THE LATENT PERIOD IN THE GROWTH OF BACTERIA.

By ALAN M. CHESNEY, M.D.

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

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The life cycle of a bacterial culture, as determined by estimations of the number of viable organisms present at various intervals, may be divided into four periods or phases, which, in the order of their appearance, may be designated: (1) latent period; (2) period of maximum rate of growth, or logarithmic period; (3) stationary period; (4) period of decline. These phases merge into one another without a sharp dividing line, and their duration varies with different species of organisms, and with the same organism under different conditions, such as temperature, nature of culture media, and still other factors.

By latent period or lag is meant the interval which elapses between the time of seeding and the time at which maximum rate of growth begins. During this time there may be slow growth, no growth, or an actual diminution of viable organisms. The present paper deals with the nature and significance of this phenomenon.

The logarithmic period, so called, is the phase of rapid growth during which the organisms are dividing regularly, so that their increase in a standard unit of time follows the law of geometric proportion, 2 bacteria giving rise to 4, 4 to 8, etc. If, during this period, the logarithms of the numbers of organisms found at varying intervals are plotted as ordinates, against time values as abscissæ, the logarithms fall upon an ascending oblique straight line (1).

This period is followed by the stationary period, in which the organisms cease to multiply at a maximum rate so that their increase in number becomes less and finally ceases; and, although they remain viable, the number present in a unit volume remains approximately constant for an appreciable length of time.

Finally the period of decline sets in, in which the number of living organisms begins to decrease, and at the expiration of several days or longer for certain bacteria living organisms cannot be demonstrated in the culture.

HISTORICAL.

The latent period in bacterial cultures was first recognized by Müller (2) in 1895 in the course of a study of the effect of high temperatures, simulating febrile conditions, upon the growth of the typhoid bacillus. Müller found that the latent period varied with the age of the culture used for seeding, being shorter for younger than for old cultures. He suggested that the latent period results from an alteration of the bacterial cells and that such bacteria transferred to a new favorable medium require time to recover from the injury sustained.

Hehewerth (3) ascertained that the duration of lag is less for *B. coli* than for *B. typhosus*, and shorter when the culture medium is one to which the organism has been accustomed.

Rahn (4) working with *B. fluorescens liquefaciens* studied the influence upon lag of the amount of bacteria used for seeding, and concluded that the larger the inoculum, the shorter the lag. Moreover, he reinoculated bacteria into bouillon from which the previous growth had been removed by filtration through infusorial earth or killed by heat, and found lag still present, although shorter than in fresh bouillon.

Barber (5) studied the rate of multiplication of single individuals of *B. coli* at different temperatures, and found that when three or four generations of bacteria had been formed in a culture, the individual members of that culture failed to show lag upon transplantation. Apparently he was the first to show conclusively that bacteria do not exhibit a latent period under these conditions.

Chick (6) found that *B. coli*, when grown in normal rabbit serum, shows lag, which varies with the temperature at which the organisms are grown, being longer when the temperature is below that of optimum growth.

The latent period has been extensively studied by Penfold (7), who has made an important contribution to the subject. He worked with *B. coli* and determined that with small seedings the lag is longer the smaller the inoculum, but with large ones variations in the size of the inoculum are without effect. He also confirmed Barber's observations, when he showed that subcultures made during the period of maximum rate of growth show no lag. In addition this author found that organisms subjected to a short application of cold during maximal rate of growth cease to multiply and that on reincubation they increase without lag. On the other hand, if the exposure to cold is long, lag appears on reincubation. Efforts to remove lag by washing the bacteria with saline were unsuccessful, as were efforts to demonstrate in the cultures the presence of heat-stable products which affect lag. Finally, he was able to show that organisms

remaining in the supernatant fluid after centrifugation of a culture continue to grow, but with a lag.

Measurement of Lag.—In estimating the rate of growth of bacteria it is convenient to speak in terms of their generation time, the formula for which was first worked out by Buchner, Longard, and Riedlin (8). If the increase always takes place by division of a single bacterium into two new bacterial cells, then, according to the law of geometric proportions $b = a \times 2n$, where b = the number of organisms obtained at the end of a given period of time T, a = the number of organisms present at the beginning of time T, and n = the number of generations which have occurred during the interval. Applying logarithms to this equation, the number of generations, or n, $= \frac{\log b - \log a}{\log 2}$. If there have been n generations in T time, obviously the generation time $G = \frac{T}{n}$.

Hence when multiplication is maximum, the generation time will be shortest, and when it ceases, or an actual decrease in a unit volume occurs, it will be infinity. It follows likewise that the generation time will vary for a single culture, depending upon the particular phase of growth during which observations are made. Consequently, estimations of generation time necessitate frequent observations upon the number of bacteria present; otherwise the results are not of great value.

The duration of lag is measured by the number of minutes or hours which intervene between the time at which the culture is seeded and the time at which maximum rate of growth commences. The onset of the maximum rate of growth corresponds to the minimum generation time.

EXPERIMENTAL.

Technique.—The bacteria employed in this study were *Diplococcus pneumoniæ*, *B. coli*, *B. prodigiosus*, and *B. fluorescens liquefaciens*. The strains of pneumococci used belonged to Types I and II[1] and were laboratory cultures which had been isolated from cases of lobar pneumonia several years previously, but subsequently had been passed through many white mice and transferred innumerable times on artificial media.[2]

The organisms were grown in beef infusion broth, made by extracting a pound of chopped beef with a liter of water over night on ice. The extract was filtered, made up to a concentration of 1 per cent peptone and 0.5 per cent sodium chloride, and boiled ½ hour. The reaction was adjusted to 0.2 per cent acid, using phenolphthalein as an indicator. It was then boiled again for 10 minutes, filtered

[1] See Dochez, A. R., and Gillespie, L. J., A Biologic Classification of Pneumococci by Means of Immunity Reactions, *J. Am. Med. Assn.*, 1913, lxi, 727.

[2] The cultures of *B. coli*, *B. prodigiosus*, and *B. fluorescens liquefaciens* were supplied by Dr. Kligler from the collections of the American Museum of Natural History.

twice through the same paper, and sterilized for 20 minutes on 3 successive days in an Arnold sterilizer. The readiness with which pneumococci grow in the different lots of media prepared in this manner has been found to vary. For this reason, to obtain comparable results with the pneumococcus it is necessary to have a large supply of a given lot of the medium. Results obtained with one lot of broth cannot be compared with those obtained with another lot.

The cultures were incubated in the dark in a water bath at approximately 37°C., in which the variation was always below 1°. Before seeding the broth was brought to 37°C. The number of organisms present in a unit volume was determined by diluting and plating, using 10 to 15 cc. of 1 per cent dextrose agar. The plate method was chosen instead of the direct method of Klein (9) which gives the total number of bacteria, dead and living, since the present study concerns itself especially with the number of living bacteria present in a culture. Dilutions were usually made in normal salt solution, but bouillon was employed when it was desired to observe growth of a few bacteria. Dilutions were carried out by tens, and so arranged that the dilution of the original culture was contained in 0.5 cc. of fluid, the unit volume. Dilutions were made with separate pipettes for each transfer of diluted culture.

During the period of maximum rate of growth of pneumococcus, chain formation is marked, and then diminishes as the age of the culture advances. Hence it might be urged that the latent period demonstrated by the plate method was apparent, rather than real, since a chain as well as a single bacterium may yield only one colony. The tendency of pneumococcus to form chains in fluid media constitutes a real objection to the use of this organism for accurate quantitative work, and renders difficult an estimation of the absolute duration of lag. However, we do not believe that the lag observed in broth culture can be explained on the basis of chain formation alone, for the plate method frequently shows that within the first 2 hours of incubation either no increase or an actual decrease in the number of viable organisms takes place. If the bacteria were multiplying rapidly during this time, although the plates showed only a slight increase or no increase at all, it would be necessary to assume that all or nearly all of the individual cells were forming chains, and that none of these chains were breaking up, an assumption which is scarcely warranted. Next, lag occurs in the case of all motile bacteria thus far investigated in which the question of chain formation can be excluded. Finally, studies which have been carried out in association with Mr. Glenn Cullen, and which will be reported in a subsequent paper, show that the increase in the hydrogen ion concentration of a broth culture of pneumococcus parallels the increase in the number of living bacteria during both the interval of latency and the period of maximum rate of growth, so that there is a lag in the growth of the culture and an equally long lag in the formation of hydrogen ions. The increase in hydrogen ion concentration observed in bacterial cultures indicates that metabolism is taking place, and hence is an expression of change in the medium produced by the agency of the bacteria themselves. It is improbable

that pneumococci carry on extensive metabolic processes independent of growth, since the bacterial cell is a unicellular organism and has a short term of life. Consequently, if, during the latent period no increase in the number of hydrogen ions is observed, it must be assumed that there is little or no metabolism taking place, and hence little or no increase in the number of bacteria.

Phases of Growth of a Bacterial Culture.

Experiment 1.—A flask containing 500 cc. of bouillon was inoculated with 0.2 cc. of an 18 hour broth culture of Pneumococcus Type II. Temperature of water bath 37°C. At frequent intervals a sample was removed for counting. The results are shown in Table I.

TABLE I.

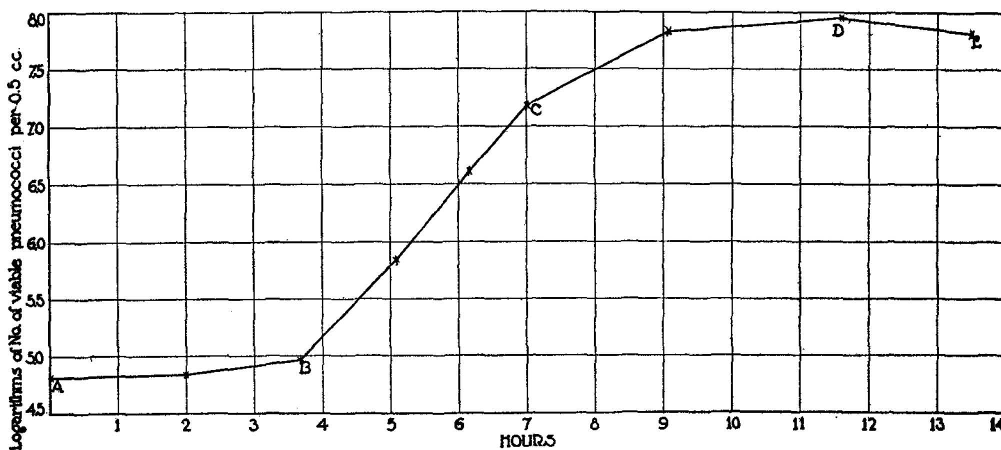
Life Cycle of a Culture of Pneumococcus Type II.

Time after seeding.	Viable bacteria per 0.5 cc.	Time.	No. of generations.	Generation time.
min.		*min.*		*min.*
0	65,000			
120	70,000	0–120	0.08	1,200.0
220	94,000	120–220	0.42	238.0
305	700,000	220–305	2.9	29.3
370	4,150,000	305–370	2.5	26.0
420	15,200,000	370–420	1.8	27.7
545	67,500,000	220–420	7.3	27.4
695	90,000,000	420–545	2.1	59.0
810	65,000,000	545–695	0.41	366.0
		695–810	0	

The table shows only a slight increase in the number of organisms at the end of 2 hours, and at the end of 3 hours and 40 minutes a single generation has not yet taken place. At the expiration of this period, a rapid increase in the number of organisms begins, which continues almost constant over a period of 200 minutes. These results are brought out more clearly by plotting a curve (Text-fig. 1), employing the time values as abscissæ and the logarithms of the number of organisms in a unit volume as ordinates.

The constant rate of increase during the period of maximum rate of growth is indicated by the fact that the logarithmic values of the number of organisms present fall upon a straight line between

the points *B* and *C*. The period of maximum rate of growth is succeeded by the so called stationary period, during which the growth is less rapid (*C* to *D* in the figure) as shown by the increase in the generation time, and finally the culture reaches the period of decline as indicated by the decrease in the number of viable organisms (from *D* onward). In this particular experiment this stage was reached about 12 hours after seeding. The experiment illustrates the four periods of growth—latent, maximal, stationary, and decline, described at the beginning of the paper. The present study relates to the nature of the first of these periods; namely, the latent period.



TEXT-FIG. 1. Growth curve of a bouillon culture of Pneumococcus Type II, at 37°C.

Latent Period.

Age of Inoculum and Latent Period.—Previous workers demonstrated the influence exerted by certain factors upon the occurrence and duration of this phase. The effect which the age of the culture used for inoculation exerts upon lag has an important bearing upon the nature of the phenomenon. Other investigators in this field all agree that the younger the culture used as inoculum, the shorter the lag. Although both Barber (5) and Penfold(7) have shown that lag is absent in subcultures made when the parent culture is growing rapidly, no attempt has been made to ascertain the particular time in the life cycle of a culture at which subcultures first display lag or

the length of time during which they fail to show it. The following experiments (Nos. 2 and 3), with pneumococcus and *Bacillus coli* as type organisms, show the behavior of subcultures made at frequent intervals during the various stages in the growth of the parent culture. The details of the experiments follow:

Experiment 2.—A flask containing 150 cc. of broth was inoculated with 0.3 cc. of a 24 hour broth culture of Pneumococcus Type I, stock strain, incubated in the water bath at 38°C., and subcultures were made one or more times during its various phases of growth. The growth of both parent culture and subcultures was observed at comparable intervals in each case by the plate method. The results are given in Tables II and III, and are represented in curves shown in Text-fig. 2.

TABLE II.

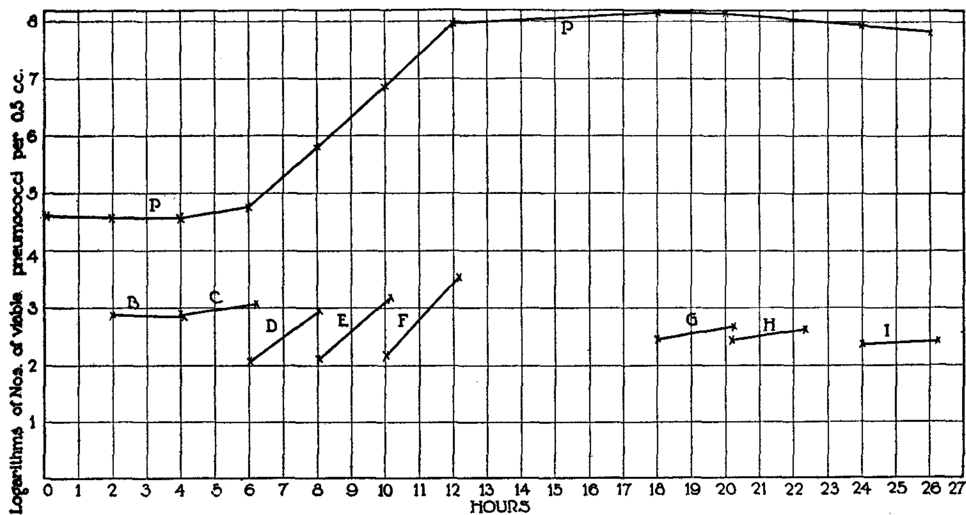
Comparison of Growth in Bouillon of Parent Culture and Subcultures of Pneumococcus Type I.

Parent culture.		Subcultures.		
Time after seeding.	Viable bacteria per 0.5 cc.	Subculture.	Time after seeding of parent culture.	Viable bacteria per 0.5 cc.
min.			*min.*	
0	41,000			
120	39,000	B	120	785
			245	713
240	38,000	C	240	766
			374	1,200
360	60,000	D	360	120
			485	898
480	625,000	E	480	127
			610	1,580
600	7,400,000	F	600	148
			730	3,490
720	95,000,000			
1,080	145,000,000	G	1,080	290
			1,215	483
1,200	136,000,000	H	1,210	273
			1,340	425
1,440	86,500,000	I	1,440	173
			1,575	263
1,560	65,000,000			

TABLE III.

Comparison of Growth of Parent Culture and Subcultures of Pneumococcus Type I, Expressed in Terms of Generation Times.

Parent culture.			Subcultures.			
Time after seeding.	No. of generations.	Generation time.	Subculture.	Time after seeding of parent culture.	No. of generations.	Generation time.
min.		*min.*		*min.*		*min.*
0– 120	0					
120– 240	0		B	120– 245	0	
240– 360	0.64	187.5	C	240– 374	0.65	206.1
360– 480	3.3	36.3	D	360– 485	2.9	43.1
480– 600	3.9	30.7	E	480– 610	3.6	36.1
600– 720	3.6	33.3	F	600– 730	4.5	28.8
720–1,080	0.6	600.0				
1,080–1,200	0		G	1,080–1,215	0.73	185.0
1,200–1,440	0		H	1,210–1,340	0.63	206.2
1,440–1,560	0		I	1,440–1,575	0.6	225.0



TEXT-FIG. 2. Growth of parent culture and subcultures of Pneumococcus Type I in bouillon at 38° C. *P* indicates parent cultures; *B* to *I* indicate subcultures.

Experiment 3.—A flask containing 50 cc. of bouillon was inoculated with two loops of a 24 hour bouillon culture of *B. coli*, and subcultures were made one or more times during the various phases of growth. The temperature of the water bath was 37°C. The growth of parent culture and subcultures was observed at comparable intervals in each case. The results are given in Tables IV and V and are represented in curves shown in Text-fig. 3.

TABLE IV.

Comparison of Growth in Bouillon of Parent Culture and Subcultures of Bacillus coli.

Parent culture.		Subcultures.		
Time after seeding.	Viable bacteria per 0.5 cc.	Subculture.	Time after seeding of parent culture.	Viable bacteria per 0.5 cc.
min.			*min.*	
0	20,000			
60	22,000	B	66	431
			129	1,390
120	64,500	C	120	120
			188	843
240	2,150,000	D	240	40
			309	349
			369	2,026
360	86,500,000	E	360	173
			426	614
525	485,000,000	F	525	107
			585	205
			645	1,257
705	970,000,000	G	705	194
			761	331
1,440	1,250,000,000	H	1,440	250
			1,508	378
			1,574	2,060
1,500	975,000,000			

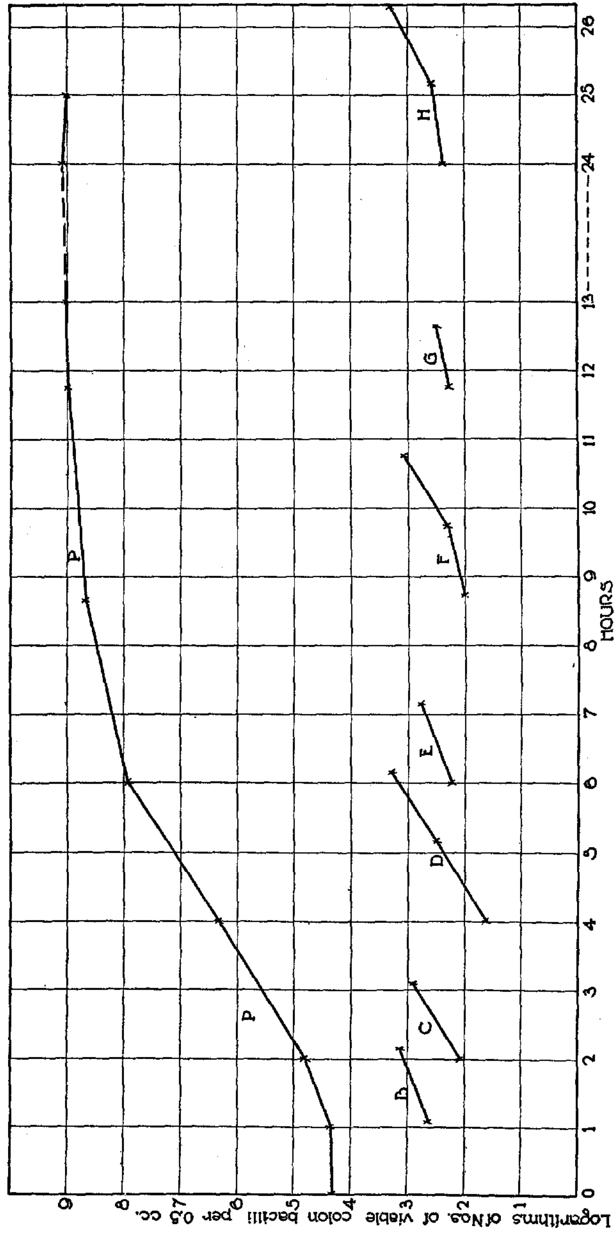
TABLE V.

Comparison of Growth of Parent Culture and Subcultures of Bacillus coli, Expressed in Terms of Generation Times.

Parent culture.			Subcultures.			
Time after seeding.	No. of generations.	Generation time.	Sub-culture.	Time after seeding of parent culture.	No. of generations.	Generation time.
min.		*min.*		*min.*		*min.*
0– 60	0.14	428.5				
60– 120	1.5	40.0	B	66– 129	1.6	39.3
120– 240	5.0	24.0	C	120– 188	2.8	24.2
240– 360	5.3	22.6	D	240– 369	5.6	23.0
360– 525	2.4	68.7	E	360– 426	1.8	36.6
525– 705	1.0	180.0	F	525– 585	0.93	64.5
705–1,440	0.36	2,041.6	G	705– 761	0.77	72.7
1,440–1,500	0		H	1,440–1,508	0.59	115.2

Experiments 2 and 3 show in both instances that if subcultures are made from the parent culture during the period of lag, the subcultures themselves show this same phenomenon. If, on the other hand, the subcultures are made while the parent culture is growing at a constant and maximum rate of growth, the subcultures show no latent period, but grow at approximately the same rate as that of the parent culture. Finally, subcultures made at a time when the parent culture is no longer growing at a maximum rate show a marked lag. This similarity in growth does not obtain if the subcultures are observed over a longer period of time, since after the latent period the subcultures repeat the entire cycle of the parent culture. Other experiments were carried out with *Bacillus fluorescens liquefaciens* and *Bacillus prodigiosus*, and identical results were obtained. They all show the importance of the relationship between the age of a culture and the presence or absence of lag in a subculture. Whether or not lag is present in a subculture depends upon the period in which the culture used for seeding happens to be at the time the subculture is made. That such was found to be the case with four different species of bacteria, motile as well as non-motile, suggests that the fact constitutes a principle applicable to all bacteria.

Occurrence of Lag in the Supernatant Fluid of Broth Culture.—The preceding experiments demonstrate that the behavior of bacteria,



TEXT-FIG. 3. Growth of parent culture and subcultures of *B. coli* in bouillon at 37°C. *P* indicates parent culture; *B* to *H* indicate subcultures.

when transferred to an environment favorable for growth, depends upon that particular period of the life cycle in which the culture happens to be at the time of transfer. The few bacteria remaining in the supernatant fluid after centrifugation of a culture at various phases in its life cycle were next studied to ascertain whether differences occurred in the behavior of the bacteria in the supernatant fluid depending upon the particular time at which the process of centrifugation was carried out.

Penfold (7) conducted one experiment with a 24 hour bouillon culture of *B. coli* and found that the bacteria which were left suspended in the supernatant fluid continued to grow on further incubation but with a definite lag. As no observations were made upon the growth of the culture which he used for centrifugation, it is impossible to say at what phase in its life cycle it happened to be at the time of centrifugation. As the supernatant fluid was kept on ice over night before incubation, a variable factor was introduced, since Penfold has shown that exposure to cold itself suffices to produce lag. No experiments seem to have been made with the organisms remaining in the supernatant fluid after centrifuging a culture during its period of maximum rate of growth.

Experiment 4.—A flask containing 500 cc. of bouillon was inoculated with 0.15 cc. of a 14 hour bouillon culture of Pneumococcus Type II, stock strain, and incubated in the water bath at 37.5°C. At three separate periods during the maximum growth of the culture, as indicated in Table VI, 50 cc. were removed and centrifuged at high speed for 30 minutes. Most of the bacteria were thrown down, leaving a clear supernatant fluid containing few organisms. The fluid was transferred to flasks which were incubated in the water bath and estimations of the number of viable organisms present were made at hourly intervals. The results are given in Table VI, and are further illustrated by curves in Text-fig. 4.

TABLE VI.

Growth of Pneumococcus Type II in Parent Culture and in Supernatant Fluids Obtained by Centrifuging at Stated Intervals.

Growth of Parent Culture.

Time after seeding.	Viable bacteria per 0.5 cc.	Time.	No. of generations.	Generation time.
min.		*min.*		*min.*
60	7,330			
120	11,000	60–120	0.58	103.4
180*	32,500	120–180	1.5	40.0
240	87,000	180–240	1.4	42.8
300*	331,000	240–300	1.9	31.5
360	887,000	300–360	1.4	42.8
420	4,850,000	360–420	2.4	25.0
480*	16,600,000	420–480	1.7	35.2
540	32,900,000	480–540	0.98	61.2
600	39,500,000	540–600	0.26	230.7
660	46,000,000	600–660	0.22	272.7
750	60,000,000	660–750	0.38	236.8

* At this point 50 cc. were removed from the culture and centrifuged at high speed for 30 minutes.

*Growth of Pneumococci in Supernatant A.**

Time after centrifuging.	Viable bacteria per 0.5 cc.	Time after centrifuging.	No. of generations.	Generation time.
min.		*min.*		*min.*
0	4,900			
120	111,000	0–120	4.5	26.6
240	1,300,000	120–240	3.5	34.2

* Obtained by centrifuging the parent culture 180 minutes after the initial seeding.

*Growth of Pneumococci in Supernatant B.**

Time after centrifuging.	Viable bacteria per 0.5 cc.	Time after centrifuging.	No. of generations.	Generation time.
min.		*min.*		*min.*
0	5,800			
120	132,000	0–120	4.5	26.6
240	610,000	120–240	2.2	54.5

* Obtained by centrifuging the parent culture 300 minutes after initial seeding.

TABLE VI—*Concluded.*

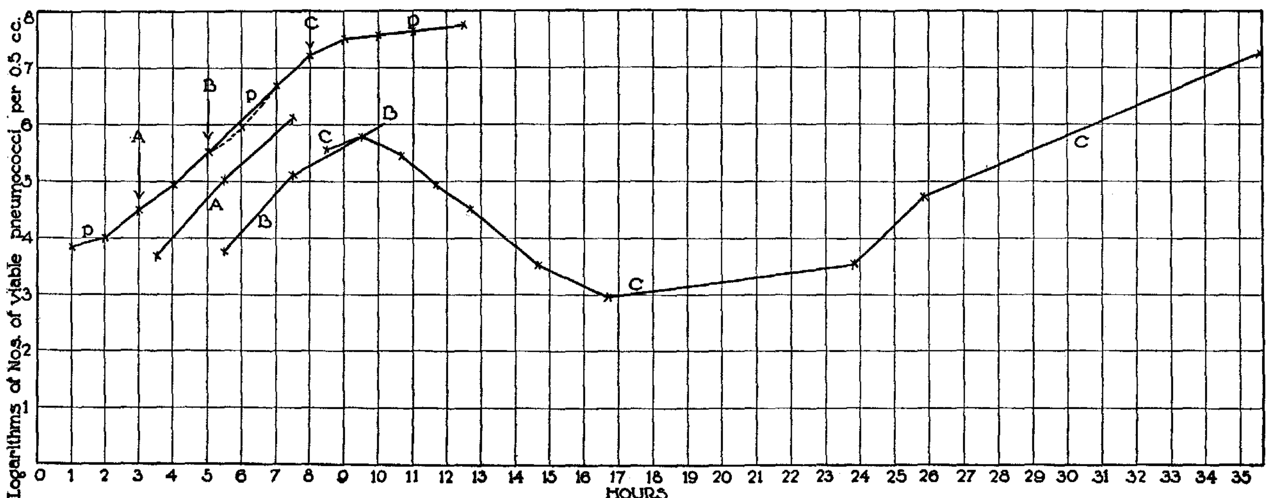
*Growth of Pneumococci in Supernatant C.**

Time after centrifuging.	Viable bacteria per 0.5 cc.	Time after centrifuging.	No. of generations.	Generation time.
min.		*min.*		*min.*
0	380,000			
60	630,000	0– 60	0.72	83.3
130	300,000	60– 130	0	
190	86,500	130– 190	0	
250	33,000	190– 250	0	
370	3,240	250– 370	0	
490	900	370– 490	0	
920	3,600	490– 920	2.0	215.0
1,040	55,000	920–1,040	3.9	30.7
1,630	17,000,000	1,040–1,630	8.2	71.9

* Obtained by centrifuging the parent culture 480 minutes after initial seeding.

Experiment 4 shows that during the early portion of the period of maximum rate of growth, the bacteria remaining in the supernatant fluid continue to increase in number at approximately the same rate as the organisms in the parent culture. If, however, the centrifugation is carried out toward the end of the period of maximum rate of growth, the viable bacteria in the supernatant fluid decrease in number over an appreciable interval of time (in this case 7 hours), then increase until finally, after 24 hours or more, there are present almost as many viable forms per unit volume as the parent culture attained. These results are illustrated graphically in Text-fig. 4, from which it is seen that the curves of growth of the bacteria in Supernatants A and B, obtained early in the period of maximum rate of growth, parallel the curve of growth of the parent culture; whereas the curve of Supernatant C, obtained at the end of the same period, does not parallel that of the parent culture, but shows, after the lapse of an hour, a fall and a subsequent rise. In other words, the bacteria in Supernatant C exhibit a marked lag. The conclusion to be drawn is that the presence or absence of bacterial lag in a supernatant fluid is dependent upon the age of the parent culture at the time centrifugation is carried out.

It is seen by reference to Text-fig. 4 that while the bacteria are



TEXT-FIG. 4. Growth of Pneumococcus Type II in parent culture and in supernatant fluids obtained by centrifuging at stated intervals. *P* indicates parent culture; *A*, *B*, and *C* indicate supernatant fluids. The arrows indicate the various times at which 50 cc. were removed from the parent culture and centrifuged.

dying the logarithms of the numbers of viable organisms, when plotted against time, fall upon an approximately straight line, indicating that the decrease in viable bacteria is a logarithmic one. It will be of interest to compare the behavior of pneumococci under such conditions with the course of destruction of bacteria when subjected to the action of disinfecting agents.

Krönig and Paul (10) showed that when anthrax spores are killed by disinfecting agents such as mercuric chloride the process is a gradual one. Madsen and Nyman (11) have confirmed these results, and have shown both from their own figures and from those of Krönig and Paul, that the process of disinfection takes place in accordance with a unimolecular reaction. Such a reaction is one in which a substance decomposes in such a manner that the amount undergoing decomposition in a unit of time bears a constant relation to the amount of substance present at the beginning of that interval of time. Chick (12) has shown that the same principle is applicable to the disinfection of anthrax spores and *B. paratyphosus* with phenol. The reaction is represented by the equation $\frac{dN}{dT} = KN$, which on integration yields $K = \frac{1}{t}\log\frac{N_1}{N_2}$ where K is the velocity constant, t is the interval of time between observations, N_1 the number of living bacteria present at the beginning of time t, and N_2 the number present at the end of time t. If the numbers of viable bacteria in a fluid medium decrease in accordance with this law, then the values determined for K should be constant when the values for N_1 and N_2 are substituted in the equation. Furthermore, the logarithms of the numbers of viable bacteria under such circumstances should fall on a descending oblique straight line when plotted against time. Text-fig. 4 shows that the bacteria in the supernatant fluid obtained at the end of the period of maximum rate of growth do decrease for several hours in such a manner that their logarithms fall upon an approximately straight line, which suggests that during this interval they are decreasing in accordance with the law of unimolecular reactions. On this account it seemed advisable to repeat Experiment 4 and obtain more frequent observations with a view of ascertaining whether the equation, which represents the course of such a reaction, can be strictly applied to the figures obtained experimentally.

Experiment 5.—A flask containing 500 cc. of bouillon was inoculated with 0.15 cc. of a 16 hour bouillon culture of Pneumococcus Type II, stock strain, and incubated in the water bath at 37.5°C. At two different intervals during the period of maximum rate of growth 50 cc. were removed and centrifuged at high speed for 30 minutes. The supernatant fluid was then removed to a flask and incubated further, and counts of both parent culture and supernatant fluids were made at frequent intervals. The results are given in Table VII and are further illustrated in the form of curves in Text-fig. 5.

TABLE VII.

Growth of Pneumococcus Type II in Parent Culture and in Supernatant Fluids Obtained by Centrifuging at Stated Intervals.

Growth of Parent Culture.

Time after seeding.	Viable bacteria per 0.5 cc.	Time.	No. of generations.	Generation time.
min.		*min.*		*min.*
0	13,100			
45	16,200	0- 45	0.3	150.0
185*	140,000	45- 185	3.1	45.1
300	915,000	185- 300	2.7	42.5
425*	11,500,000	300- 425	3.6	34.7
485	19,000,000	425- 485	0.7	85.7
560	39,100,000	485- 560	1.0	75.0
815	61,000,000	560- 815	0.6	425.0
1,220	100,000,000	815-1,220	0.7	578.5
1,575	113,000,000	1,220-1,575	0.17	2,088.2
1,880	80,000,000	1,575-1,880	0	

* At this point 50 cc. of culture were removed and centrifuged at high speed for 30 minutes.

*Growth of Pneumococci in Supernatant A.**

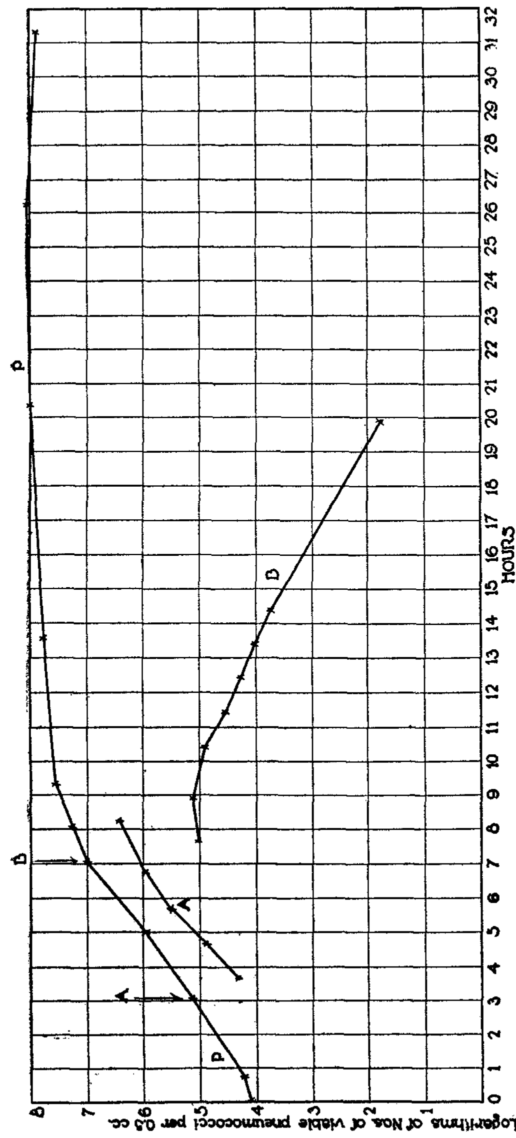
Time after centrifuging.	Viable bacteria per 0.5 cc.	Time after centrifuging.	No. of generations.	Generation time.
min.		*min.*		*min.*
0	21,300			
60	77,000	0- 60	1.8	33.3
120	335,000	60-120	2.1	28.5
185	885,000	120-185	1.4	46.4
275	2,650,000	185-275	1.5	60.0

* Obtained by centrifuging parent culture 185 minutes after initial seeding.

*Growth of Pneumococci in Supernatant B.**

Time after centrifuging.	Viable bacteria per 0.5 cc.	Time after centrifuging.	No. of generations.	Generation time.
min.		*min.*		*min.*
0	106,000			
75	132,500			
165	80,000			
225	35,000			
285	18,800			
345	10,900			
405	5,400			
735	60			
2,190	Flask cloudy; shows many pneumococci.			

* Obtained by centrifuging parent culture 425 minutes after initial seeding.



TEXT-FIG. 5. Growth of Pneumococcus Type II in parent culture and in supernatant fluids obtained by centrifuging at stated intervals. *P* indicates parent culture; *A* and *B* indicate supernatant fluids. The arrows indicate the various times at which 50 cc. were removed from the parent culture and centrifuged.

In Table VIII are shown the values of K obtained when the numbers of living bacteria in the second supernatant fluid are substituted in the equation $K = \frac{1}{t} \log \frac{N_1}{N_2}$. In this table are also shown the successive values for N_2 obtained by substituting for K its mean value, and for N_1 the number of bacteria found to be present when the organisms had begun to decrease. Opportunity is thus afforded to contrast the calculated theoretical values with those actually found by experimentation. This contrast is also illustrated in Text-fig. 6 in which the theoretical values are plotted in the form of a curve and the results determined by experimentation are designated as dots surrounded by circles.

TABLE VIII.

Application of the Behavior of Pneumococcus Type II in Supernatant B to the Law of Unimolecular Reactions.

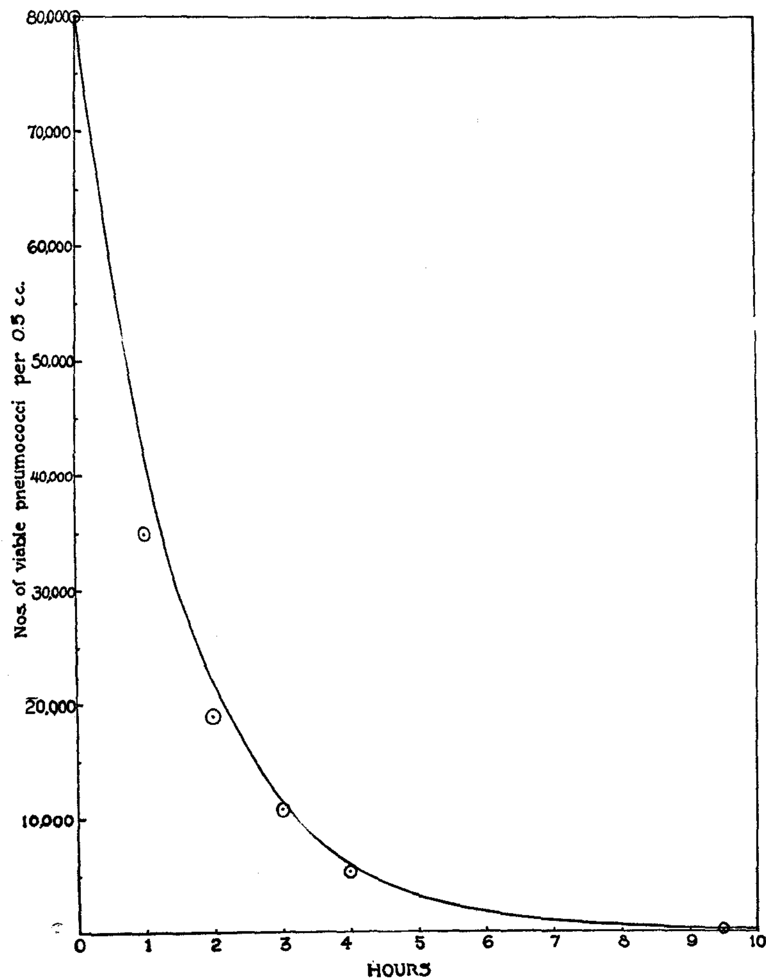
Time after seeding.	Viable bacteria per 0.5 cc.	Time.	Values of K calculated from equation $K = \frac{1}{t} \log \frac{N_1}{N_2}$ assuming $N_1 = 80,000$.	Successive values of N_2 assuming $N_1 = 80,000$ and $K = 0.0046$.
min.		*min.*		
625	80,000			
685	35,000	625– 685	0.0056	42,000
745	18,800	685– 745	0.0042	22,000
805	10,900	745– 805	0.0038	11,600
865	5,400	805– 865	0.005	6,100
1,195	60	865–1,195	0.003*	180
2,650	Flask cloudy; shows many pneumococci.			

Mean value of $K = 0.0046$

* The value for the constant K was not calculated when small numbers of bacteria were present, since Chick (12) has shown that in disinfection of *B. paratyphosus* when the culture has reached this stage the velocity of the reaction is slightly greater than one would expect, and the formula cannot be strictly applied.

Experiment 5 confirms Experiment 4 in that it shows that the pneumococci in the supernatant fluid at the beginning of the period of maximum rate of growth continue to multiply at the same rate as those in the parent culture, whereas the bacteria in the supernatant fluid obtained at the end of the period of maximum rate of growth do not continue to grow as do those in the parent culture, but exhibit a marked lag. This experiment shows also, as suggested by Experi-

ment 4, that the bacteria in the second supernatant fluid decrease in number in accordance with the law of unimolecular reactions, since the values obtained for K are quite constant, considering the experi-



TEXT-FIG. 6. Curve showing that the decrease of pneumococci in the supernatant fluid of a broth culture, centrifuged at the end of the period of maximum rate of growth, follows the law of unimolecular reactions. The solid line represents the curve of the calculated values; the dots surrounded by circles represent the values found by experimentation.

mental error necessarily introduced in counting. The closeness with which the course of the destruction of the bacteria follows that of a unimolecular reaction is illustrated by Text-fig. 6 in which the numbers of living bacteria found by experiment fall quite close to or upon the curve which represents the course of such a reaction. It may be said, then, that most of the pneumococci remaining in the supernatant fluid after centrifugation of a culture at the end of the period of maximum rate of growth die within a limited period of time in the course of which they follow the same law as do other bacteria when subjected to the action of disinfecting agents.

The fact that such a close parallelism exists between the behavior of bacteria subjected to the action of disinfectants on the one hand, and the behavior of the pneumococci in the supernatant fluid of broth cultures on the other, suggests that there are present in the supernatant fluid injurious substances that destroy large numbers of the bacteria and arrest the growth of still others. These hypothetical substances must be the products of bacterial growth and demonstrable in every broth culture of pneumococcus. But since the supernatant fluid does not become sterile and ultimately growth is resumed, it follows that either these injurious substances are destroyed or have their effect neutralized, or that the bacteria become adapted to them.

If pneumococci, when grown in broth, form substances inhibitory to their growth, it might be expected that these substances would be present in greatest concentration when the culture had reached the summit of growth. If, however, such substances are unstable on standing at 37°C. as suggested by Experiments 4 and 5, then when a broth culture of pneumococcus is incubated for some period beyond that of maximum growth, they should no longer be present in as great a concentration as that existing at the time when the culture was at its summit. Consequently, if the fluid portion of a culture which has undergone prolonged incubation is reinoculated with the same strain of bacteria, at least partial growth should result, provided the nutritive elements of the medium have not been exhausted. The next experiment was carried out to determine this point.

Experiment 6.—A flask containing 500 cc. of bouillon was inoculated with a culture of Pneumococcus Type II, stock strain, and incubated in the water bath at 37°C. for 3 days. Previous experiments (Nos. 1 and 2) have shown that at this time the culture has passed the summit of its growth, and that the bacteria are dying or are nearly all dead. The culture was then centrifuged until a clear supernatant fluid was obtained and this fluid was removed by a pipette to another container. Cultural tests showed only a few viable bacteria. These, however, died off rapidly on further incubation and at the end of 24 hours the fluid was found to be sterile. 50 cc. of this sterile fluid, representing the supernatant of a 4 day culture of pneumococcus, were inoculated with 1 cc. of a culture of the same strain which itself was in the period of maximum rate of growth (7½ hours old). 50 cc. of unused bouillon from the same original lot were inoculated with a like amount of the same culture, thus constituting a control. Both flasks were incubated at 37°C. in the water bath and counts made at frequent intervals. The results are given in Table IX.

TABLE IX.

Comparison of Growth of Pneumococcus Type II in the Supernatant Fluid of a 4 Day Culture of Pneumococcus Type II and in Unused Broth.

Growth in supernatant fluid of 4 day culture.					Growth in unused broth.			
Time after seeding.	Viable bacteria per 0.5 cc.	Time.	No. of generations.	Generation time.	Viable bacteria per 0.5 cc.	Time.	No. of generations.	Generation time.
min.		*min.*		*min.*		*min.*		*min.*
0	4,400				3,000			
120	212,000	0–120	5.5	21.8	110,000	0–120	5.1	23.5
240	2,700,000	120–240	3.6	33.3	925,000	120–240	3.07	39.0
300	5,750,000	240–300	1.09	55.0	2,125,000	240–300	1.2	50.0

Experiment 6 shows that if actively growing pneumococci are reinoculated into bouillon in which the same strain has already grown until the culture has sterilized itself (in this case after incubation at 37°C. for 4 days), they continue to multiply for several hours so that many organisms are formed. If substances are produced in a bouillon culture of pneumococcus which ultimately cause the death of many of the bacteria, then this experiment indicates that after prolonged incubation (in this case 4 days) they are no longer so potent as they were earlier, since they are not able to prevent the formation of several millions of bacteria per unit volume when an actively growing culture of the same strain is reinoculated.

Behavior of Pneumococci in Filtrates of Broth Cultures.—Experiments 4, 5, and 6 suggest that there are formed in broth cultures of pneumococcus substances which have an inhibitory or bactericidal action upon the bacteria themselves and that they lose potency when the cultures are allowed to incubate at 37°C. over a period of several days. In view of these facts, filtrates taken at different periods of growth were tested. Experiments have been reported in the literature which deal with the effect of filtrates of bacterial cultures upon the bacteria themselves when reinoculated into the filtrates. Apparently, however, the question has not been investigated from the standpoint of the relation of the age of a culture to the injurious action of its filtrate. Moreover, in previous work the cultures used for reinoculation have all been of an age sufficient to show a definite lag in the control medium, and for that reason the results are somewhat difficult of interpretation. The following experiment shows that the bactericidal action of a filtrate is dependent in part upon the age of the culture from which it is obtained.

Experiment 7.—A flask containing 1,000 cc. of bouillon was inoculated with 0.2 cc. of an 18 hour broth culture of Pneumococcus Type II, stock strain, and incubated at 37.5°C. At frequent intervals counts were made. At intervals of 1, 2, 4, and 6 days 100 cc. of the culture were removed and filtered through a Berkefeld filter. The same filter was used in each case after careful washing and sterilization. The filtrates were collected separately, and kept on ice until all were assembled and then inoculated with equal amounts of an actively growing (4 hour) culture of the same strain of pneumococcus, and incubated at 37.5°C. Bacterial counts were made at frequent intervals. The results are given in Tables X and XI.

TABLE X.

Growth of the Original Pneumococcus Type II Culture, from Which Definite Amounts Were Removed at Stated Intervals for Filtration.

Time.	Viable bacteria per 0.5 cc.	Time.	No. of generations.	Generation time.
hrs.		*hrs.*		*min.*
0	36,600			
1	34,500	0–1	0	
2	36,100	1–2	0.066	909.0
3	41,900	2–3	0.2	300.0
4	57,000	3–4	0.44	136.3
6	310,000	4–6	2.4	50.0
7	710,000	6–7	1.1	54.5
8	2,100,000	7–8	1.5	40.0
9	5,900,000	8–9	1.4	42.8
11.5	145,000,000	9–11.5	4.5	33.3
13	229,000,000	11.5–13	0.6	150.0
14	270,000,000	13–14	0.2	300.0
16	275,000,000	14–16	0.02	6,000.0
21	292,000,000	16–21	0.08	3,750.0
24*	231,000,000	21–24	0	
29.5	140,000,000	24–29.5	0	
48*	38,500,000	29.5–48	0	
75	970,000	48–75	0	
96*	2,280	75–96	0	
144*	0	96–144	0	

* At this point 100 cc. of culture were removed and filtered through a Berkefeld filter, the same filter being used in each case.

TABLE XI.

Comparison of Growth in Filtrates Obtained from a Culture of Pneumococcus Type II at Varying Periods in Its Life Cycle (See Table X).

4 hour culture of same strain of pneumococcus used for inoculation.

Time.	Viable bacteria per 0.5 cc.			
	1 day filtrate.	2 day filtrate.	4 day filtrate.	6 day filtrate.
min.				
0	1,350	1,540	1,380	1,570
60	2,300	6,250	8,800	10,600
120	4,000	11,700	28,700	44,000
180	8,500	19,800	58,000	82,000
245	8,400	28,000	82,000	127,000
330	9,000	34,000	111,000	173,000
420	8,800	46,000	169,000	225,000
515	5,000	39,000	243,000	322,000
605	3,950	35,000	330,000	254,000
695		18,000	363,000	167,000
1,350	8	570	104,000	91,000
	Clear.	Clear.	Slight cloud.	Slight cloud.

The results show that the filtrates obtained at varying intervals after the culture has passed its period of maximum rate of growth, differ in the deleterious effect which they exert upon the growth of the same strain of pneumococcus and that this inhibitory action is most marked at the time when the culture has reached the summit of its growth, after which it becomes progressively less as the culture is allowed to stand at 37.5°C., being least marked when the culture has sterilized itself.

Toxic Substances and Lag.—The experiments show that lag is a phenomenon which bacteria exhibit when the culture used for inoculation has passed the period of maximum rate of growth, and indicate that at this time substances are present in the fluid portion of a culture which exert a deleterious effect upon the bacteria, causing the death of many and temporarily inhibiting the growth of others; furthermore, that these substances are not permanent but disappear or lose their effect in part as the culture becomes older. If this interpretation of the experiments is the correct one, then, by exposing actively growing pneumococci for several hours to the action of a culture filtrate containing these deleterious substances in their maximum concentration, the bacteria should become injured and the injury should be manifested by a failure to grow at a rapid rate immediately upon inoculation into a new medium. In other words, a definite lag should be induced upon otherwise actively growing organisms by this procedure. The exposure would have to be carried out at temperatures below those at which the bacteria grow rapidly, hence in the ice box.

Experiment 8.—A 24 hour bouillon culture of Pneumococcus Type II, stock strain, was filtered through a Berkefeld filter and the filtrate found to be sterile. 1 cc. of a 6 hour broth culture of the same strain was inoculated into 50 cc. of this filtrate and a like amount inoculated into a flask containing 50 cc. of unused bouillon of the same lot as that from which the culture filtrate was obtained. The flasks were immediately placed in the ice chest for 18 hours; their contents were then centrifuged separately at high speed for 20 minutes and the supernatant fluids were removed. The sedimented bacteria were suspended in small amounts of isotonic salt solution and inoculated separately into two flasks containing 50 cc. of unused broth warmed to 37°C. The latter were incubated and the numbers of viable bacteria determined at frequent intervals. The results are given in Table XII.

TABLE XII.

Comparison of Growth of Pneumococcus Type II in Broth, after Exposure to 24 Hour Culture Filtrate and after Exposure to Unused Broth at 0°C. for 18 Hours.

Time.	After exposure to filtrate of 24 hour broth culture.				After exposure to unused broth.			
	Viable bacteria per 0.5 cc.	Time.	No. of generations.	Generation time.	Viable bacteria per 0.5 cc.	Time.	No. of generations.	Generation time.
min.		*min.*		*min.*		*min.*		*min.*
0	18,250				7,600			
60	28,200	0- 60	0.62	97.0	19,000	0- 60	1.02	58.0
110	55,000	60-110	0.93	53.0	61,000	60-110	1.6	31.5
180	170,000	110-180	1.6	43.7	312,000	110-180	2.3	30.4
240	409,000	180-240	1.2	50.0	1,070,000	180-240	1.7	35.3

Experiment 8 demonstrates that, by exposing actively growing pneumococci to the action of a culture filtrate of the same strain for a period of 18 hours at 0°C., it is possible to impose upon such bacteria a generation time almost twice as great as that shown by the control organisms. In other words, an increased lag has been induced upon such bacteria by exposing them to an environment in which they have been previously grown.

DISCUSSION.

Several views have been advanced to explain the nature of lag. The fact that bacteria, when inoculated into suitable nutritive media, do not immediately increase at the maximum rate of which they are capable, indicates that there is a lack of complete adjustment between the bacterial cell and its environment, and that this discrepancy must first be corrected before rapid growth can ensue. Obviously, one of two factors, cell or medium, must be at fault. It follows that in order that bacterium and nutritive medium may become completely adjusted to one another a change must take place in one or both of the factors. Lag, then, would represent the time necessary for such a change to be effected.

Keeping these points in mind, the possible causes of lag may be considered under two heads, according to whether the medium or

the bacterial cell is responsible. In other words, the cause may be extracellular or cellular in origin.

Extracellular Theory of the Nature of Lag.—According to this conception, the medium as such is not available for immediate growth and must first be altered by the bacteria themselves in one of the following ways: (1) by the agency of an extracellular enzyme whose function is to split up complex non-available food substances into simpler ones suitable for immediate utilization by the bacterial cell; or (2) by the elaboration of chemical substances which either exert a stimulating effect upon the metabolic processes of the bacteria (as suggested by Rahn (4)) or neutralize preformed toxic substances already present in the medium.

If the causes of lag are extracellular, then lag should always be present no matter what the age of the culture used for inoculation. That such is not the case is demonstrated by the fact that subcultures made from a parent culture when the latter is growing at a rapid rate continue to grow at the same rate and show no lag whatever. This fact, first indicated by Barber (5), later more clearly shown by Penfold (7) to be the case for colon bacilli, and further extended in our experiments with other species, disposes of the extracellular theory of lag and puts the responsibility for failure to grow upon the bacteria themselves.

Cellular Theory of the Nature of Lag.—Since bacterial lag is not due to a fault in the medium, it follows that the cause must be sought in the bacterial cell itself. In Experiments 2 and 3 it was shown that bacteria do not exhibit lag until the culture from which the inoculation has been made has ceased to grow at a maximum rate. When a culture has passed the period of maximum rate of growth the bacteria have changed in some manner since they are no longer able to multiply at once at a rapid rate when transferred to suitable medium. The nature of this change must constitute the cause of lag. The alteration which the bacteria undergo when a culture passes the period of maximum rate of growth may be explained by one or the other of the following reasons. (1) The bacterial cell may lack the proper concentration of intermediate products essential to the synthesis of bacterial protoplasm, a view suggested by Penfold (7); or (2) the bacterial cell may have sustained an injury to those functions or

parts of the cell which are concerned with the maintenance of bacterial metabolism. This conception was suggested by Müller (2) who first described the phenomenon of bacterial lag, although his experiments were not sufficient to establish his view conclusively.

The first idea is not substantiated by the facts brought out in Experiments 4 and 5 which demonstrate that when a broth culture of pneumococcus is centrifuged at the end of the period of maximum rate of growth, the bacteria in the supernatant fluid on further incubation exhibit a marked lag, whereas the bacteria in the original culture continue to grow quite rapidly. Consequently, there is a marked discrepancy in the behavior of the bacteria in the culture as a whole and of those in the supernatant fluid. Why should not the bacteria in each case behave in the same fashion? The lag which is manifested by the pneumococci in the supernatant fluid cannot be due to a lack in those organisms of sufficient intermediate products of metabolism, since the bacteria in the parent culture continue to grow rapidly for several hours at least.

When a culture is centrifuged those bacteria remaining in the supernatant fluid are exposed to the action of all the soluble substances that have been formed as a result of the growth of the culture as a whole. If such metabolic products are toxic for the bacterial cells, it would seem that their action would be stronger when the proportion of metabolic substances to bacterial cells has been increased. It would therefore seem not impossible that the marked lag which is manifested by the pneumococci under these conditions is due to an excess of deleterious substances which cause the death of large numbers and temporarily inhibit the growth of others.

The injurious substances may represent waste products from the cells or unused portions of the molecules of food substances. In any event, the amount present in the fluid is directly proportional to the number of bacteria that have been formed. For that reason they would not be present in any marked amount during the early portion of the period of maximum rate of growth and consequently the bacteria in the supernatant fluid obtained at that time would not show lag. Experiments 4 and 5 show that such is actually the case.

Additional evidence for the view that there are present in the sup-

ernatant fluid obtained at the end of the period of maximum rate of growth substances that exert an injurious effect upon the bacteria is supplied by Experiment 5, which shows that during the time that the organisms in the supernatant fluid are succumbing they do so in accordance with the law of unimolecular reactions, and in this respect their behavior is identical with that of other bacteria when subjected to the action of disinfecting agents.

Although the pneumococci in the supernatant fluid show a marked lag, many of them being killed, the fact that growth is resumed and that ultimately several millions are formed, indicates that the factors responsible for the injurious action have ceased to exist as such. This result may have been brought about through absorption of the substances by the bacterial cells, through spontaneous decomposition, or through decomposition by extracellular enzymes; or, on the other hand, through adaptation of the bacteria to the substances in question.

The idea that the substances are lost through absorption by the cells, or through decomposition or neutralization, is supported by Experiment 6, which shows that after a culture of pneumococcus has sterilized itself through incubation at 37°C. for 4 days, actively growing bacteria when reinoculated continue to grow for several hours. Additional evidence is offered by Experiment 7 which shows that culture filtrates of pneumococci manifest actual loss in inhibitory action, the longer the culture stands at incubator temperatures.

Finally, the view that broth cultures of pneumococcus contain substances that have the property of exerting a direct deleterious effect upon the bacteria themselves is supported by Experiment 8, which demonstrates that it is possible to impose upon actively growing pneumococci an increased generation time by exposing them at ice box temperatures to the action of a filtrate of a 24 hour broth culture of the same strain.

To sum up: (1) Bacterial lag represents an alteration of the bacterial cell. (2) The alteration is concerned with that function or structure of the cell which is essential to metabolism and hence growth of the cell, and is to be regarded in the nature of an injury. (3) The injury is due to the exposure of the cell to direct or indirect products of its own metabolism. (4) The injury may be sufficient

to bring about the death of the cell or it may merely inhibit growth temporarily. (5) The alteration is not always permanent. The bacteria may overcome or survive the condition, or the agents responsible for producing it may suffer actual destruction or be rendered inert. (6) Inasmuch as different media vary in their suitability for bacterial growth, the injury is likely to be most marked when bacteria are grown in a medium which is least favorable.

Stated briefly, it is supposed that if bacteria upon inoculation into suitable media do not begin to multiply immediately at the maximum rate of which they are capable, it is because they have sustained an injury from their former environment.

This view is in accord with the facts elicited by the experiments reported in this paper. It is essentially the view suggested by Müller, although, as already stated, his experimental evidence was meager. It was also advanced by Barber who thought he could distinguish differences in the morphology of the individuals of a 24 hour culture of colon bacilli and those of a culture that was growing rapidly.

The work of Moore (13) lends strong support to this conception of the nature of lag. He has shown that the blood serum of rabbits that have received certain amounts of ethylhydrocuprein (optochin) acquires the property of destroying pneumococci *in vitro*. If smaller amounts of the drug be administered, however, the serum is no longer able to kill the pneumococci, but increases the duration of lag.

The idea that bacteria form toxic substances which in turn inhibit the growth of the organisms is of course not a new one and has given rise to two opposing schools of opinion (14). The evidence here presented indicates that such substances are formed, and that they exert a direct injurious effect upon the microorganisms. Furthermore, these substances appear much earlier than is generally supposed, and on this account it would seem necessary to obtain accurate knowledge of the growth curve of a culture before the terms "old" or "young" are applied to it. Failure to do so has in the past led to erroneous conclusions as to the nature of lag. What is most important to recognize is the fact that the members of a bacterial culture vary considerably in their ability to grow in a given medium, depending upon the particular phase in which the original culture happens to be at the time of transplantation. The bearing

of this fact upon the possibility of variations in virulence of the members of a culture at different stages in its growth is at present under investigation.

Inasmuch as the bacteria in a culture which has passed its period of maximum rate of growth seem to have sustained an injury, it is suggested that in testing the efficacy of any bactericidal agent, actively growing cultures should be used, for if organisms already injured are employed, the result would be a summation of effects due to the agent and those represented by the injury to the bacteria, hence exaggerated ideas might be derived concerning the efficacy of the agent tested. Evidence in favor of this view is offered by the work of Chick (12) who has shown that the individuals of a 3 hour culture of *Bacillus paratyphosus* are more resistant to the action of antiseptics than those of an 18 hour culture of the same strain.

In order to preserve cultures over a long period of time, it has been found best to put on ice those which are in the period of maximum rate of growth, indicated in the case of broth cultures of pneumococcus by the presence of slight turbidity. Such bacteria retain their viability over a longer period than those of 18 hour cultures, hence necessitate less frequent transfers and give greater assurance of recovery, a factor of no little importance in the care of large collections of cultures.

CONCLUSIONS.

1. Cultures of *Diplococcus pneumoniæ, Bacillus coli, Bacillus fluorescens liquefaciens* and *Bacillus prodigiosus,* when grown in meat infusion broth exhibit an initial latent period when the culture used for inoculation is no longer growing at its maximum rate; if, however, the culture is growing at its most rapid rate the bacteria, upon subculture, show no latent period but continue to multiply at the same rate as that of the parent culture.

2. If broth cultures of pneumococcus are centrifuged at the beginning of the period of maximum rate of growth, the bacteria remaining in the supernatant fluid continue to grow at a rapid rate upon further incubation; if, however, the culture is centrifuged at the end of the period of maximum rate of growth, those bacteria which remain in the supernatant fluid show a prolonged latent period, during

which many of the organisms die. While the death of these bacteria is taking place the process follows closely the law of unimolecular reactions.

3. Actively growing pneumococci inoculated into the supernatant fluid from a 4 day culture of the same strain continue to grow rapidly for an appreciable time after inoculation.

4. Filtrates from 24 hour cultures of pneumococcus inhibit the further growth of actively growing pneumococci when the latter are inoculated into such filtrates. This inhibitory action of the filtrates is lost in part as the culture from which the filtrate is obtained is allowed to incubate longer.

5. Actively growing pneumococci, after exposure at low temperatures to the action of the filtrate of a 24 hour broth culture of the same strain, show a greater lag than the controls.

6. The foregoing facts offer strong support for the view that lag is an expression of injury which the bacterial cell has sustained from its previous environment.

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