88. *Nitrogen Utilisation and Growth of Coliform Bacteria. Part III. Nitrogen Utilisation and Lag Phase.*

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Considerable lag phases in a growth medium consisting of glucose, magnesium, phosphate, and ammonium sulphate may be shown by untrained *Bact. coli*, trained *Bact. coli* which has been allowed to "age", and strains of *Bact. lactis ærogenes* which have been allowed to age or have been irradiated. In all these cases the lag seems to be largely connected with the need for the production from glucose of four- and five-carbon skeletons which can be aminated to give key amino-acids.

The lag may be reduced almost to zero by allowing the cells to remain for the appropriate time in contact with a glucose medium lacking any added nitrogen source, and in the full medium no detectable consumption of ammonia occurs before the very end of the lag, when there are indications that traces of glutamic and aspartic acids appear in the solution. Additions of these acids shorten lags in ammonium sulphate media considerably, glutamic acid being the more effective. (The growth rates with glutamic acid or aspartic acid as sole source of nitrogen are, however, much lower than those observed with ammonium sulphate, the amino-acids being capable of initiating growth by mechanisms which are not the optimal.) Certain carbon compounds which are obvious possible precursors of glutamic or aspartic acid, especially α-ketoglutaric acid, approach the corresponding amino-acids in efficiency in shortening lag, but are never quite as effective as glutamic acid itself.

Ammonia Consumption in Lag Phase.—An old culture of the strain *Bact. coli* M was washed and suspended in glucose–phosphate–magnesium sulphate medium containing ammonium sulphate at $44{\cdot}5 \pm 0{\cdot}5$ mg./l. The ammonia content of samples was determined by Conway and O'Malley's method (*Biochem. J.*, 1942, **36**, 655) at intervals during the lag phase, with results recorded in Table I, which shows that the actual consumption of ammonia before growth sets in is too small to detect.

Table I.

Time (mins.).	log *n*.	NH_3, mg./l.	Time (mins.).	log *n*.	NH_3, mg./l.	Time (mins.).	log *n*.	NH_3, mg./l.
5	2·41	44·5±0·5	238	2·41	45·2	415	2·55	40·0
50	2·41	45·2	290	2·41	44·5	470	2·67	28·4
140	2·41	41·4	350	2·41	45·2	530	2·96	4·7
200	2·41	44·9						

In view of the small ammonia consumption indicated by this last experiment the following test was made. Inocula from an old culture were transferred in parallel to two media, one complete except for the glucose, the other complete except for the ammonium sulphate. After various intervals of time the media were completed, and the lag before the onset of growth was determined. The results are recorded in Table II, which shows that for the medium containing

Table II.

	Medium initially lacking added nitrogen.		Medium initially lacking glucose.	
Time of completion of medium (minutes).	Lag measured from inoculation.	Lag measured from completion of medium.	Lag measured from inoculation.	Lag measured from completion of medium.
5	363	358	387	382
38	351	313	436	398
67	290	333	—	—
187	386	199	545	358
225	379	154	554	329

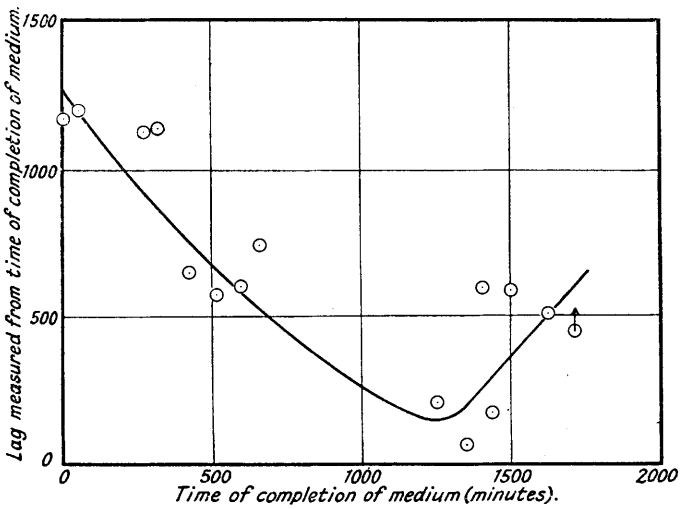
glucose but lacking ammonium sulphate the total lag as measured from the time of *inoculation* is nearly constant, while for the medium lacking glucose there is a constant lag as measured from the time of *completion*. In other words, either no nitrogen is needed for the preparatory processes of the lag phase or the extremely minute amounts contained in the glucose as impurity or carried over in the cells of the inoculum are all that is required.

The results of a similar experiment with a parent culture showing a longer lag are given in Fig. 1. The inoculations were made into medium lacking any added nitrogen. The curve shows that almost the total preparation for growth may be made in the glucose medium before the ammonium sulphate is added, the lag, as measured from the time of *completion* of the medium, reaching a minimum value of almost zero.

Influence of Glutamic Acid and Aspartic Acid on Lag Phase and Growth Rate.—A partition chromatography method to be described later gave indications of the formation of traces of glutamic and aspartic acids at the end of the lag just before growth began in an ammonium sulphate–glucose medium. This led to a series of experiments on the effect on the lag of additions of these substances (these experiments being of course independent of the validity of the chromatographic observations which prompted them).

FIG. 1.

Effect on lag of standing in nitrogen-free medium.



The strain *Bact. coli* M, trained in asparagine, which was referred to in Part I, was inoculated into eight different media in parallel at various times after growth of a parent culture had begun. These media contained glucose, phosphate, magnesium sulphate, together with one of the following nitrogen sources : (1) ammonium sulphate, (2) ammonium sulphate and glutamic acid (G), (3) ammonium sulphate and aspartic acid (A), (4) ammonium sulphate with both glutamic and aspartic acids, (5) asparagine, (6) asparagine and glutamic acid, (7) glutamic acid (G), (8) aspartic acid (A). Where the amino-acid was an addition the concentration was approximately 500 mg./l. ; where the sole source, it was equivalent in nitrogen to the ammonium sulphate.

TABLE III.

		ΔL.						
Time.	L in Am_2SO_4.	Am_2SO_4 + G. (1 − 2).	Am_2SO_4 + A. (1 − 3).	Am_2SO_4 + A + G. (1 − 4).	Asparagine. (1 − 5).	Asparagine + G. (1 − 6).	G. (1 − 7).	A. (1 − 8).
0	0	0	0	0	0	0	0	0
393	110	90	40	90	40	85	105	60
1188	590	260	130	340	170	340	350	190
1398	650	200	160	310	150	290	235	130

The first set of inoculations was made when the count of the parent culture was 195×10^6 cells/ml., the lag at this stage being zero in all the media. As the parent aged, the lag increased most rapidly in the medium (1) containing ammonium sulphate alone, and least rapidly in (4) which contains both glutamic and aspartic acids. Glutamic acid shortens the lag more than aspartic acid but the two effects may be more or less additive. In Table III are recorded the lags, L, in minutes, for the medium (1) and the shortening, ΔL, observed in the various other media.

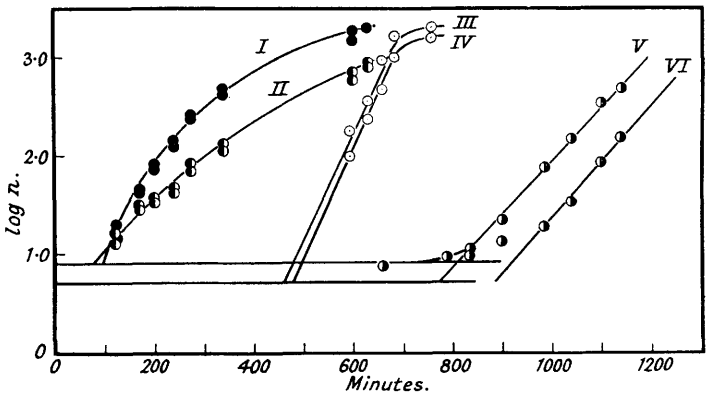
In Table III it is to be noted that (1 — 5) agrees rather closely with (1 — 3) and (1 — 6) with (1 — 4). Thus the results for asparagine are substantially the same as those for ammonium sulphate with addition of aspartic acid.

The rate of growth in media containing aspartic or glutamic acid as the only nitrogen source

is much lower than in those containing ammonium sulphate or asparagine. The mean generation time with glutamic acid alone is initially about 67 minutes, increasing slightly as growth proceeds. With aspartic acid alone the mean generation time is about 72 minutes for cultures showing a short lag. If the age of the parent culture is such that the lag is long, the subsequent growth is more rapid. It appears, therefore, that aspartic acid may *initiate* growth by a

FIG. 2.

Growth in various nitrogen sources (Bact. coli).



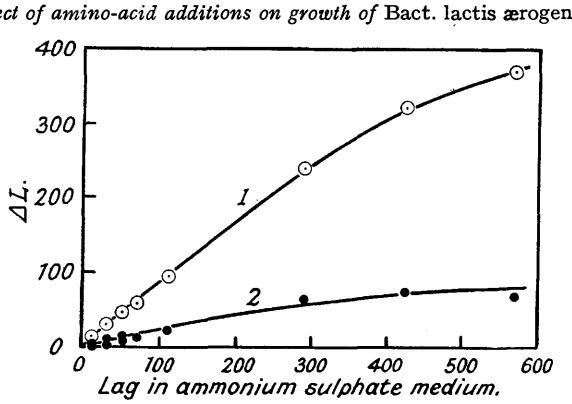
I. *Glutamic acid plus aspartic acid.*
II. *Glutamic acid.*
III and IV. *Asparagine.*
V and VI. *Aspartic acid.*

mechanism which is not the optimum one. During the longer lags other intermediates are formed which permit growth by a more rapid mechanism. Indeed, occasionally a long lag, in media containing traces of aspartic acid, is followed by growth according to a composite curve, a period of slow multiplication being superseded at a certain point by a more rapid one.

Some of the lag and growth rate relations for the strain *Bact. coli* (M) are shown in Fig. 2.

FIG. 3.

Effect of amino-acid additions on growth of Bact. lactis ærogenes.



Upper curve: glutamic acid or glutamic–aspartic acid mixtures.
Lower curve: aspartic acid.

Similar experiments were next made with a strain of *Bact. lactis ærogenes* which has been the subject of previous study in various other respects. Once again, parallel inoculations were made from a parent culture at different times into a series of media.

The nitrogen sources were respectively ammonium sulphate and ammonium sulphate with addition of small amounts of aspartic acid, glutamic acid or a mixture of both. The results are shown in Fig. 3. In general, glutamic acid shortened the lag phase considerably, whereas

aspartic acid had relatively little effect, and the mixture of the two was not appreciably better than glutamic acid alone.

Influence of Related Non-amino-compounds on Lag Phase.—Although aspartic acid and glutamic acid shorten the lag phase, it is not clear whether this effect is due to the actual amino-acids or to their carbon skeletons. Consequently, it was decided to test the influence on lag of suitable acids which are structurally related to these amino-acids but contain no nitrogen.

The most likely substances are the corresponding keto-acids, oxaloacetic and α-ketoglutaric. The latter was prepared by the method given in " Organic Syntheses ". The product was a faintly yellow crystalline solid of m. p. 109° (sharp), which is the value given in the literature. Oxaloacetic acid was prepared in a mixture with malic acid by controlled oxidation of the latter with hydrogen peroxide (Fenton and Jones, *J.*, 1900, **77**, 75). The product was a faintly yellow crystalline solid containing about 30% of oxaloacetic acid.

The strain *Bact. coli* M, trained to asparagine, was inoculated into seven different media in parallel at various times after growth in the parent cultures had begun. The time of inoculation is counted from the moment when the count of the parent cultures was 200×10^6 cells/ml. The media contained glucose, phosphate, magnesium sulphate and ammonium sulphate, together with one of the following additions (concentration approximately 500 mg./l.) : (1) no addition, (2) aspartic acid, (3) glutamic acid, (4) malic acid, (5) " oxidised malic acid ", (6) α-ketoglutaric acid, (7) " oxidised malic acid " and α-ketoglutaric acid. Each culture tube contained 26 ml. plus the volume of the additions, and 1 g./l. of ammonia as ammonium sulphate. The results are recorded in Table IV. The lags, L, and differences in lag, ΔL, are given in minutes.

Clearly, glutamic acid is the most efficient (1 — 3) and more so than α-ketoglutaric acid (1 — 6), but quite an important part of the lag reduction is due to the carbon skeleton. Malic acid and aspartic acid are more or less equivalent and the least efficient tested. Oxaloacetic acid is of intermediate efficiency.

Long Lag of Bact. coli *Untrained to Ammonium Salt Media.*—Since the lag which normally develops as the culture ages has been shown to be specially concerned with the utilisation of the carbohydrate, the very long lag shown by certain strains of *Bact. coli* when first transferred

TABLE IV.

Time of inoculation (minutes).	L (1).	ΔL. (1 — 2).	(1 — 3).	(1 — 4).	(1 — 5).	(1 — 6).	(1 — 7).
Series I.							
0	0	0	0	0	0	0	0
500	200	45	185	45	115	130	130
670	200	30	100	30	80	90	90
840	250	30	150	60	115	120	130
1290	290	80	170	30	70	140	120
1680	290	55	150	60	90	110	90
1900	290	50	140	50	85	90	85
Series II.							
780	435	95	285	75	165	195	165
1260	610	270	470	280	340	420	410

to an ammonium salt medium (Part I) seemed likely also to depend upon a difficulty in the appropriate metabolism of glucose. The strain *Bact. coli* H was therefore subjected to further investigation.

Inocula of about 10^6 cells per c.c. from a fresh bouillon culture were transferred to ammonium salt media and to similar media containing α-ketoglutaric acid, at approximately 500 mg./l. The lags in the former were 95—116 hours, but in the latter only 6. In another experiment similar inocula were transferred to media with the following additions, and exhibited the lags given in parentheses : (1) No addition (96 hours), (2) asparagine (40 hours), (3) malic acid (19·5 hours), (4) oxaloacetic acid (22 hours), (5) α-ketoglutaric acid (20 hours).

A further confirmation of the reason for this phenomenon of long lag was provided by various strains of *Bact. lactis ærogenes* isolated by A. R. Peacocke from a culture which had been irradiated with ultra-violet light. These strains showed the phenomenon of a long lag in the ammonium salt medium, and this lag was largely removed by addition of α-ketoglutaric acid. The results will be reported in another paper.

Discussion.—The results described have shown that during the lag phase extremely little

nitrogen is required but that the carbohydrate substrate must be present. This suggests that it is the breakdown of the carbon source to give the appropriate kind of molecules which is of key importance. In this connextion it is worth noting that magnesium ions, which are catalysts for phosphorylations, are also necessary during this period (Pett and Wynne, *Biochem. J.*, **1933**, **27**, 1660; Lodge and Hinshelwood, *J.*, 1939, 1692).

From the effects on the lag phase of the various additions it seems quite likely that during this time various compounds are formed from the glucose and that molecules of special importance are those with a five-carbon skeleton. When these reach a sufficient concentration nitrogen can be taken up to give amino-acids and growth starts.

If a substance corresponding to a relatively early link in the metabolic and synthetic chain is added to the medium, it may be as effective as its corresponding amino-acid in shortening the lag, the time required for amination being of no importance compared with other stages. Thus malic acid and aspartic acid have about the same influence. The more effective additions, however, will be those which correspond to later stages of the sequence. Thus, ready-made α-ketoglutaric acid is very active. When the other steps have been by-passed as completely as possible, the amination step itself becomes relatively important, and, accordingly, glutamic acid is even more effective as an addition than the α-ketoglutaric acid from which it could be formed by amination.

A key rôle, it would seem, must be assigned to glutamic acid itself, since of all the substances tested it is the most efficient in reducing the lag. It is superior in this respect to its keto-precursor, but there is little doubt that the prior formation of a keto-group is important, since a given carbon skeleton containing it is more effective than one without.

These conclusions bring the present work into close relation with various well-known facts connected with the biochemistry of yeast, muscle and other systems.

The long lag exhibited by certain strains of *Bact. coli* when first transferred to ammonium salt media is also due largely to an inability to produce required compounds at an adequate rate from the glucose. As these substances are required at definite concentrations before growth can proceed, the apparent inability to utilise the ammonia arises. It is probable that the cells fail to produce the four- and five-carbon compounds at first, and are handicapped consequently in the later stages of the metabolism. The same difficulty may appear with *Bact. lactis ærogenes* after irradiation with ultra-violet light.

The interrelation between the three parts of this work is now clear. In Part I the training of *Bact. coli* to utilise ammonia was shown to be connected with the elimination of a long initial lag. In Part III this lag, as well as the lag developed in older cultures of trained bacteria, was shown to be largely connected with the production from glucose of carbon compounds of well-known biochemical significance such as oxaloacetic acid and α-ketoglutaric acid. The amination of these compounds appears to occur with comparative readiness to give amino-acids such as aspartic acid and glutamic acid which were shown in Part II to be directly used by the cell and not to serve as mere sources of ammonia. On the other hand, acids such as glycine when provided as sole nitrogen sources largely yield ammonia for aminating key carbon compounds. From these considerations follows the somewhat complicated relation between deaminase activity and growth rate in various amino-acids.

We are indebted to Messrs. R. G. Otter and G. Stafford for assistance in the preparations involved in this work.

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