51. *Physicochemical Aspects of Bacterial Growth. Part IX. The Lag Phase of* Bact. Lactis Aerogenes.

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1. A method is worked out for determining from total counts of bacterial cultures the lag, *i.e.*, the time elapsing between inoculation and the establishment of the logarithmic growth phase.

2. When *Bact. lactis aerogenes* is inoculated in to an artificial medium containing asparagine as nitrogen source, the lag first increases regularly with the age of the inoculum, and then settles down to a more or less constant value.

3. When a medium containing ammonium sulphate as nitrogen source is used, the lag is considerable for very young cultures (" early lag "), falls rapidly to a minimum value approaching zero, and then increases again as in the asparagine medium (" late lag ").

4. The early lag in the ammonium sulphate medium is diminished by traces of asparagine, but only insofar as these are themselves enough to support some independent growth. It is diminished by lowered concentration of glucose in the medium.

5. Sterile filtrates from an actively growing culture approaching the minimum lag (para. 3) remove the early lag. This shows that a diffusible lag-removing substance is produced in the solution.

6. This being so, the lag should be less, the greater the number of cells transferred in the inoculum, even when the amount of the old medium transferred is kept constant (as can be arranged by the use of a centrifuge). This is confirmed, and an approximate quantitative expression for the early lag can be established on the assumption that all cells make their contribution to a common store of the lag-removing substance.

7. With asparagine and with alanine the early lag is absent, and, correspondingly, the lag is independent of the inoculum size.

The lag phase of bacterial cells is known to depend upon their age (Penfold, *J. Hyg.*, 1914, **14**, 215; Stern and Frazier, *J. Bact.*, 1941, **42**, 479). The object of the experiments to be described was to study quantitatively the rate of development of lag with age.

We define lag as follows : n_0 is the initial number of living cells, n the number after a total time t, and k the growth rate constant. When logarithmic growth has once started, n_0 increases to n in a time t_1 given by $2{\cdot}303 \log n/n_0 = kt_1$. The lag is taken as $(t - t_1)$. If the transition from lag phase to logarithmic phase is not abrupt, we still define the lag formally by this relation.

Method.—The culture used was *Bact. lactis aerogenes*, grown at 40·0° in various media, heart broth, the glucose–phosphate–ammonium sulphate medium described in Part I (J., 1938, 1930), and a similar medium in which ammonium sulphate was replaced by asparagine or alanine. The synthetic media will be referred to briefly as ammonium sulphate medium, alanine medium, and asparagine medium, respectively. Counts were made by hæmocytometer as previously described. " Aerated " media are those through which a gentle stream of air of normal carbon dioxide content was bubbled.

True and Apparent Lag.—Direct viable counts could not be made in the number and with the accuracy required. Total counts of living and non-living cells were therefore made. This raises the following problem. If the total initial number of cells is n_0' of which n_0 are living, then from the definition given above, the time taken for n_0 to increase to n_0' will be reckoned as part of the lag, giving an apparent value greater than the true one. Before proceeding, it was necessary to investigate the effect of ignoring the difference between n_0 and n_0'.

Indirect method of determining viable counts and distinguishing between true and apparent lag. Let n_0 be initial number of living cells, x_0 the number of dead cells, n the total number at time t, and L the true lag. Neglecting the death of organisms during the period of growth itself, which will usually be small, we have $n = x_0 + n_0 e^{k(t-L)}$, whence

$$\mathrm{d} \ln n/\mathrm{d}t = (1/n)\mathrm{d}n/\mathrm{d}t = kn_0 e^{k(t-L)}/n = k(n - x_0)/n$$

If all the cells were living d ln n/dt would be k : in general we call it k_{apparent} ($k_{\text{app.}}$) and find $k_{\text{app.}} = k(1 - x_0/n)$ or

$$x_0/n = (1 - k_{\text{app.}}/k) \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad (1)$$

The true and apparent mean generation times are in the inverse ratio of the corresponding values of k.

Usually, growth curves are measured over the range where n is very much greater than n_0 or x_0, so that the apparent *m.g.t.* is indistinguishable from the true value, as shown by equation (1). If, however, we work with a

very large inoculum, and if an appreciable fraction of the cells are dead, then $k_{app.}$ will vary with n and the growth curve will no longer be logarithmic. From the value of $k_{app.}$ at any value of n, the value of x_0 can be calculated. Two examples of the application of this method follow.

In an experiment with a bouillon culture which showed lag, the real *m.g.t.*, determined from measurements with small inocula, was 22·2 mins. When bouillon was given a heavy inoculum (hæmocytometer count = 48), the growth curve was logarithmic with an apparent *m.g.t.* of 22·7 mins. at $n = 90$. From equation (1) it follows that $x_0 = 2$, or that 96% of the inoculum was living.

In the second example a slightly different procedure was followed. The experiments were made in an aerated asparagine medium, in which the inoculant had developed a lag of 300 mins. Growth curves were determined for a series of inoculum sizes, and the apparent *m.g.t.* values obtained from the slope of the curves of log n against t, at $n = 100$ and $n = 220$ respectively. The following table gives these values together with those calculated from equation (1) according to two hypotheses : (*a*) that the lag is real and the culture wholly living ($x_0 = 0$), and (*b*) that the true lag is zero and that the apparent lag is entirely due to the discrepancy between the total initial count and n_0. It is evident from the results that hypothesis (*a*) is very near the truth, *i.e.*, that a high percentage of the inoculum consisted of living cells.

Inoculum size.	Apparent *m.g.t.* $n = 220$.	Calc. (*a*). $n = 100$.	Apparent *m.g.t.* $n = 220$.	Calc. (*b*). $n = 100$.	Observed *m.g.t.* $n = 220$.	$n = 100$.
6·5	29·3	29·3	30	31	28	28
18	29·3	29·3	32	35·5	29	29
54	29·3	29·3	39	64	31·5	31·5
171	29·3	29·3	130	—	33	33

One may conclude, therefore, that with these cultures, typical of those which are not too old, the apparent lag really represents the delay in the onset of the logarithmic phase.

This ceases to apply when the cultures are very old or when they are kept in an unsuitable medium. The following experiments illustrate the fact that the very long lags sometimes observed with sub-cultures from such a medium are fictitious. An unaerated ammonium sulphate culture was inoculated at different ages into the aerated asparagine medium. Inocula of increasing age showed rapidly lengthening apparent lags, and finally appeared to have become quite sterile. The size of living inoculum from which growth could have occurred during the apparent lag period was calculated. It was found that the last culture to grow before sterility was reached would have grown from something of the order of a single cell. Thus one appeared to have been measuring, not the lengthening of the true lag, but the death rate of the organisms, since the hypothesis that this was occurring led to the prediction that the culture would become sterile at the observed point. Some typical results are given below :

Series I.			Series II.		
Age, mins.	Appt. lag, mins.	Fraction assumed alive.	Age, mins.	Appt. lag, mins.	Fraction assumed alive.
1220	20	0·63	720	17	0·68
1490	30	0·50	1023	60	0·23
1880	103	0·09	1515	750	3×10^{-8}
2274	860	2×10^{-9}	2130	inf.	0
2982	inf.	0			

In these experiments the inoculum size was 1·5 (hæmocytometer reading) which corresponds to 5×10^7 organisms per culture tube : thus the calculated living count of the last tube to grow was 0·1 cell in series I and 1·5 cells in series II. Since there are statistical fluctuations in death rate when the numbers are small, these results may be taken to represent fairly enough the passage over the border between viability and sterility.

With the above methods of discrimination available, it is believed that the lags recorded in the following sections truly represent the times taken for the living cells to develop from the resting phase to the phase of logarithmic growth.

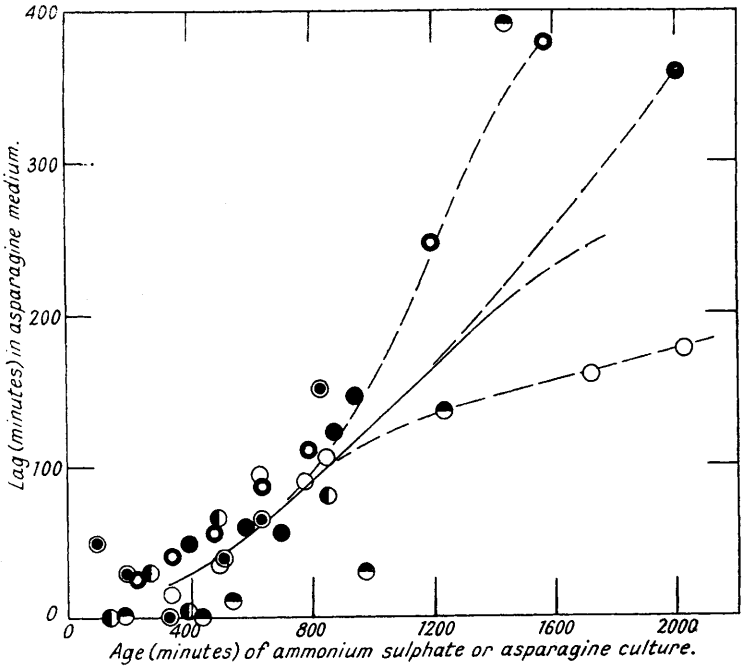
Development of the Lag with Time.—(a) *Early stages of development.* In the early stages the lag is fairly reproducible, and seems to be the same definite function of the age of the culture whether the ageing takes place in an asparagine or in an ammonium sulphate medium (Figs. 1 and 2). The ages are measured from the times at which the experimental count had the arbitrary value unity, which in fact corresponds fairly closely to the beginning of the logarithmic phase.

The lag measured in the ammonium sulphate medium at 40·0° is, for very young cells, of a considerable length, falls in the course of about 500 mins. to nearly zero, and then increases again rather rapidly. The phenomena occurring before and after the minimum will for brevity be referred to as early lag and late lag, respectively.

When cultures, whether aged in the asparagine or in the ammonium sulphate medium, are inoculated into asparagine there is little or no sign of early lag. The lag starts from zero for the youngest inocula and at an age of about 1500 mins. has increased to about 240 mins. In the ammonium sulphate medium in the 1500 mins. of ageing subsequent to the minimum the lag increases to about 500 mins., *i.e.*, it develops rather more rapidly than the lag measured in asparagine.

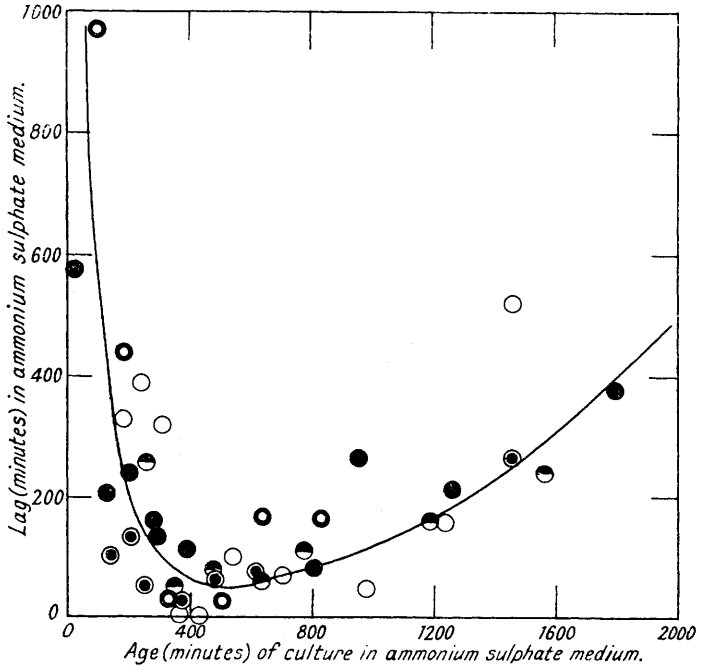
The development of the lag, as measured in the asparagine medium, is progressive during the period of growth of the parent culture and becomes slower as the stationary phase sets in. Qualitatively at least there

FIG. 1.



Black-centred circles } *cultures ageing in ammonium sulphate medium.*
Horizontally divided circles }
Others: cultures ageing in asparagine medium.

FIG. 2.



Black-ringed circles } *culture ageing in ammonium sulphate medium.*
Open circles
Full circles }
Half-shaded circles } *culture ageing in asparagine medium.*
Black-centred circles }

is a correspondence between the development of the lag in the new medium and the cessation of active growth in the old. During the stationary phase the cells shrink in size: during the lag they swell again, as has been noted by other authors working with other organisms.

A few experiments were made with cells grown in and sub-cultured for test in an alanine medium. The general behaviour was similar to that found in the asparagine medium as the following numbers show :

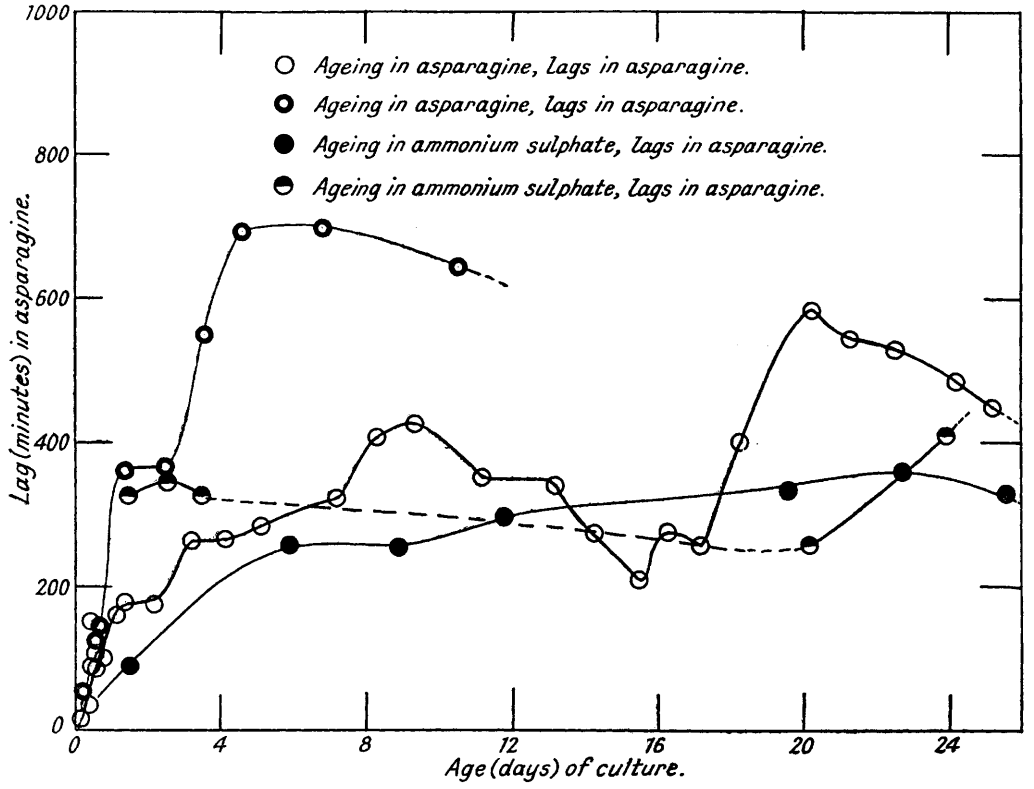
Age (mins.) ...	220	282	387	480	615	720	915	1465	3125	4410	5730	7372	8095
Lag (mins.) ...	28	28	39	29	49	40	19	146	282	512	646	640	558

The rate of development of lag (measured in ammonium sulphate medium) when the bacteria aged in heart broth is indicated by the following numbers (means of several determinations) :

Age (days)	1·0	2·0	4·0
Lag (mins.)	110	210	380

(b) *Later stages of development.* These are shown in Fig. 3. The lag is less reproducible than in the early stages. It rises rapidly at first and then settles down to a more or less constant level about which it shows

FIG. 3.



irregular fluctuations, due to causes at present unknown. This level itself varies from culture to culture within certain limits, as may be seen in the figure. Although the operation of an unknown factor is evident, it is also clear that the cells tend to settle down to a constant resting condition—a state from which they must be brought before division will again occur. [Observations have been published (Martin, *J. Gen. Physiol.*, 1932, **15**, 691) which indicate that there are two phenomena at least in lag : in the initial stages the bacteria appear to be in a resting condition with a very low oxygen consumption rate per g. of bacterial nitrogen. In the later stages oxygen consumption rate increases, but division is still delayed. This question will be returned to later.]

Factors influencing Early and Late Lag in the Ammonium Sulphate Medium.—(1) *Glucose concentration.* Both early and late lag are reduced when the glucose in the medium is diluted, the former relatively more than the latter, as is shown by the numbers in Table I.

TABLE I.

Early lag : age of inoculant 200 mins.						
Concn. of glucose (relative)	1·0	0·8	0·6	0·4	0·2	0·05
Lag (mins.)	837	763	661	510	253	241
Late lag : age of inoculant 28 hours.						
Concn. of glucose (relative)	1·0	0·8	0·6	0·4	0·2	0·05
Lag (mins.)	1281	1132	918	920	921	916

(2) *Filtrate from a young culture.* The early lag is reduced or even abolished by the addition of a filtrate, sterilised by heating to 100°, from young actively growing cells. An aerated ammonium sulphate culture was

divided into two parts, one being filtered and sterilised. The other was used as inoculant for more ammonium sulphate medium to which various amounts of the filtrate were added. The results are in Table II. In a second experiment (see Table III) an ammonium sulphate culture which had just grown to the point of zero lag was filtered, and the filtrate added to media with a very young inoculant.

TABLE II.

Vol. of inoculum, 0·1 c.c.					
Vol. of filtrate, c.c.	0	0·1	0·25	0·5	1·0
Lag (mins.)	182	106	46	70	12

TABLE III.

Filtrate, c.c.	0	0	0·1	0·5	0·75	1·0
Lag (mins.)	243	376	160	170	180	47

In contrast with this, the filtrate appears to have comparatively little influence on the late lag, the cause of which must be different (see Table IV).

TABLE IV.

Filtrate from young ammonium sulphate cultures : inoculant 3 days old.							
Filtrate, c.c.	0			0·1	0·5	1·0	1·5
Lag (mins.)	480	560	605	545	585	495	630
Filtrate from cultures of zero lag : inoculant 1 day old.							
Filtrate, c.c.	0			0·1	0·1	1·0	
Lag (mins.)	470	470	459	410	410	392	

(3) *Size of inoculum.** The above results indicate that a diffusible substance is produced during growth which can remove early lag. We should therefore expect the lag to depend upon the inoculum size : first, because some of the original medium is transferred with the inoculum, and, secondly, because larger numbers of organisms will more rapidly build up the required concentration of the lag-removing substance in the new medium. These expectations are confirmed.

Aerated ammonium sulphate culture was used as inoculant (*a*) at count $n_1 = 132$ and (*b*) at count $n_1 = 960$: the following results were obtained. (The " calc." values in Table V refer to the theory outlined in the next section.) v is the volume in c.c. of inoculum and n_0 the initial count.

TABLE V.

	(*a*).				(*b*).		
v.	n_0.	Lag (obs.).	Lag (calc.).	v.	n_0.	Lag (obs.).	Lag (calc.).
0·1	0·57	185	185	0·01	0·40	>450	645
0·2	1·13	0	0	0·03	1·20	260	260
0·3	1·70	−30	0	0·10	4·0	50	50
0·5	2·83	− 8	0	0·20	8·1	10	0

In the above experiments the amount of medium transferred increased in proportion to the number of cells in the inoculum. To investigate the effect of number while the amount of active substance was kept constant, an aerated ammonium sulphate culture was grown to a small count and then centrifuged. The clear medium was removed by a pipette, and the residue shaken to give a heavy suspension. By mixing the latter with the clear medium in different proportions for use as inoculum, n_0 could be varied while v was kept constant. The results are in Table VI.

TABLE VI.

n_0	2·54	0·338	(0·161	0·127)	0·144	(mean)	0·055
Lag (obs.)	136	804	(1030	970)	1000	(mean)	1220
Lag (calc.)	136	640			950		1220

Volume of inoculum 0·1 c.c. throughout.

Even with comparatively old ammonium sulphate cultures the lag is still dependent upon the inoculum size :

Vol. of inoculum, c.c.	0·005	0·01	0·02	0·03	0·04	0·11	0·22
Lag (mins.)	3000	3000	1500	3000	1320	920 760	625

(4) *Additions of asparagine.* The early lag is reduced by small additions of asparagine to the ammonium sulphate medium : w is the weight in g. $\times 10^{-6}$ of asparagine added to the 26 c.c. of culture solution.

w	0				2·5	5	15	50	100	250	500
Lag (mins.)	400	404	427	444	435	387	377	260	120	90	90

* Compare other works on inoculum size from different points of view : Penfold, *loc. cit.*; Mueller, *Proc. Soc. exp. Biol. Med.*, 1939, **40**, 632.

The reduction seems, however, only to become marked when the asparagine is concentrated enough to contribute independently to the growth: there is no early lag in asparagine and the cells which it allows to grow themselves contribute to the removal of the lag in the others.

w	10	100	500
n_{24}	26	47	170

n_{24} is the count after 24 hours' growth in a medium containing the asparagine with no other nitrogen source. It may be compared with $n_{24} = 2000$ for the standard ammonium sulphate medium.

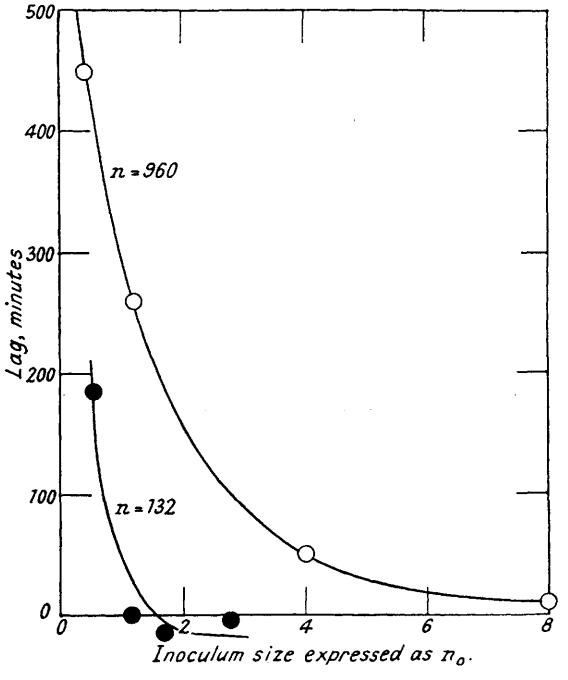
Theory of the Early Lag in Ammonium Sulphate Medium.—We suppose that the lag ends when the concentration c of some active substance reaches in each cell a critical value c'. We write:

$$c = \alpha v + \beta n_0 t + \gamma t$$

where v is the volume of old medium transferred with the inoculum, and αv the concentration of the active substance thereby set up: α will be a function of the count n_1 of the inoculant medium (so long as the cells have not been separated from their original medium by centrifuging or otherwise), and n_0 is the number of cells per c.c. of the new medium, so that $\beta n_0 t$ is the contribution to c which they have made in time t by generating active substance in the medium. γt represents the concentration built up in a given cell without the contribution of the others. (The simple summation of the last two terms is an approximation, but will not be far from the truth.) When $c = c'$, $t = L$, whence

$$L = (c'/\beta - \alpha v/\beta)/(n_0 + \gamma/\beta) \quad . \quad . \quad (2)$$

FIG. 4.



From this equation we deduce the following: (*a*) When n_0 is constant but v varies, as when filtrate is added, the lag should decrease linearly with increase of v; this is at least qualitatively true (see Tables II and III). (*b*) When v is constant and n_0 varies (centrifuge experiments above), L assumes a form such as

$$L = 382/(n_0 + 0{\cdot}258) \quad . \quad . \quad . \quad (3)$$

which has been used to express the results in Table VI. (*c*) In Table V, n_0 and v vary together: moreover, α will be different for Table V (*a*) and Table V (*b*). Taking from (3) the value of γ/β, we can express the results in Table V (*b*) by $L = (448 - 2350v)/(n_0 + 0{\cdot}258)$. From Table V (*a*), to give the correct value for the lag for the smallest n_0, α/β must be taken as 2950. Taking $c'/\beta = 448$ from Table V (*b*) and combining it with (3), we find $\alpha/\beta = 660$. As the various calculated numbers show, these assumed constants reproduce the results in the tables as nearly as can be expected, so that we may regard the relative values of α/β as approximately correct. β should be a constant but α should depend upon the amount of active substance in the inoculant medium. It should therefore vary with the count n_1 of the latter. The relation found is:

n_1	25	132	960
α/β	660	2950	2350

This would indicate that the amount of active substance in the medium increases at first and then remains steady or declines, as would be expected on general grounds and from the fact that the lag increases again after a certain stage.

Contrast between Ammonium Sulphate and Asparagine or Alanine.—The early lag was little in evidence in the asparagine or alanine media. In confirmation of the views of the last section, the lag has been shown to be independent of the inoculum size with these media. An aerated asparagine medium was made, at two different ages, the inoculant of a new asparagine medium, with the following results:

Count of parent medium.	n_0 for new medium.	Lag, mins.	Count of parent medium.	n_0 for new medium.	Lag, mins.
1960	24·9	56	3500	29·6	350
	8·3	40		14·8	490
	2·5	28		5·9	350
	0·83	50		1·8	338
				0·6	345

Similar results were found with a young culture from alanine transferred to fresh alanine.

Discussion.—During the early lag a diffusible substance appears in the solution : it may be transferred with inocula or with sterile filtrates and reduce lag in fresh media. Each cell helps to end the lag of the others, whence the inverse relation, in ammonium sulphate media, of inoculum size and lag. Since early lag is not found in asparagine or alanine media, the active substance can plausibly, though not necessarily, be assumed to be a nitrogen compound, synthesised easily from amino-acids but less easily from ammonium sulphate.

The ending of the lag phase must depend upon two separate factors : the cells must begin to increase in size, and the growing cells must become capable of dividing. Either of these processes might become the rate-determining one. Which is the more important in the above experiments is not yet clear : the matter is under investigation.

After passing through a condition in which they show no lag, the cells age and the lag increases. The ageing may involve the decay of an active substance, but morphological changes occur also. The cells shrink in size, and there may be more profound internal changes. The curve showing the development of late lag could be interpreted as the decay curve of a chemical substance in the cells, but since filtrate has little effect on late lag, the structural changes must also have a profound influence.

The development of the lag does not continue indefinitely, but there is a tendency for it to settle down and then fluctuate about a mean which is maintained over a fairly long period. The existence of something like a limiting state is more striking than the fact that it is not a particularly stable one. Some hypothesis is needed to explain why there is this roughly steady value and why it changes with very slight changes of conditions. One may suppose that during the development of the lag various chemical and structural changes occur in parallel, and that the chemical balance of the organism in the steady, resting state depends upon the relative speeds of these processes. This same balance determines the leeway to be made up when the cell is placed in a new medium and hence the actual magnitude of the lag. This also is a starting point for further investigation.

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