50. *Physicochemical Aspects of Bacterial Growth. Part VIII. Growth of* Bact. Lactis Aerogenes *in Media containing Ammonium Sulphate or Various Amino-acids.*

By R. M. Lodge and C. N. Hinshelwood.

When *Bact. lactis aerogenes* is grown in a liquid synthetic medium containing ammonium sulphate or various amino-acids as the sole source of nitrogen: (*a*) The maximum population of cells which the medium supports varies very widely with the nitrogen source. (*b*) For certain nitrogen sources this population is greatly increased by aeration of the solution; for others not. (*c*) In contrast with this diversity, the growth rates all fall within a comparatively narrow range. (*d*) After a slight mutation had occurred, the growth rate in the ammonium sulphate medium increased by about 40% and the maximum population now showed a positive response to aeration, previously absent. (With certain amino-acids there was no such change in behaviour.)

It is suggested that the maximum population is in part controlled by the formation of an inhibiting substance removable by a cellular oxidation; that the earliest steps in the utilisation of the amino-acids are not usually rate-determining; and that the mutation involved a slight change in a delicate balance of these and other reactions. Similar considerations help to explain occasional departures from logarithmic form of the growth curve.

The different phases of bacterial growth, namely, lag, logarithmic and stationary phases, are separately influenced by such factors as concentrations of medium constituents, pH, and the presence of foreign substances (Parts IV, V, and VI; J., 1939, 1683, 1692; 1940, 1565). It is to be hoped that the detailed investigation of these effects will assist in the analysis of some of the physicochemical mechanisms underlying cell growth.

In the experiments to be described the nitrogen source in the growth medium was varied. *Bact. lactis aerogenes* was grown in a solution containing glucose (38·5 g./l.), potassium dihydrogen phosphate (3·46 g./l.), brought to pH 7·12 with sodium hydroxide, and magnesium sulphate (0·038 g./l.) together with various nitrogen sources, ammonium sulphate (0·96 g./l.) or different amino-acids giving an equivalent supply of nitrogen. The culture was that used in previous work: it was usually subcultured, just before use, in the artificial medium, from which it was inoculated into the culture medium by a calibrated pipette of about 0·1 c.c. Cell counts were made in a hæmocytometer as previously described. The number (n) tabulated below is the number in 16 small squares of the counting grid: multiplied by $1{\cdot}25 \times 10^6$ it gives the number per c.c.

In the first series of experiments the artificial medium was inoculated with two or three loops of a bouillon culture and incubated overnight. Next morning 0·1 c.c. of the young culture, which was counted, was transferred to a tube of new medium. The mean generation time (*m.g.t.*) and the steady population attained at 24 hours (n_{24}) were determined; n_0, the initial count, was known from the count of the inoculum and the ratio of volumes of inoculation pipette and growth medium. The curves of the logarithm of the count, n, against time usually showed a well-defined straight portion from which the *m.g.t.* could be calculated. By extrapolating to the time where $n = n_0$, the length of the lag phase could be read off.

The results may be grouped as follows.

(1) Under the conditions of these experiments the lag was nearly zero with different amino-acids, but with ammonium sulphate it was variable and sometimes considerable. This fact is illustrated in Fig. 2, in which

log n/n_0 is plotted against time; where the line passes through the origin the lag is zero; otherwise the intercept on the time axis where log $n/n_0 = 0$ gives the lag. The whole question of lag is dealt with more fully in the next paper.

(2) The maximum population of cells in media containing different amino-acids varies widely. With certain amino-acids, though not with others, it also depends in the most marked way upon whether or not a current of air (normal carbon dioxide content) is passed through the medium (Table I). As shown in Part IV (*loc. cit.*),

TABLE I.

Nitrogen source.	n_{24}. Solutions aerated.	n_{24}. Solutions not aerated.
Glycine	34, 63 (110) *	122
Alanine	1470, 1040, 1390, 815 (2500)	235, 40, 125
α-Amino-*n*-butyric acid	20	11
,, ,, ,,	790	72
α-Amino*iso*butyric acid	23, 22	18, 16
α-Amino-*n*-valeric acid	300	230
Valine	1230, 1205 (1440)	55
Leucine	740, 740 (740)	72
Cysteine	46, 48	50, 53
α-Amino-octoic acid	124	—
Aspartic acid	2100, 2240	630
Glutamic acid	1570, 1550 (2900)	50
Asparagine	4800	1000
Ammonium sulphate	560 (2140, 1780)	530 (470, 530)
,, ,,	550	540
$(NH_4)_2SO_4$ + glycine	—	485
$(NH_4)_2SO_4$ + valine	—	380

TABLE II.

Mean generation times.

(The solutions were aerated but in the early stages of growth aeration made little difference to the *m.g.t.*)

Nitrogen source.	*m.g.t.* (mins.).	Nitrogen source.	*m.g.t.* (mins.).
Ammonium sulphate	46 (32)	Aspartic acid	39
Glycine	49	Glutamic acid	40
Alanine	41 (40)	Asparagine	30·5 (29·2)
Valine	Early 40 (—)	Leucine	36
,,	Late 100 (114)	α-Amino-octoic acid	<42

* The values in parentheses were measured after the slight mutation of the bacterium.

the setting in of the stationary phase is determined, according to conditions, either by the exhaustion of food or by the accumulation of substances adverse to growth (including adverse pH). The early setting in of the stationary phase with some of the unaerated amino-acids is not due to exhaustion or to wastage of nitrogen in side reactions: this is proved by the following experiment. n_{24} for aerated alanine was 1470, for unaerated alanine only 125. On subsequent aeration of the latter the count rose to 1390. The cessation of growth in the first instance was therefore probably due to the accumulation of an inhibitor removable by aeration. This hypothesis can reasonably be extended to explain the early cessation of growth with some of the other amino-acids even in presence of air: here we have only to assume that the inhibiting product is not capable of sufficiently rapid removal by oxygen.

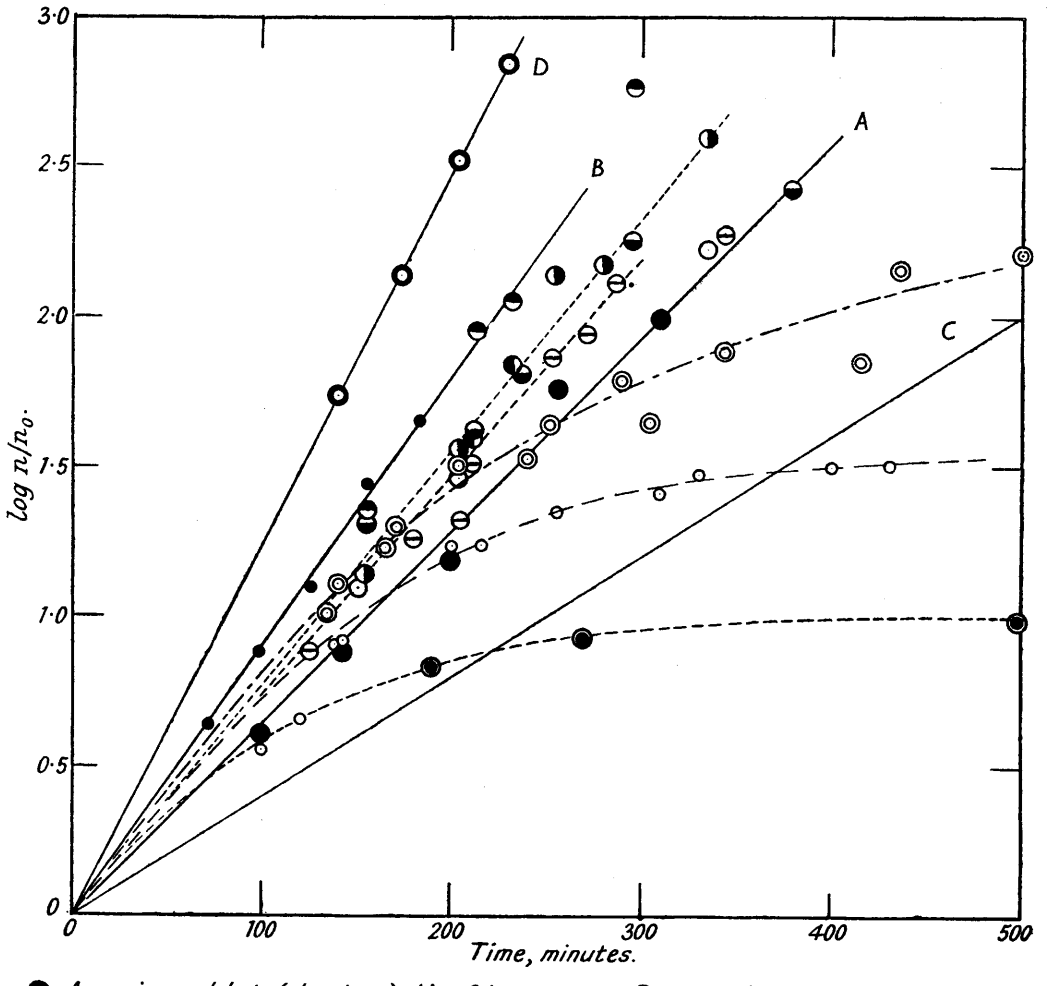
The removal is probably an oxidation and not the blowing off of a volatile product, since growth rates and stationary populations are practically independent of the actual rate of aeration. When two nitrogen sources are present together, one giving by itself a high value of n_{24} and the other a low, the actual value is close to the higher. For example, ammonium sulphate unaerated gave $n_{24} = 535$, glycine and valine gave respectively 122 and 55. Ammonium sulphate in presence of the two respective amino-acids gave 485 and 380. Either the ammonium sulphate inhibits toxin formation from the amino-acids, or else the substances which they produce and which would inhibit their own utilisation fail to affect the processes by which ammonium sulphate is utilised in the cell. A similar effect in which amino-acids which support a small n_{24} fail to inhibit growth to large n_{24} values in others is illustrated in Fig. 2.

One might have supposed that in all these experiments the limiting factor was oxygen supply, but this hypothesis would not easily explain the very great differences between the various amino-acids, and the fact that, with some, growth responds to aeration, and with others not.

It might have appeared profitable to try correlating the above-mentioned differences with the structure of the amino-acids. The following observations seem, however, to show that they depend upon a very delicate quantitative balance of the enzyme processes of the cell. Some months after the first experiments were made the bacteria underwent a slight mutation—not detectable by any qualitative criterion and only shown in slight changes in growth rate (see later). With ammonium sulphate, aeration, which had previously made little difference to n_{24}, now had a marked effect, the values of n_{24} for the unaerated and the aerated cultures being on the average 500 and 2000 respectively. In some of the other cases there was little change.

(3) Although the values of n_{24} varied widely for the different nitrogen sources, the mean generation times all fell within a comparatively restricted range. This is shown in Fig. 1 and Table II. In the early experiments ammonium sulphate gave a *m.g.t.* of 45′ and the fastest growth occurred in asparagine where the *m.g.t.* was 29′. As seen in Fig. 1, change of the amino-acid affected the *m.g.t.* far less than a change to another medium such as bouillon, lactose–phosphate or lactose–tartrate. Where the value of n_{24} is small the *m.g.t.* is hard to determine, but from the composite plot in Fig. 1 it looks probable that the initial growth rates with very many

FIG. 1.



- ● *Ammonium sulphate (slow type). Line OA.*
- ◎ *dl-Valine (two curves).*
- ○ *Meat extract ('heart broth'). Line OD.*
- ◉ *α-Aminoisobutyric acid.*
- ◒ *l-Leucine.*
- ◓ *Asparagine.*
- ◑ *Aspartic acid.*
- ◐ *dl-α-Aminooctoic acid.*
- ⊖ *dl-Alanine (two curves).*
- ○ *Glutamic acid.*
- ○ *Glycine.*
- ● *Ammonium sulphate (fast type) Line OB.*

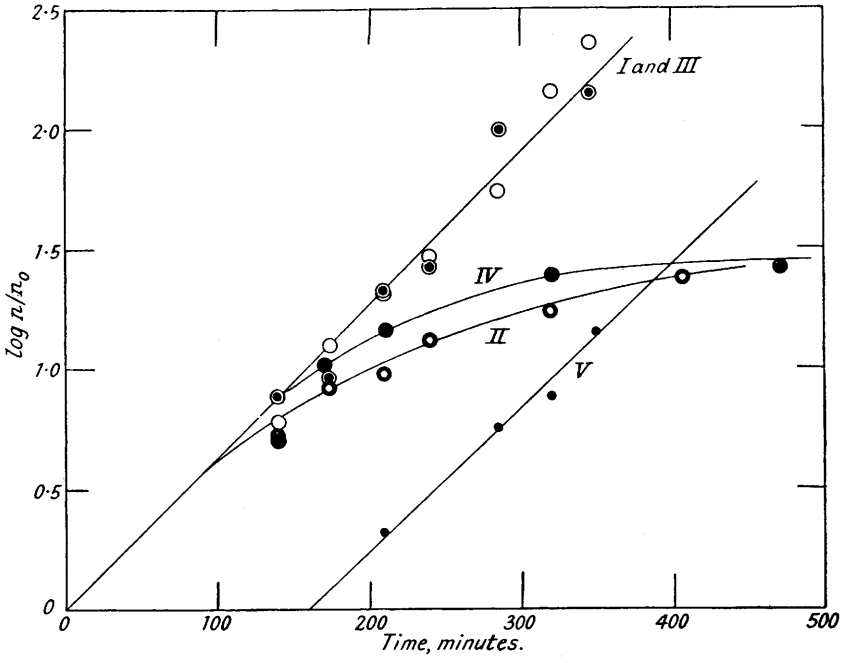
of the amino-acids are really very closely similar even where the *m.g.t.* is not accurately tabulated in Table II. Mixtures of several amino-acids gave no particular advantage, as is shown by the curves in Fig. 2.

(4) After sub-culturing for about a year, the *Bact. lactis* was observed to have undergone a slight mutation. (Plating out and application of qualitative tests showed that no infection had occurred.) The n_{24} values for several amino-acids in the glucose–phosphate medium were unchanged (Table I) but there was now a marked influence of aeration upon growth in the ammonium sulphate medium. Aeration now increased n_{24} (see Table I), and, whereas formerly aerated and unaerated cultures had both shown an *m.g.t.* of 45′, aerated growth was

now speeded up (average *m.g.t.* = 32′), while unaerated growth was slower (*m.g.t.* 40′ or longer). The *m.g.t.* with alanine (aerated) was 40′, *i.e.*, not much change; two determinations with glutamic acid gave 46′ and 46′ (again little change), whereas leucine gave considerably slower growth. The growth curve originally obtained with valine was non-logarithmic (Fig. 1). Initially the *m.g.t.* was about 40′ but this lengthened and over a considerable range before the stationary phase set in it remained constant at about 100′. After the mutation, values found were 114′ and 112′. In Fig. 1 the lines *OA* and *OB* represent the growth rates in ammonium sulphate before and after the mutation. It is to be noted that practically all the rates in different amino-acids lie *within* this range. *OC* refers to growth in a lactose–phosphate–ammonium sulphate medium.

(5) Usually the growth curve was of well-defined logarithmic form from the end of the lag phase nearly to the point at which the rapid turn over to the stationary phase occurred [Fig. 3 (*a*), (*b*)]. There were, however, occasional exceptions, significant enough to be worth noting and discussing. (i) A *gradual* transition from the lag to the true logarithmc phase. This effect was specially in evidence with ammonium sulphate after the mutation. It is illustrated in Fig. 3 (*d*). (ii) An arrest in growth for a time [Fig. 3 (*e*)], after which it is

FIG. 2.



I. *Aspartic acid, glutamic acid, and asparagine.*
II. *Glycine, α-aminoisobutyric acid, and α-amino-octoic acid.*
III. *All six of the above.*
IV. *Glycine with traces of tyrosine and tryptophan.*
V. *Ammonium sulphate (showing lag).*

(*All solutions unaerated.*)

resumed, sometimes even making up lost time, as in Fig. 3 (*c*). (iii) Growth slows down long before the stationary phase is reached and continues with a lengthened *m.g.t.*, as in Fig. 1 for the growth in valine.

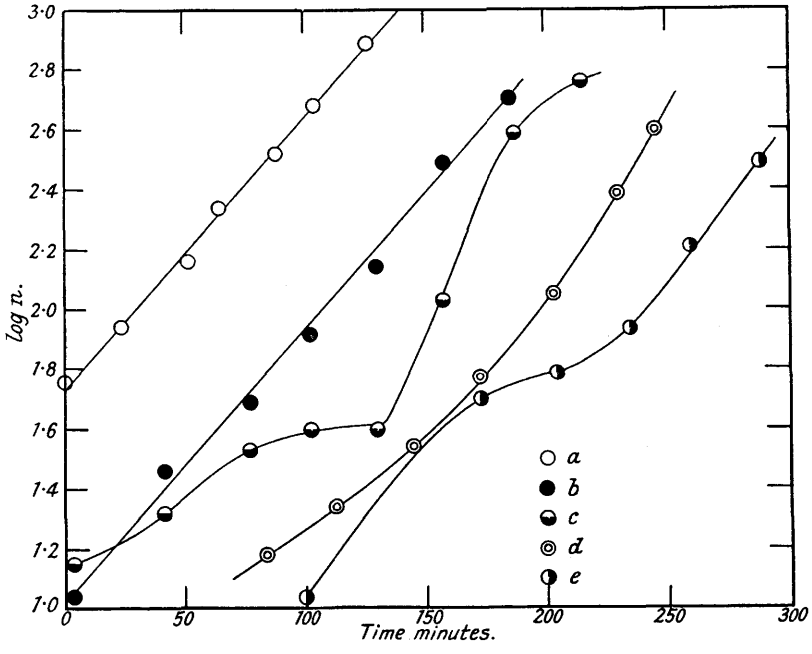
Discussion.—The facts described above are best interpreted in the light of the hypothesis that growth depends upon a delicately balanced series of reactions, one or other of which may become rate-determining according to circumstances.

In bouillon the *m.g.t.* is only 18′. In glucose–phosphate media with ammonium sulphate or amino-acids, this rate is not nearly reached, slower processes being rate-determining. In growth, something must be derived from the carbon source and something from the nitrogen source, and the intermediate derived from one of the sources will tend to be more readily available than that from the other. Since the growth rates with different nitrogen sources form a fairly compact group, one may conclude that the nitrogen-containing intermediate is the more readily available. In so far as the mutation of the organism tended somewhat to increase the differences between the *m.g.t.*'s with various nitrogen sources, it would mean that the relative rates of the two classes of reaction have been slightly shifted.

With some unaerated amino-acids growth ceases early: this effect is best explained by assuming the formation of an inhibitor, removable by aeration. The hypothesis that this removal is due to an oxidative mechanism developed *within the cell*, helps to link together a number of facts. It ascribes the change in reponse to aeration (after the minor mutation of the organism) to an improvement in an intracellular mechanism. Logically this

change could equally well be explained by assuming that a different and more easily removable inhibitor is produced after mutation.

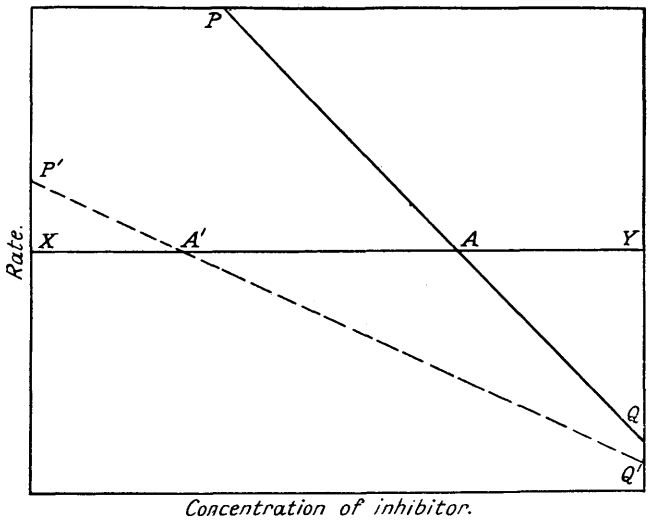
FIG. 3.



The first hypothesis, however, has the advantage of offering also a partial explanation of the anomalous growth curves referred to above (Fig. 3).

Suppose among the growth-determining processes there are two, with rates depending upon the concentration of a certain inhibitor as in Fig. 4. If the pair are affected by the inhibitor according to the curves XY and PQ, the former will be rate-determining until the concentration of inhibitor corresponds to the point A, after which the other takes control. If PQ is steep, and especially if, by the time A is reached, the number of organisms is large, the rate then falls off very rapidly and there is an almost abrupt transition to the stationary phase. This is a common occurrence. If, however, the two reactions correspond to XY and $P'Q'$ then the deceleration is more gradual, since it sets in at A', which is reached when there are fewer cells and the rate of inhibitor formation is still small. Such a state of affairs may well correspond to the valine type of curve (Fig. 1).

FIG. 4.



The cell mechanisms responsible for the removal of this inhibitor may themselves well depend upon the building up of intermediate products. The removal reaction could therefore itself show a lag and we might have the abortive setting in of a stationary phase, subsequently disturbed by the development of the removal reaction. This state of affairs seems to correspond to curve (*c*) in Fig. 3. What determines the occasional lag in the inhibitor-removing mechanism is not clear: it may be immeasurably small traces of impurity such as grease. The fact that the temporarily inhibited growth sometimes makes up for lost time as in Fig. 3 (*c*) is interesting, and could be explained as follows. During normal growth a steady concentration of some intermediate substance is established: to this corresponds the normal *m.g.t.* The inhibitor now comes into play and prevents the utilisation, but not the formation, of this intermediate, which therefore builds up to an abnormally high concentration in the cell. As soon as the inhibitor-removing reaction comes into play the intermediate begins to be utilised again and, being at

an abnormally high concentration, gives rise to an abnormally high growth rate until the normal balance is restored.

The type of curve shown in Fig. 3 (*d*) will depend upon the gradual development of the inhibitor-removing reaction.

That the same organism may show the various types of curve in an apparently random manner may be explained by the influence of uncontrollably small amounts of impurities, but also by the fact that the state of the enzymes must be a function of the age and previous history of the cell and may therefore show variations from test to test great enough to influence the exact quantitative balance between these various delicately adjusted processes.

PHYSICAL CHEMISTRY LABORATORY, OXFORD UNIVERSITY. [*Received, January 5th,* 1943.]