86. *Nitrogen Utilisation and Growth of Coliform Bacteria. Part I. Adaptation to Growth in Ammonium Salt Media.*

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Certain strains of *Bact. coli* which grow readily in a glucose–phosphate–asparagine medium require training before they will grow with ammonium salts as sole nitrogen source. The training process is shown to be associated chiefly with the overcoming of a long initial lag. The phenomena belong to the type encountered in the training of *Bact. coli mutabile* to lactose or of *Bact. lactis ærogenes* to D-arabinose.

This series of studies is concerned with the utilisation of ammonia and of amino-acids by coliform bacteria.

Some of these bacteria, for example, certain strains of *Bact. coli*, utilise ammonium salts in growth with great difficulty until they have been " trained ". The method of training usually employed in this laboratory has been to cultivate the bacteria in media containing mixtures of asparagine and ammonium sulphate as the nitrogen source, and, in the course of successive sub-cultures, to reduce the proportion of asparagine gradually to zero.

The first problem to which it seemed proper to give attention was the nature of the adaptive process involved in this training to grow with ammonium salts as the sole nitrogen source.

Apart from its bearing upon the question of nitrogen metabolism, this problem is of interest in connexion with the systematic study of adaptive processes in general.

These processes, which hitherto have been investigated chiefly in relation to changed carbon sources, may conform to several different patterns. In one type, exemplified by *Bact. coli mutabile* and lactose (Massini, *Arch. Hyg.*, 1907, **61**, 250; Postgate and Hinshelwood, *Trans. Faraday Soc.*, 1946, **42**, 45) and by *Bact. lactis ærogenes* and D-arabinose (Cooke and Hinshelwood, *ibid.*, in the press; Jackson and Hinshelwood, *ibid.*, in the press), there is on the first sub-culture into the medium with the new source a very long lag (several days) which, once growth has taken place, does not occur in subsequent sub-cultures. This may be referred to as Type I. In Type II, exemplified by *Bact. lactis ærogenes* with glycerol (Lodge and Hinshelwood, *Trans. Faraday Soc.*, 1944, **40**, 571; Cooke and Hinshelwood, *ibid.* in the press) or with nitrate as a new nitrogen source (Lewis and Hinshelwood, *J.*, 1948, 824), there is no abnormal lag, and growth, in proper conditions, starts at once, but at a rate which is well below optimal and improves in successive sub-cultures. This type is often characterised, in the early stages of the training, by growth curves showing discontinuities.

The usual procedure did not show clearly whether training to utilise ammonia belonged to Type I or to Type II or whether it conformed to yet another type. Experiments show that, in fact, it conforms rather closely to Type I.

Training Experiments.—The methods used in the work to be described were similar to those given in previous papers from this laboratory. The bacteria were grown in aerated synthetic media at 40·0°. The most informative experiments were those made with a pure strain of *Bact. coli*, referred to as H, isolated by Dr. R. L. Vollum who kindly placed it at our disposal. This strain proved very suitable for the investigation.

Parallel inocula were made into two glucose–phosphate–magnesium sulphate media, containing respectively asparagine and ammonium sulphate as nitrogen sources. *Bact. coli* being normally fully adapted to use the former, the *difference* in lags, ΔL, provides a criterion of

the degree of adaptation to ammonia. The asparagine media undoubtedly produce ammonia during growth and so are equivalent to mixed media. At the first pair of parallel sub-cultures ΔL was 1780 minutes. Inocula were then taken from the ammonia medium during logarithmic growth, and transferred in parallel to the asparagine and to the ammonia medium: ΔL was now 10 minutes only. It thus seems that once the very long initial lag in the ammonium salt medium has been overcome it need not be traversed again (Type I).

The question now arises as to what occurs during the conventional training in a mixed ammonia–asparagine medium. The figures in Table I show that the training achieved in this process depends essentially upon the successive *periods of rest* in the asparagine medium, and that the actual *growth* in asparagine is irrelevant. This result is completely parallel to what has been found with *Bact. lactis ærogenes* and D-arabinose (Cooke and Hinshelwood, *ibid.*, in the press; Jackson and Hinshelwood, in the press).

Parallel sub-cultures were then made from a bouillon culture of H into an alanine medium and an ammonium salt medium.

TABLE I.

Bact. coli (Strain H), sub-cultured in asparagine, and tested for lag in the ammonium salt medium at each stage. Re-inoculation in the asparagine medium made during growth to eliminate stationary phase.

Number of passages in asparagine medium.	Sum of lags in asparagine (mins.) (I).	Sum of growth times in asparagine (mins.).	Lag (mins.) before growth in test in ammonium salt medium (II).	I + II.
0	0	0	5460	5460
1	3680	560	1060	4740
2	4010	980	980	4990
3	4310	1390	730	5040
4	4610	1530	400	5010

The first sub-culture directly into ammonia was attended by a lag of 52 hours. Passage through alanine in serial sub-cultures merely allowed this time to be passed with intermissions for growth rather than in one stretch, as is shown by Table II.

TABLE II.

Total time in (hours).	Time of active growth in alanine (approx.) (hours).	Total resting time (hours) in alanine (I).	Lag in ammonium sulphate medium (hours) (II).	I + II.
0	0	0	52	52
40	4	36	23	59
65	8	57	1	58

The conclusion is that before ammonia can be utilised the bacterial substance must spend the required length of time at rest in the medium without an alternative nitrogen source to allow growth by another mechanism.

Essentially similar results were obtained with two other strains of *Bact. coli*. With a strain, M, for example, three sub-cultures in the asparagine medium had reduced the lag in ammonia to within 16 minutes of that in asparagine, the initial value of ΔL having been nearly 8000 minutes. When M was inoculated for the first time into the ammonia medium growth was slow and erratic, even after the lag of 9000 minutes. A second pair of parallel sub-culutres into ammonia and asparagine (of cells which had not been passed through asparagine at all) gave a value of ΔL of 140 minutes only. This confirms once more that the behaviour conforms to Type I.

Even when the initial long lag has been completely removed by standing in the ammonia medium, or by suitable sub-culturing in the mixed medium, the bacteria are not necessarily fully adapted in respect of growth rate (mean generation time) or in respect of the power to multiply to the maximum possible population in the given medium. This normally requires more prolonged training (an argument, incidentally, against the view that the disappearance of the lag is due simply to the selection of a " mutant ").

With H, for example, the first growth rate in ammonia corresponded to a mean generation time of 47 minutes, and the next to 47 minutes also. After repeated sub-culture the value settled down to 42 minutes.

Rather more detailed observations were made with M. Some of these are recorded in Table III.

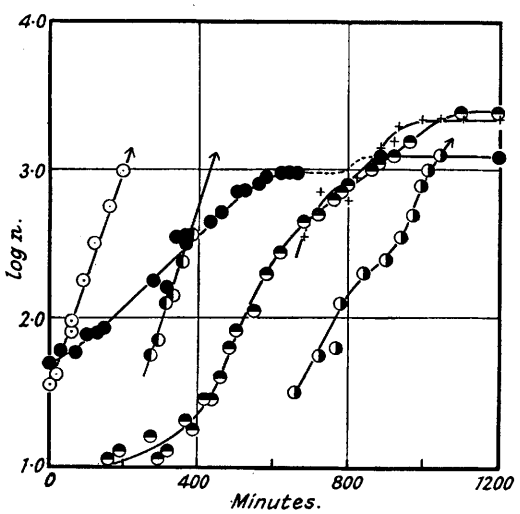
The results in Table III refer to serial sub-cultures in asparagine (with the small amounts of ammonia inevitably present during growth in this medium). When the strain M was

TABLE III.

Strain M. Trained in asparagine (+ ammonia).
Tested in asparagine and in ammonium sulphate.

Number of sub-cultures in asparagine.	Test in asparagine. M.g.t.* (mins.).	Total population.‡	ΔL (mins.).	Test in ammonium sulphate. M.g.t. (mins.).	Total population.‡
0	44	1100	8000	Erratic and † long	
1	29	2000	85	33	140
2	—	2000	—	—	400
3	—	2000	16	34	1100
4	—	2000	—	—	2000
5	29	2500	−35	29	2500
13	—	2500	20	29	2500
20	29	2500	10	29	2500

* M.g.t. = mean generation time.
† Formation of filamentous cells.
‡ The unit is $1{\cdot}25 \times 10^6$ cells/ml.



Successive sub-cultures in the ammonium sulphate medium, showing the development of the true logarithmic growth.

○ *30th Sub-culture.* ◐ *20th Sub-culture.*

The other (non-linear) curves represent early sub-cultures with irregular growth.

N.B.—*Time scales have arbitrary zeros.*

sub-cultured in the ammonium salt medium alone, the history of the training was somewhat different. The mean generation time was at first in the neighbourhood of 60 minutes, and by the twentieth sub-culture had settled down to a steady value of 38 minutes. The true logarithmic form of growth curve is only established as the training becomes complete (see Fig.). The history of the training has here, rather remarkably, a definite influence on the final equilibrium state. The two trained sub-strains were tested by Dr. Vollum, to whom we are indebted for the information that in all the usual diagnostic tests they showed identical behaviour, apart from a tendency of the strain trained directly to ammonia to yield mucoid colonies on deoxycholate–citrate agar. There is thus no question of infection by a foreign organism.

Preservation of the parent strain M in bouillon caused gradual training to ammonia—in all probability in a way corresponding to that shown in amino-acid media. It was of some interest to ascertain to what extent this change affected the whole population. The culture was therefore plated and nine colonies were selected and tested for lag in the ammonium sulphate and the asparagine medium respectively, the difference, ΔL, serving as a criterion of training to the former. The results (expressed in minutes) were: 20, 50, 50, 100, 100, 150, 150, 200,

ca. 1000, which may be compared with the original value of several thousand minutes. The training is therefore spread over the bulk of the population.

Conclusion.—The general conclusion to be drawn from all the experiments described is that adaptation to ammonia is essentially an adjustment whereby a long initial lag is overcome, the utilisation of ammonia depending upon the presence of certain intermediates not necessarily present during growth in other circumstances. Growth in asparagine almost certainly exposes the cells to the action of ammonia, but it is only during the resting periods rather than during active growth in asparagine that the processes leading to the termination of the lag in ammonia utilisation can go on undisturbed.

Once this lag is over, growth with ammonia utilisation takes place and, strictly speaking, this is the period when the modifications in cell material constituting the real adaptation occur. These growth-linked changes ensure that the long lag need not be traversed again.

The elimination of the long initial lag does not complete the adaptation, and the optimum growth rate throughout the logarithmic phase is only reached after further sub-cultures in the ammonia medium. In the intermediate stages the growth curves show arrests indicating that more than one process is in need of further adjustment. This observation and the fact that training, at a quite early stage, is distributed over the bulk of the population, are on the whole more easily compatible with the hypothesis of direct adaptation than with that of spontaneous mutations.

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