25
	10th "	10	25	25
	11th "	9	25	25
	12th "	8	25	25
	13th "	8	25	25
	14th "	8	25	25

The animals used for these experiments came from special stock kept at The Rockefeller Institute, and known to have been free from spontaneous mouse typhoid for a period of years. It was hoped that these animals would show no heightened resistance to the infection, and would present an ideal object for the study to be undertaken.

75 mice of approximately 25 gm. weight each were divided into three equal groups. To each mouse of the first group (Protocol II, *A*) was given, by means of a stomach tube, 0.5 cc. of an 18 hour broth culture of *B. pestis caviæ* (M. T. II), diluted to contain about 3,000,000 bacteria in each dose. The mice of the second group (Protocol II, *B*) each received about 3,000,000 bacteria (in 0.5 cc. volume)

from an 18 hour broth subculture from the eleventh generation of a resistant W-Little R_{11} on agar. The mice of the third group (Protocol II, *C*) each received 0.5 cc. of broth containing about 3,000,000 bacteria of an 18 hour broth subculture of W-178 R_{11}. Each animal was placed in an individual jar, its ordinary ration was supplied daily, and the litter was changed three times per week. No symptoms were noted until the 4th or 5th day after the feeding of bacteria, when certain animals in Group A appeared ill, and from the 6th day deaths began to occur, as indicated on Protocol II. The experiment was interrupted on the 15th day, after no deaths had occurred among the animals for 3 days. At this time all the animals receiving resistant bacteria (Groups B and C) were living, whereas only eight animals out of twenty-five (about 32 per cent) survived the feeding of the original M. T. II.

Susceptibility to Infection by B. pestis caviæ (M. T. II) of Mice Surviving the Feeding of Resistants.

In the next two experiments the resistants of the thirteenth and fourteenth generations on agar were used. The method was the same as in the first experiment and the results may be summarized by stating that the mortality of controls (corresponding to Group A on Protocol II) was 62 and 66 per cent respectively, whereas all the animals but two of those fed the resistants remained alive. That these two deaths did not arise from mouse typhoid infection is rendered probable by the fact that at the autopsy the blood and internal organs were found to be free from *B. pestis caviæ*.

Since the failure of resistants to infect mice was contrary to what was expected on the basis of earlier reports in the literature (1–5, 11), it was thought advisable to ascertain the susceptibility of the surviving mice in the above experiments to infection with the parent culture M. T. II; if these animals prove susceptible to subsequent infection with M. T. II, their earlier resistance to W-Little R_{14} and W-178 R_{14} respectively can be ascribed to a change in virulence in these cultures.

Accordingly, twenty mice each of those (twenty-five) surviving feeding with W-Little R_{14} and W-178 R_{14} respectively (corresponding to Groups B and C on Protocol II) were divided into two groups. The first subgroup of ten mice from each group received a suspension of an 18 hour broth culture of virulent M. T. II by mouth, the other by intraperitoneal injection. Similarly, half of twenty normal mice were given the same bacterial suspension by mouth, and the other half by intraperitoneal injection. The animals were cared for exactly as in the earlier ex-

periments, except that those injected intraperitoneally were placed in larger containers—five mice in each. Protocol III illustrates the results obtained.

The remaining eight mice surviving the feeding of resistants[2] were killed and examined for signs of invasion by the bacteria which had been fed to them. As in the case of two mice of this series which died

Protocol III.

Susceptibility of Surviving Mice to Infection by M. T. II.

	Mice surviving the feeding of resistants 15 days previously				Normal controls	
	Survivals of Group B (fed W–Little R_{14})		Survivals of Group C (fed W–178 R_{14})			
No. of mice	10	10	10	10	10	10
Mode of infection	By mouth	Intraperitoneal	By mouth	Intraperitoneal	By mouth	Intraperitoneal
No. of virulent bacteria given (about)	5,000,000	2,000,000	5,000,000	2,000,000	5,000,000	2,000,000
No. of deaths per day: 1st day		3		4		3
2nd "		3		3		6
3rd "		1		3		1
4th "		2				
5th "		1			1	
6th "			1		2	
7th "	2		3			
8th "	1				1	
9th "	3		1		2	
10th "						
11th "	1					
12th "			1		1	
13th "						
14th "						
No. of survivals	3	0	4	0	3	0

earlier, the internal organs of these mice were sterile, but the intestinal contents showed the presence of bacteria resembling the parent strain M. T. II in their lack of ability to ferment lactose, in the production of H_2S, and in their immunologic properties. They differed

[2] Twenty mice of each series of twenty-five were used in the preceding experiment, and one animal in each group died from intercurrent cause, thus leaving four mice in each group (see experiment Protocol 11).

only in their power to resist lysis by homologous lytic agents, thus establishing their identity with the material fed to the mice. In other experiments of similar nature, the intestinal contents yielded an occasional colony susceptible to lysis; the bulk of bacteria fed to the mice seemed, however, to have remained in the intestinal contents without becoming susceptible to lysis, and without entering into the blood for at least 14 days. If this be proven to be the case with larger experimental material, it would suggest the loss of invasive power by the resistants.

Virulence of Resistants by Intraperitoneal Injection.

The fact that mice surviving the feeding of resistants were still susceptible to subsequent infection by the virulent parent M. T. II strain, together with the finding that the resistants lack invasive power, would indicate that their failure to kill animals in earlier experiments was due to this change in virulence, as suspected. However, we thought it of interest to inquire whether this change in virulence was limited to their loss of invasive power only, or to some more radical change. For this reason we compared their power to infect mice by parenteral route with that of the parent strain M. T. II. Accordingly, three series of fifteen mice each were given, by intraperitoneal injection, varying doses of the original M. T. II, W-Little R_{14}, and W-178 R_{14} respectively, as indicated on Protocol IV, and the time of death of each mouse was noted. It will be seen from this protocol that whereas all the mice receiving the original M. T. II injection were dead before the expiration of 5 days (after injection), only three mice out of a total of thirty animals injected with resistants were dead up to the 10th day, when the experiment was terminated. We are inclined to attribute the death of these three mice to other causes rather than to the infection, particularly since in two subsequent experiments analogous to the one just described—except that the dose of bacteria was doubled and trebled respectively—only one mouse died, out of a total of 60 receiving resistant bacteria intraperitoneally, whereas the mortality of mice receiving the original M. T. II culture was invariably 100 per cent. These experiments, in our opinion, indicate that at least in the case of *B. pestis caviæ* isolation of cultures resistant to lysis by bacteriophage results in obtaining an avirulent strain of this organism.

Protocol IV.

The Loss of Virulence by the Resistant Subcultures of M. T. II as Tested by Intraperitoneal Injection.

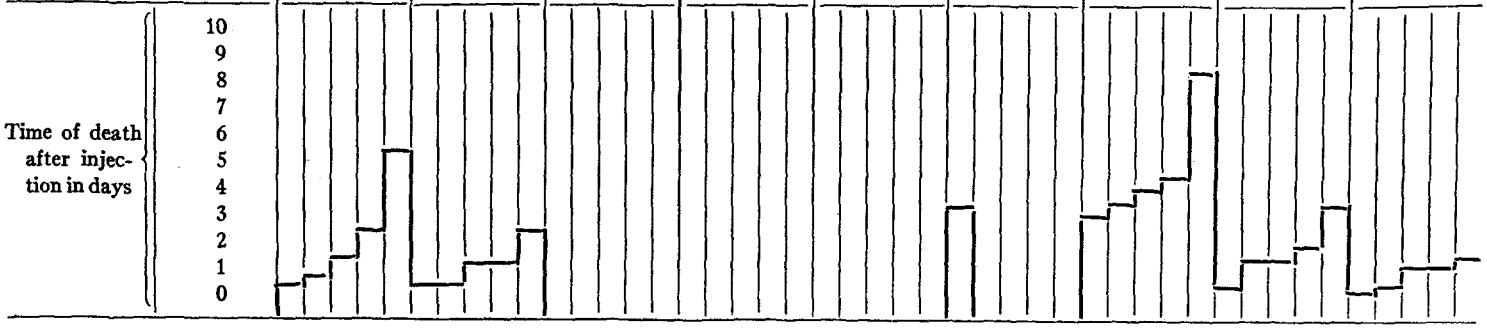
	Original M. T. II (susceptible to lysis)			W-Little R_{14} (resistant to lysis)			W-178 R_{14} (resistant to lysis)		
No. of bacteria given intraperitoneally*	777,000	1,900,000	7,000,000	735,000	2,300,000	7,150,000	700,000	2,167,000	6,800,000
No. of mice injected	1 2 3 4 5	1 2 3 4 5	1 2 3 4 5	1 2 3 4 5	1 2 3 4 5	1 2 3 4 5	1 2 3 4 5	1 2 3 4 5	1 2 3 4 5
No. of mice surviving after 10 days	None	None	None	All	All	Four	Four	All	Four
Time of death after injection, in days: 10th day									
9th "									
8th "									
7th "									
6th "									
5th "									
4th "									
3rd "									
2nd "									
1st "									
0									

* These figures show the bacterial count on agar plates poured immediately after injection of animals.

Protocol V.

Virulence of the Reverted W-Little R_{14} by Intraperitoneal Injection.

	A			B			C		
				W-Little R_{14} (resistant variant)					
	Original culture of M. T. II			Grown for 18 hrs. on broth			After 20 daily passages on broth		
Character of growth on broth	Diffuse			Agglutinated			Diffuse		
Susceptibility to lysis by lytic agent W–Little	Susceptible			Not susceptible			Susceptible		
No. of bacteria given intraperitoneally*	966,000	2,900,000	8,700,000	1,366,000	4,100,000	12,300,000	967,000	3,100,000	9,700,000
No. of mice injected	1 2 3 4 5	1 2 3 4 5	1 2 3 4 5	1 2 3 4 5	1 2 3 4 5	1 2 3 4 5	1 2 3 4 5	1 2 3 4 5	1 2 3 4 5
No. of mice surviving after 10 days (virulence)	None	None	None	All	All	Four	None	None	None



* These counts were obtained by plating samples of suspensions of bacteria immediately after injection.

Virulence of W-Little R_{14} after Its Recovery of Susceptibility to Lysis.

It is well known that bacteria isolated from the overgrowth of lysed cultures usually revert to the original type and become susceptible to lysis after a more or less prolonged cultivation on media free from lytic agent. Since the preceding experiments indicated that there may exist an interdependence between the lack of susceptibility to lysis and the loss of virulence in the culture of *B. pestis caviæ*, it seemed desirable to find out whether the return of susceptibility to lysis will be followed by a return of virulence in such cultures.

For this purpose the fourteenth generation of a resistant strain (W-Little R_{14}) was subcultured from agar into broth, and from the latter daily transfers to broth were continued for 20 days, after which the culture thus obtained was tested for its susceptibility to lysis, as well as for its virulence to mice, by the intraperitoneal route. For the sake of comparison parallel tests were made with the culture of the original M. T. II, as well as with an 18 hour broth culture obtained by inoculating into sterile broth a small loop from the old agar slant W-Little R_{14}, which presumably retained its resistance to lysis by the phage W-Little. The procedure followed in this experiment was entirely analogous to that of the preceding experiment (Protocol IV), except that in addition to other tests, each culture was subjected to a control test of its susceptibility to lysis. The results of the virulence test are given in Protocol V. Similarly, the twentieth passage in broth from W-Little R_{14} proved capable of killing mice when given by mouth (Protocol VI).

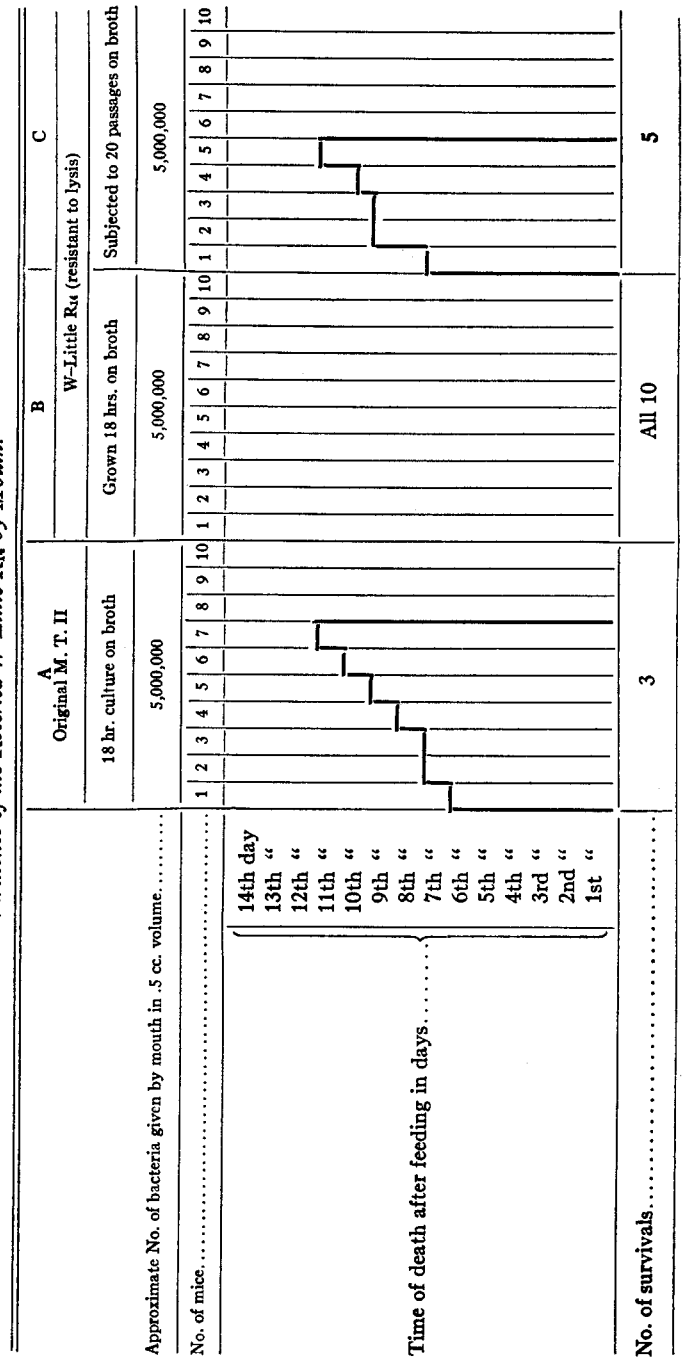
It is evident, then, that when full reversion to susceptibility to lysis, after prolonged cultivation in broth and in the absence of bacteriophage, has taken place, the resistant strain W-Little R_{14}, which remains avirulent and resistant to lysis on the first subculture in broth (Protocols V, *B* and VI, *B*), tends to become as virulent as the parent culture of M. T. II, from which it originally was derived.[3]

In order to ascertain more closely the relationship between the return of virulence and the susceptibility to lysis of the culture, the experiment was repeated and the test of susceptibility, as well as of virulence, was made daily.

[3] It should be noted incidentally that simultaneously with these changes in virulence and susceptibility to lysis, the appearance of growth on broth changed from being agglutinated in the first subculture from agar to the diffuse growth characteristic of the parent culture M. T. II.

Protocol VI.

Virulence of the Reverted W-Little R_{14} by Mouth.



For this purpose we returned to the agar culture W-Little R_{14} (kept on ice all the time), and made a subculture from it into broth. After 18 hours of incubation at 37°C. the resulting broth culture was tested for its virulence and susceptibility to lysis. A little later on the same day, a second subculture in broth was made from the first to be tested next day (after 18 hours of growth) and so on for several days (Protocol VII).

Only the intraperitoneal method of injection was used for testing virulence in this experiment, as it was deemed more convenient, both because of simplicity and the shorter incubation period. When it was observed that the animals died regularly, at a rate approximating that of the mortality of mice injected with the original M. T. II culture, the experiment was interrupted. Results of this experiment indicate that on the sixth transfer in broth the resistant culture W-Little R_{14} became susceptible to lysis, and at the same time it regained its pathogenicity.

Non-Reversion to Susceptibility of W-178 R_{14}.

As stated in the early part of this paper, resistant strains obtained by us could be roughly divided into two groups, judging by the macroscopic appearance of their respective growth in broth. Since it has been observed further that resistants secured by means of the phage W-Little are susceptible to lysis by the phage W-178 (but not *vice versa*, see Protocol I), we undertook to ascertain whether resistants obtained by the action of the latter "stronger" phage would also undergo reversion to susceptibility and recover virulence if grown in the absence of phage. However, all attempts in this direction have thus far been unsuccessful. The cultures have remained resistant to lysis, and when tested for virulence, after nearly 200 successive passages in broth over a period of 10 months, mice survived an injection of 3,000,000 bacteria intraperitoneally. Throughout the period resistant bacteria exhibited their original characteristics as regards fermentation and antigenic properties, and were found to be free from bacteriophage (not "lysogenic"). Occasionally, when grown on agar plates with a corresponding bacteriophage, some of these cultures gave rise to a few "pale" plaques which were quickly overgrown by resistants. In such cases corresponding broth cultures have, on occasion, shown a slight increase in the titer of the phage, but at

Protocol VII.

Respective Rate of Recovery of Virulence and of Susceptibility to Lysis by the Resistant Subcultures of W-Little R_{14}.

No. of passages on broth.....	First					Second					Third					Fourth					Fifth					Sixth					Seventh					Eighth					Ninth					Tenth				
Susceptibility to lysis by W-Little phage.....	Resistant					Resistant					Resistant					Resistant					Resistant					Susceptible quickly overgrown					Susceptible but quickly overgrown					Susceptible but overgrown					Susceptible					Susceptible				
No. of bacteria given intraperitoneally*.....	5,100,000					4,750,000					6,200,000					5,700,000					5,200,000					6,300,000					4,900,000					5,300,000					5,000,000					4,700,000				
No. of mice.....	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
Time of death after injection, in days: 14 days																																																	
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* The numbers recorded above were obtained by plating suspensions immediately after injection.

no time was it possible to isolate the few susceptible individuals which must have appeared temporarily in such cultures, to be immediately overgrown by the resistants or to be destroyed by the phage. Since our first observations concerning the behavior of resistants obtained

Protocol VIII.

Attempt to Cause Reversion in the Presence of Antiphage Serum.

No. of transfers previous to test	Culture medium	Character of growth	Susceptibility to lysis as tested	
			In broth	On 1 per cent agar
1	Plain broth	Diffuse	—	—
	Broth + serum	Agglutinated	—	—
2	Plain broth	Diffuse	—	—
	Broth + serum	Agglutinated	—	—
4	Plain broth	Diffuse	—	—
	Broth + serum	Agglutinated	—	—
6	Plain broth	Diffuse	—	—
	Broth + serum	Agglutinated	—	—
7	Plain broth	Diffuse	—	—
	Broth + serum	Agglutinated	—	—
10	Plain broth	Diffuse	—	—
	Broth + serum	Agglutinated	—	—
17	Plain broth	Diffuse	—	—
	Broth + serum	Agglutinated	—	—
25	Plain broth	Diffuse	—	—
	Broth + serum	Sediment and diffuse overgrowth	—	—
32	Plain broth	Diffuse	—	—
	Broth + serum	Sediment and diffuse overgrowth	—	—

by means of bacteriophage W-178 (18), we have noted a similar failure of resistants to revert to susceptibility when other "strong" phages were used for their production. Although in every instance the resistants were tested and found not to carry phage, we attempted

to grow them in the presence of an antibacteriophage serum, in the hope that we might thus induce a reversion.

In these experiments a resistant strain, isolated by means of a phage P.I.D. and carried for several months without reversion, was employed. Starting from an agar slant culture of this strain P.I.D.-R_{15}, we have made two sets of daily transfers, the first into plain broth and the second into broth containing 0.02 cc. of antibacteriophage serum for each cc. of the medium. From time to time cultures of resistants thus obtained were tested for their susceptibility to lysis in broth as well as in agar. This procedure was continued for a month without leading to reversion, as illustrated on Protocol VIII.

DISCUSSION.

According to the original conception of d'Hérelle and his collaborators, the production of resistants is the result of an increase in the resistance of bacteria against the invasion of bacteriophage. They consider this process analogous to the development of active immunity in higher forms, after exposure to infection (19). In accordance with this theory, resistants might be expected to be more virulent, and actual observation seems to have confirmed the expectations in many instances (2–7). However, our experiments fail to support this notion since, at least in the case of *B. pestis caviæ*, resistants have been found repeatedly to be devoid of virulence. On the other hand, only cultures susceptible to lysis have been found to be pathogenic. In a measure, as cultures of resistants recover their susceptibility to lysis, they again become virulent. The cultures which fail to become susceptible to lysis, under the conditions of the experiment, remain avirulent. The fact that some resistant strains may not become susceptible, after having been carried free from bacteriophage for 200 successive transfers in broth, seems to indicate that the production of resistants is not a phenomenon of active "hereditary" immunization (12), but rather a result of irreversible variation, somewhat analogous to that observed in pneumococcus cultures (13, 14). It has been shown by Arkwright (15), Gratia (16), and others that cultures of bacteria of the colon-typhoid group can be normally dissociated into a number of variants of which at least two are manifestly different in the appearance of their colonies. Gratia (17) found later that the variants normally possess different resistance

to lytic agents and may exhibit varying rates of growth (3). Thus, a bacterial culture may be considered in its cross-section (as indeed any other "population") as composed of individuals approaching a certain type, but occasionally lacking or taking on exaggerated characteristics. Depending upon the conditions of growth, some of the variants may find themselves favored by environment and may become quantitatively dominant. On the contrary, if the environment is changed so that it becomes incompatible with life or the multiplication of a certain type of variant, the latter is eliminated more or less completely, and the whole cross-section of the bacterial population in the culture changes accordingly, with more or less noticeable changes in the biologic activity of the culture as a whole. In the case of a highly specific, "weak" bacteriophage which displays activity only toward a comparatively narrowly defined type of individuals in the culture (as, for example, the phage W-Little), only the most susceptible individuals carrying the potential characteristic of virulence are destroyed, and if such a culture is allowed to grow in the absence of bacteriophage, the few remaining closely related individuals, which may carry the potential characteristic of virulence, begin to multiply anew and to produce the original cross-section of the culture. If, however, a phage of less specificity is used (as, for example, the "strong" phages W-178 or P.I.D.), it may happen that all the individuals carrying the characteristic of virulence are destroyed, and a permanently avirulent culture results.

SUMMARY AND CONCLUSIONS.

Resistants isolated from the overgrowth of cultures of *B. pestis caviæ* (M. T. II) lysed by various strains of specific bacteriophage proved to be avirulent when administered to mice by feeding, or by intraperitoneal injection.

These cultures remained resistant to the action of bacteriophage so long as they were carried on agar. When transferred to broth, however, one group of resistants, namely, those isolated by means of "weak" phages, became susceptible to lysis after five to seven daily passages. The other group of resistants, isolated from the cultures lysed by one of the "strong" phages, failed to become susceptible to lysis even after nearly 200 passages in broth.

Simultaneously with the recovery of susceptibility, the cultures of the first group regained a degree of virulence comparable to that of the parent culture of *B. pestis caviæ*. The cultures of the second group of resistants have failed thus far to recover virulence (10 months after isolation). The latter cultures, apart from lack of both virulence and susceptibility to lysis, are identical with the parent culture of *B. pestis caviæ*, as indicated by biochemical and antigenic properties.

Our findings offer evidence in favor of the view that resistant strains result from selection among variants already existing in the parent culture and do not arise through the inheritance of specific immunity properties produced by the action of phage.

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