165. *The Growth of Coliform Bacteria in Media containing Nitrate and Nitrite. Part I. Adaptation to Growth with Nitrate and Nitrite as Nitrogen Sources.*

By P. R. Lewis and C. N. Hinshelwood.

Coliform bacteria reduce nitrate to nitrite and utilise the nitrite in growth. The relative rates of formation and consumption of nitrite vary considerably among strains. In less adapted strains of *Bact. lactis aerogenes* the nitrate reduction is the slowest step but the rate increases rapidly on training by serial subculture in nitrate media. In the trained state all the strains tested grew at a rate determined by the consumption of the nitrite, the product : rate of nitrite consumption × mean generation time being approximately constant. High concentrations of nitrite exert an inhibitory effect : this can be overcome by training the cells in presence of the appropriate nitrite solutions. There is no inhibition by corresponding concentrations of nitrate.

Training separately to nitrate and to glycerol produces the same final result as training simultaneously to the combination of substrates.

With strains of *Bact. coli* not initially trained to an ammonium sulphate–glucose medium, training to this confers *pari passu* a considerable measure of training to nitrate, suggesting that the adaptation of the nitrate–nitrite reducing system is a concomitant to the adaptation of some part of the normal dehydrogenase system of the cells as it becomes trained to the artificial medium.

(1) *Introduction.*—The most satisfactory hypothesis about the adaptation of bacteria to growth in a new culture medium is that changes occur in the nature or proportions of certain cellular enzymes (for arguments and references see Hinshelwood " Chemical Kinetics of the Bacterial Cell ", Oxford, 1946). In this connexion it is of interest to combine a study of some typical adaptive process with a direct investigation of the changes which actually occur in the appropriate enzymes. In the first part of the work to be described the adaptive process studied was the training of coliform bacteria to a medium in which the ammonium sulphate normally used as a source of nitrogen was replaced by sodium nitrate or sodium nitrite.*

The plan followed was to study the changes in growth characteristics accompanying adaptation by serial subculture in media containing nitrate or nitrite, and then, for various suitably chosen strains of cells, to measure the rate of enzymic reduction of nitrate to nitrite and the rate of consumption of nitrite. In any given experiment the difference between these two rates determines the rate at which nitrite accumulates in the nitrate medium. The results show which stage—the reduction of nitrate to nitrite or the further utilisation of nitrite—is the slower; what relative changes in these reactions occur during training; and how the absolute rate of one or other is related to the growth of the cells. These matters are considered in Section 5.

During these studies it became clear that the reduction of nitrite or nitrate to a form in which the nitrogen is available for the synthetic reactions is closely linked with the normal dehydrogenase mechanisms of the cell, and this idea came more and more into prominence in the later part of the work. In this the experiments were extended to include the study of the rôle of hydroxylamine and of ammonia which are likely reduction products of nitrite.

Neither hydroxylamine nor ammonia attains to any considerable concentration in the nitrite or nitrate media during growth. To compare the rate of utilisation of ammonia with its possible rate of formation from nitrate, experiments were made with media containing mixtures of ammonium salts and nitrate or nitrite.

It proved that, while ammonia is being utilised by the cells, the reduction of nitrite and nitrate is inhibited. The nature of the inhibition was therefore studied, and proved to be connected with the different oxidation–reduction conditions prevailing during normal ammonia utilisation and nitrate or nitrite reduction respectively.

Three phenomena—namely, the inhibitory action in question, a characteristic influence on nitrate reduction of the aeration conditions, and the Pasteur effect—all appear to be explicable on a common kinetic basis. This involves a common link in the carbohydrate–dehydrogenase cycle of the cell and in the nitrogen synthesis reactions. With this one assumption the various phenomena appear to be derivable from general kinetic principles in a way which is discussed in Part II (following paper).

* Pollock (*Brit. J. Exp. Path.*, 1946, **27**, 419) has studied the reduction of nitrate by a strain of *Bact. coli*, but was concerned mainly with lag phenomena, rather than with progressive changes accompanying serial subculture, or with the mode of reduction during active cell division.

(2) *Experimental Methods.—Cultures and media.* The methods used to determine the growth characteristics (lag, growth rate, and total population) were as previously described (Hinshelwood and Lodge, *Proc. Roy. Soc.*, 1944, *B*, **132**, 47) except that the bacterial population was usually determined by measuring the turbidity (Monod, " La Croissance des Cultures Bactériennes ", Paris, 1942) of the sample with a Spekker absorptiometer which had been calibrated against hæmacytometer counts. The counts, n, are given in terms of hæmacytometer readings; $n \times 1{\cdot}25 \times 10^6$ gives millions/ml. The scale is such that a culture showing the first signs of turbidity has $n = 5$—10, while one fully grown has n from 1000 to 3000.

In studying the effect of continued growth in a given medium on the properties of a bacterial strain, the latter was regularly subcultured (0·2 ml. of the full-grown culture being transferred to fresh medium) and the properties of the strain examined at suitable intervals. This process is known as " training ". Each subculture is approximately equivalent to 7 successive divisions of the cells, its serial number being a measure of the length of the training.

Strains of *Bact. lactis aerogenes* and of *Bact. coli* were normally cultured in a medium of the following composition: potassium dihydrogen phosphate, 3·46 g./l.; magnesium sulphate, 0·38 g./l.; glucose, 20 g./l.; and ammonium sulphate, 0·96 g./l.; brought to pH 7·12 with sodium hydroxide.

They were then trained to media in which the ammonium sulphate had been replaced by sodium nitrate (1 g./l.) or sodium nitrite (1 g./l.). These are in future referred to as the " nitrate " and the " nitrite " medium respectively.

At intervals during training, the growth rates in the nitrate and the nitrite medium were determined. The rates at which these salts were reduced were measured by inoculating in parallel into two test media containing respectively nitrate and nitrite, and determining the concentration of nitrite prevailing at each stage during growth. From this the rates of nitrate and nitrite reduction per cell could be calculated. Where nitrate reduction was slower than removal of nitrite, experiments were also made with a third medium in which both salts were supplied initially.

The compositions of the three test media used were as follows: (i) sodium nitrite, 100 mg./l.; (ii) sodium nitrate, 1000 mg./l.; (iii) sodium nitrite, 40 mg./l.; sodium nitrate, 1000 mg./l.; the other constituents being as in the standard medium referred to above, with omission of ammonium sulphate.

The media were inoculated simultaneously either from a fully grown culture, or from a suspension of centrifuged and washed cells.

Determination of nitrite. Samples were withdrawn at intervals, and heated to 100°. The concentration of nitrite was determined by the reaction with amino-G acid and α-naphthylamine in acid solution, which gives an azo-dye with a permanganate-like colour (Stieglitz and Palmer, *J. Pharmacol.*, 1934, **51**, 398). The tint is proportional to the amount of nitrite, though only over narrow ranges of concentration, since not one, but several, dyes may be formed according to the nitrite concentration. Beer's law is, however, obeyed over the range of 4—10 mg./l. of sodium nitrite in the test sample, and the sample was diluted when necessary to bring the concentration within this range.

The compositions of the reagents were modified as follows:

(i) Amino-G acid, 0·18 g.; glacial acetic acid, 180 ml.; made up to 1 l. with glass-distilled water.

(ii) α-Naphthylamine, 1·8 g.; glacial acetic acid, 150 c.c.; made up to 1 l. with glass-distilled water.

To a 5 ml. portion of the test solution heated to near 100°, 5 ml. of each reagent were added [(i) being added first]. The mixture was kept at 40° for 40 minutes and then allowed to cool. The colour so formed is reproducible and stable for several hours.

Since the colour is affected by the number of bacteria present, several series of standards containing different amounts of nitrite and suspension were made up for each experiment. The more turbid samples were compared with the standards by eye and the less turbid in a Duboscq colorimeter. Quite accurate determinations could be made provided that the turbidities of sample and standard were approximately equal, and that the concentrations lay within 25% of one another.

(3) *Calculation of Reaction Rates.*—The calculation of the results depends upon the following considerations:

Let $a_{t_1}^{t_2}$ = amount of nitrite removed between times t_1 and t_2 and n_t = count at time t.

Since rate of removal of nitrite depends upon the number of cells present and on the time interval

$$\mathrm{d}a = k' n_t \, \mathrm{d}t$$

whence

$$a_{t_1}^{t_2} = k' \int_{t_1}^{t_2} n_t \, \mathrm{d}t$$

If this integral can be evaluated, k' can be found. Two methods were used according to circumstances: (i) graphical integration, and (ii) integration with the assumption of logarithmic growth.

(i) n_t is plotted against time and the area under the curve computed for successive time intervals.

$$\Delta_{t_1}^{t_2} = \int_{t_1}^{t_2} n_t \, \mathrm{d}t$$

Hence

$$k' = \frac{a_{t_1}^{t_2}}{\Delta_{t_1}^{t_2}}$$

This method was used whenever growth was not logarithmic.

(ii) When growth was logarithmic:

$$n_t = n_0 e^{kt}$$

where $k = \ln 2/T$, T being the mean generation time (*m.g.t.*).

Hence,
$$\int_{t_1}^{t_2} n_t \, dt = \left[\frac{n_0}{k} e^{kt}\right]_{t_1}^{t_2} = \frac{n_0}{k}(e^{kt_2} - e^{kt_1})$$
$$= T(n_{t_2} - n_{t_1})/\ln 2$$

Hence
$$k' = \frac{a_{t_1}^{t_2} \times \ln 2}{T(n_{t_2} - n_{t_1})}$$

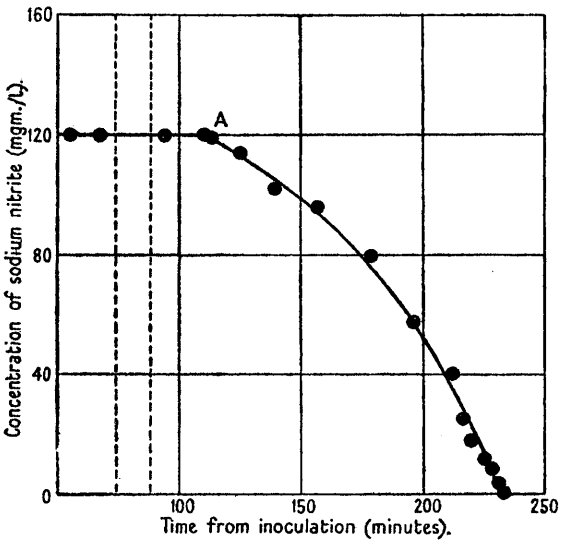
In the following, rates are expressed in terms of mg./l. of sodium nitrite removed or formed in 100 minutes by cells assumed to remain at a constant count of 150. Hence

$$\text{Rate} = k' \times 15 \times 10^3.$$

These will be taken as the standard units in all tables and diagrams.

FIG. 1.

The removal of nitrite by washed cells.



The curve is the theoretical curve drawn with the assumption of a constant rate of removal of nitrite of 86 units.

Growth began at the point A.

The rate so determined was normally plotted either against time, or else against the number of divisions, x_t, which a given culture has undergone at a time, t. x_t is given by the expression, $\log(n_t/n_0) = x_t \log 2$.

In order that the results of the main series of tests may be more concisely summarised in later sections, the course of typical experiments will first be described once and for all.

(4) *Typical Behaviour.*—(a) *In the nitrite test medium.* After inoculation there was an interval during which the concentration of nitrite remained unchanged. Growth then started, and, simultaneously, the concentration of nitrite began to fall, reaching zero after 1—2 hours. During the removal of nitrite growth was logarithmic, but as soon as its concentration fell to zero, the rate of division dropped abruptly. Fig. 1 shows the drop of nitrite concentration as a function of time and also a curve calculated on the assumption that the rate of nitrite removal during logarithmic growth remains constant at 86 units.

Control experiments showed exact proportionality between the initial count of a suspension and its activity. The age of a suspension, estimated from the time of centrifuging, had little effect on the results. The age of the parent culture from which the suspension was prepared had, within moderate limits, also little effect but was always carefully controlled to within 1 hour of the cessation of logarithmic growth.

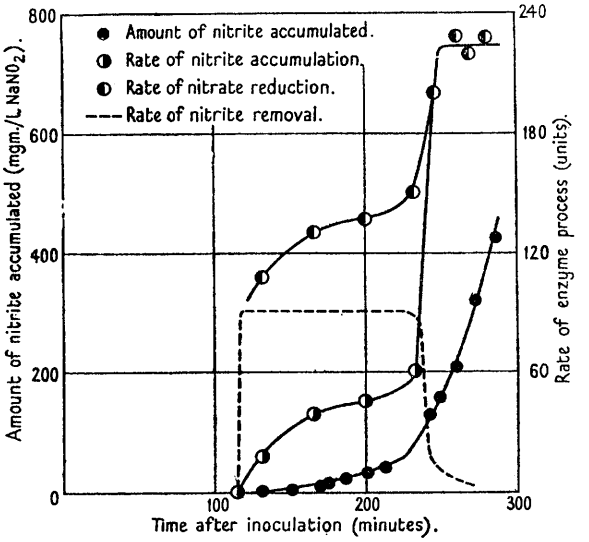
(b) *In the nitrate test medium.* The behaviour in the nitrate test medium varied

considerably with the experimental conditions and with the history of the inoculum. It can be conveniently summarised under three headings.

With strains of *Bact. lactis aerogenes* having a low growth rate in nitrate, no significant amounts of nitrite accumulated during growth, though a just detectable concentration (1—2 mg./l.) appeared before growth started, only to disappear again when it did.

FIG. 2.

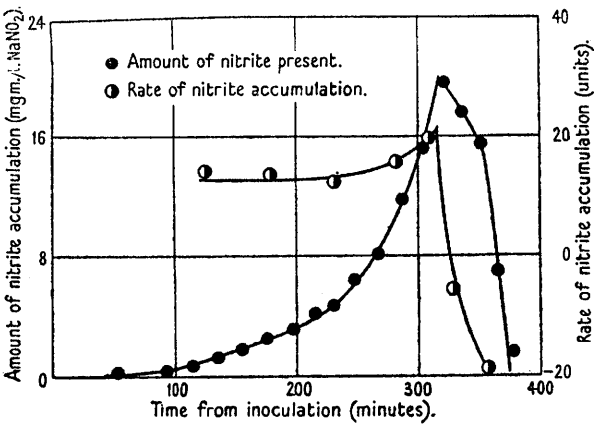
Behaviour of washed cells, trained to nitrate, in a nitrate medium.



With other strains, having a higher growth rate in nitrate, nitrite accumulated during logarithmic growth. Under the conditions of most of the experiments the nitrite concentration continued to increase during the logarithmic phase, and after the end of this it rose sharply. Fig. 2 gives the results of a typical experiment : it shows (*a*) the actual concentration of nitrite, (*b*) the rate of accumulation of nitrite (per cell), and (*c*) the rate of reduction of nitrate to nitrite, estimated on the assumption that the latter is removed at the same rate as in the nitrite test medium.

FIG. 3.

Behaviour of trained cells transferred from a fully grown culture medium and grown in a nitrate medium.



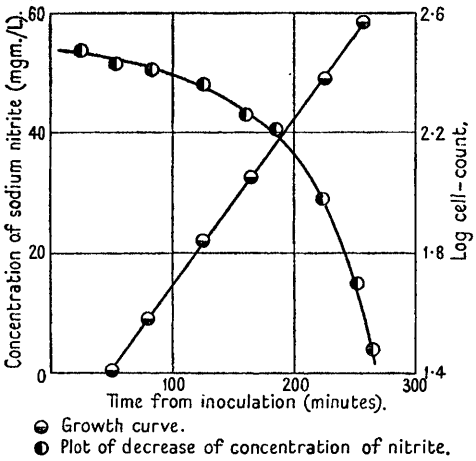
The above mentioned experiment was carried out in a 175 ml. flask with moderate aeration. In corresponding experiments carried out in 7″ × 1″ test-tubes, nitrite still accumulated during growth, but at a slower rate. Just before the end of logarithmic growth its concentration fell rapidly to zero, as shown in Fig. 3. The reason for this difference in behaviour is thought to lie

in the different rates of aeration. (The effect of the rate of aeration on the enzymic behaviour has been studied in some detail and is discussed later.) Under controlled experimental conditions, however, the behaviour was reproducible.

(c) *In a mixed medium.* Fig. 4 shows the growth curve of an untrained strain (which consumed nitrite faster than it could produce it from nitrate), and the net consumption of nitrite which occurred throughout the logarithmic phase in the mixed medium. From the net removal of nitrite in this medium and from the corresponding rate in a medium containing no nitrate the rate of reduction of the latter can be calculated.

FIG. 4.

The disappearance of nitrite from a medium containing both nitrite and nitrate, inoculated with untrained cells.



(5) *Experiments on the Adaptation of* Bact. lactis aerogenes *to Nitrate and Nitrite.*—(a) *Growth-rate experiments.* In the first series of experiments a strain of *Bact. lactis aerogenes* was serially subcultured in the standard nitrate medium, and its growth characteristics were determined at intervals.

Even on the first transfer to the nitrate there was little difference between the observed lag and that for a parallel subculture into the ammonium sulphate medium in which the cells had been previously cultivated. Fifteen subcultures in nitrate made little difference to this state of affairs. In some experiments with very young cultures the lag of the untrained cells was actually less in the nitrate medium, but, on the whole, the lag–age relationships were very similar to those found in the ammonium sulphate medium, and it would appear that changes in lag, in sharp contrast with certain other cases, play little part in the adaptive phenomena

The mean generation time was 66 minutes on the first subculture in nitrate and then fell in the manner shown in Fig. 5.

In a first subculture in the nitrite medium the mean generation time was 100 minutes and then fell gradually to the same final value as that reached in nitrate (Fig. 5). Since growth in nitrite was slower than that in nitrate, it seemed at first sight that the former could not be an intermediate in the utilisation of the latter. This idea proved, however, illusory. Nitrite at the concentrations used in the standard medium was found to have a specific inhibitory effect on growth. At lower concentrations of nitrite the mean generation time drops to values as low as 53 minutes (Fig. 5). The principal effect of training cells by serial subculture in the test nitrite is simply to adapt them to resist this specific drug action of the nitrite itself. The training has little effect on the growth rate in nitrite solutions which are sufficiently dilute.

Serial subculture in nitrate confers some immunity to nitrite, 105 passages through the former being roughly equivalent to 25 through the latter. From this it is reasonable to assume that at some stage during growth in a nitrate medium the cells are subjected to the action of nitrite, which thus appears in fact to be an intermediate in the utilisation of the former.

Determination of growth rates at nitrate concentrations in the range 0·2—5 g./l. showed no dependence, and the growth rate in ammonium sulphate was unaffected by addition of nitrate. Thus there is no inhibitory effect of nitrate corresponding to that of the nitrite.

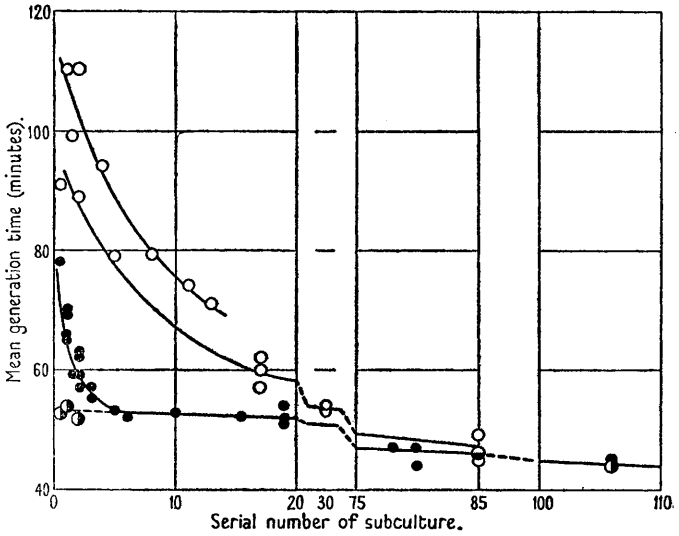
With the untrained cells in nitrate the reduction of nitrate to nitrite appears to be the slowest step. After several subcultures in nitrate the enzyme responsible for this reduction seems to have expanded, and the slowest step finally seems to be the removal of the nitrite. In the trained state all the cells grow at the rate characteristic of that initially found for media containing dilute nitrite.

(b) *Enzyme activities.* The same strain of cells was next tested for its power of reducing nitrate, and of consuming nitrite, as explained in Section 2.

The specific rate of nitrite utilisation was found, for the untrained cells, to be 86 units. In the mixed nitrate–nitrite test medium the net removal rate for nitrite was 27, and, since the gross removal rate was 86, the rate of formation of nitrite from nitrate was 59 (Fig. 4). In a medium containing nitrate only no appreciable accumulation of nitrite occurred.

FIG. 5.

The adaptation of Bact. lactis aerogenes *to growth in nitrate and in nitrite media.*



Open circles—trained in nitrite, tested in nitrite (two series). Black full circles—trained in nitrate, tested in nitrate. Vertically divided circles—trained in nitrate, tested in dilute nitrite. Horizontally divided circles—trained in nitrite, tested in dilute nitrite.

After 14 subcultures in the nitrate medium the rate of nitrate reduction increased, and, in the nitrate test medium, nitrite accumulated at a net rate of 14 units. The rate of utilisation of nitrite had not changed appreciably (88 units approx.). Thus the total rate of nitrate reduction increased on training from 59 to 88 + 14 = 102 units.

As appears from Fig. 5, the initial rapid adaptation to nitrate is followed by a phase in which the training shows a slow further improvement. Several strains were tested before and after this phase of the training (including strains which were found for some reason, presumably connected with their racial history, to start with a partial adaptation to nitrate). They showed little further change in the ability to reduce nitrate to nitrite, but a gradual improvement in the power of removing nitrite. In Table I, for example, is shown, for various stages of growth, the total rate of nitrate reduction by a strain initially partially trained and after 80 further subcultures in nitrate, which are seen to have caused little change.

TABLE I.

n.	Original strain.	Same strain after 80 subcultures in nitrate.
	Rate of nitrate reduction.	
100	114	114
150	122	122
215	138	139
300	rising rapidly to *ca.* 230	
	Rate of nitrite utilisation.	
50—120	94—96	100—102

In the experiments on nitrate reduction the rate always increased rapidly towards the end of the growth—which under the conditions of the experiment occurred when the cells in the initial heavy suspension had suffered about 2 successive divisions. This effect is shown in Fig. 2. When standard rates have been referred to above they are those characteristic of the early stages of the growth, before the suspension has seriously multiplied.

(c) *Experiments with glycerol in place of glucose as a carbon source.* No reduction of nitrate or consumption of nitrite occurs when the carbon source is omitted from the medium. Thus the reduction process is linked with the carbon utilisation and is presumably coupled with some dehydrogenase system concerned in the latter.

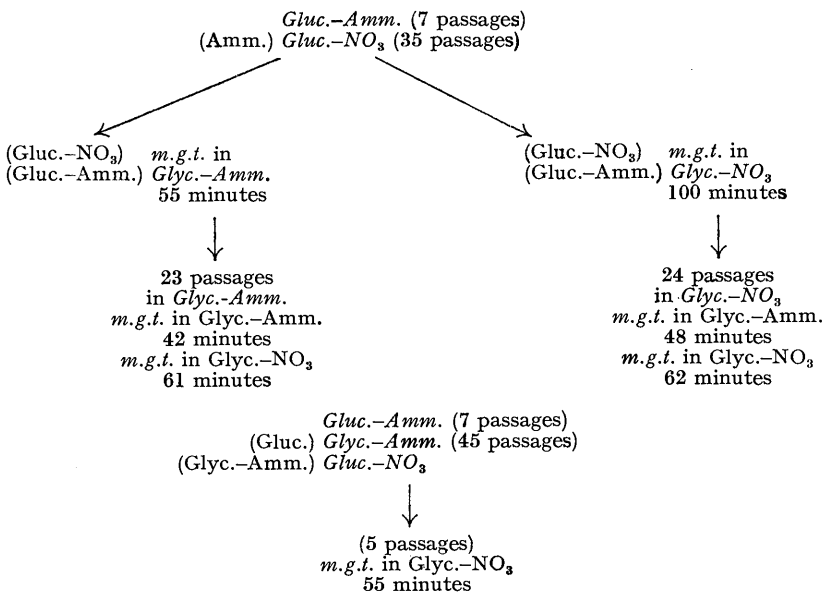
The experiments now to be described had two objects, namely to investigate whether training to glycerol–ammonium sulphate media confers training to glycerol–nitrate media, and to find what changes in enzyme activity occur during training to the new combination of substrates. This should throw some light upon the connexion between the enzymes that reduce glycerol or glucose and those which reduce nitrate.

The conclusion is that if cells have first been trained to nitrate in the absence of glycerol, then training to a glycerol–ammonium sulphate combination confers adaptation to a glycerol–nitrate medium as rapidly as does growth in the glycerol–nitrate medium itself. The order of training to glycerol and to nitrate is unimportant, and the adaptations to nitrate and to glycerol appear to be independent of one another. The method of training to glycerol was as described in previous papers (Lodge and Hinshelwood, *Trans. Faraday Soc.*, 1944, **40**, 571; Cooke and Hinshelwood, *ibid.*, 1947, **43**, 733). The above summarised statement is illustrated by the results in Table II.

TABLE II.

Glycerol–nitrate training experiments.

Key to abbreviations: (1) Cells have already been trained to the substrates shown in parentheses, *e.g.*, (Gluc.–NO_3). (2) Cells are being trained to the combination in italics, *e.g.*, ***Glyc.–Amm.*** (3) The number of passages through the training medium is given in parentheses.



Experiments on the enzyme activity of a strain partially adapted to a glycerol–nitrate medium gave a nitrite utilisation rate of 103 units with glycerol and of 100 units with glucose. The rate of nitrate reduction appeared to be appreciably greater with glycerol, the value starting from about 190 units in the early stages of the test and rising still higher.

(6) *Experiments with* Bact. coli *and* Bact. coli mutabile.—The investigation of *Bact. coli* is complicated by the fact that it must first be trained to grow in the ammonium sulphate medium. Two strains were used, one of *Bact. coli commune* and one of *Bact. coli mutabile*. The results with both were closely similar.

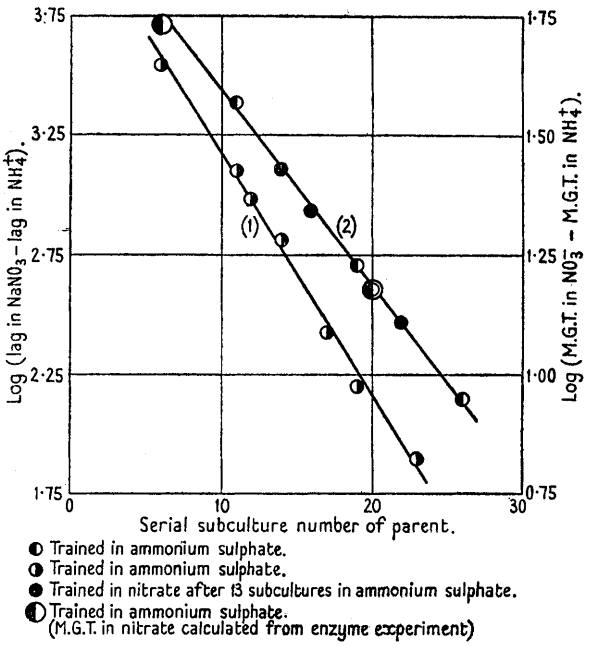
The most marked apparent difference between these and the *aerogenes* strain lies in the

existence of a considerable lag in the earlier subcultures in nitrate. This lag gradually diminishes on serial subculture until it becomes equal to that in ammonium sulphate. In this connexion a rather surprising fact appears, namely that prolonged serial subculture in ammonium sulphate trains the *Bact. coli* strain to nitrate as efficiently as a corresponding number of subcultures in nitrate itself. This is shown by the results in Fig. 6, where the adaptation is shown as a function of the total number of subcultures in either medium. (The logarithmic scale is for convenience only.)

The training to ammonium sulphate is completed sooner than that to nitrate, a culture just trained to the former requiring a considerable number of further subcultures before becoming fully trained to the latter. Direct training in the nitrate medium (with gradual elimination of an initial asparagine source) gives adaptation both to nitrate and to ammonia, the final mean generation times in the two media being 38 minutes and 37 minutes respectively (*mutabile* strain) after 9 passages through nitrate.

FIG. 6.

Training of Bact. coli.



(1) *Log Δ (lag) against serial subculture number of parent.*
(2) *Log (m.g.t. in* $NaNO_3$—38) *against serial subculture number of parent.*

Since the rate of training to nitrate is the same in both media, the natural conclusion is that what occurs here is the development not of a nitrate- or nitrite-specific reducing system, but one which is capable of reducing these ions as an incidental to its normal function. In other words, that the nitrate–nitrite adaptation is here the concomitant to the adaptation of some part of the normal dehydrogenase system as the cells become trained to the synthetic medium.

The enzyme tests (with both the *commune* and the *mutabile* strains) showed certain contrasts with *Bact. lactis aerogenes*. The rate of nitrate reduction was always greater than the rate of consumption, so that nitrite accumulated in the medium during growth in nitrate. The cells were never observed in the condition corresponding to the untrained state of the *Bact. lactis aerogenes*, where nitrate reduction was the slowest step. This difference is hardly of great significance since strains of *Bact. lactis aerogenes* were later encountered in which a certain initial degree of adaptation to nitrate (acquired during their past history in an unknown manner) was evident.

The rates of nitrite consumption by the *mutabile* strain are as follows :

after short training in ammonium sulphate medium . .	66 units
after prolonged training in ammonium sulphate medium .	118 units
after training in nitrate medium	138 units

The rate of reduction of nitrate was always greater, and for the nitrate-trained strain showed a value, steady during growth, of 185 units, having shown, after short training in ammonium sulphate only, a value of about 155 units.

After 6 subcultures in ammonium sulphate the *Bact. coli commune* strain showed a rate of nitrite consumption of about 60 units, and after 20 subcultures this had risen to 99, the rate of nitrate reduction being, at this stage, 150—170 units.

(7) *Correlation of Growth Rate and Rate of Nitrite Consumption.*—The rate of growth is directly proportional to the rate of consumption of nitrite. This is shown by the constancy of the product : rate of nitrite consumption × mean generation time for the *Bact. coli mutabile* in nitrate at various stages of training, for *Bact. coli commune*, and for trained *Bact. lactis aerogenes*.

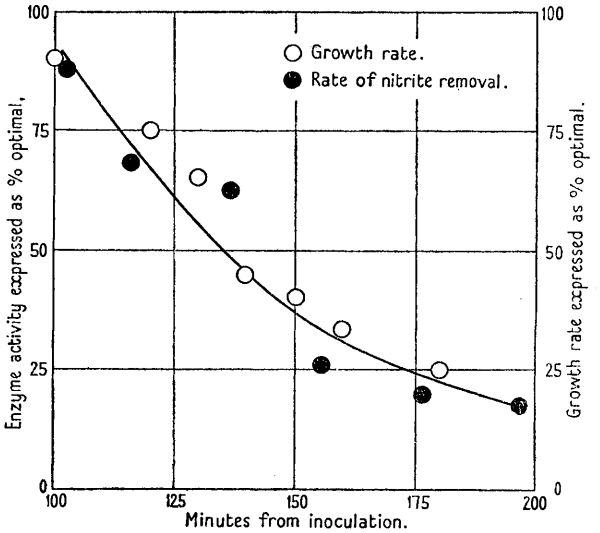
	Rate of nitrite consumption.	M.g.t.	Product.
Bact. coli mutabile	66	82	$5{\cdot}41 \times 10^3$
	118	45	$5{\cdot}31 \times 10^3$
	138	38	$5{\cdot}24 \times 10^3$
		Mean :	$5{\cdot}3 \times 10^3$

	Rate of nitrite consumption.	M.g.t.	Product.
Bact. coli commune	99	53	$5{\cdot}25 \times 10^3$
Bact. lactis aerogenes (trained)	86	53	$4{\cdot}56 \times 10^3$
	87	52	$4{\cdot}52 \times 10^3$
	90	51	$4{\cdot}59 \times 10^3$
	95	49	$4{\cdot}65 \times 10^3$
	102	54	$4{\cdot}59 \times 10^3$
		Mean :	$4{\cdot}6 \times 10^3$

For the untrained *Bact. lactis aerogenes* the product of nitrate reduction rate and mean generation time gave approximately this value, *viz.*, 4·55 and 4·65.

FIG. 7.

Enzyme activity and growth rate in magnesium-deficient nitrate medium.



Further evidence of the correlation between the growth rate and the rate of nitrite consumption appeared in the course of some experiments on growth in a medium deficient in magnesium. In such a medium the growth rate is lower than normal and drops during growth. In Fig. 7 the growth rate and the rate of nitrite removal are both plotted as percentages of their respective optima. The points lie on one curve. This shows the correlation in question, which does not appear when the nitrate reduction rate is plotted.

The proportion of nitrite which is usefully incorporated into the nitrogenous material of the cell is constant. This appears from the following results. With *Bact. coli mutabile* there is an exact linear relation between the total increase in cell count and the total amount of nitrite removed, 1 mg./l. of sodium nitrite giving an increase of 1·95 in the hæmacytometer reading.

If one assumes that this is a constant, then the product: mean generation time × nitrite removal rate is easily shown to be $5{\cdot}33 \times 10^3$, in good agreement with that found from the enzyme experiments. With *Bact. lactis aerogenes* the mean value of this product is $4{\cdot}6 \times 10^3$, *i.e.*, for a given mean generation time the nitrite removal rate is lower in the ratio 4·6 : 5·3. Thus the increase in hæmacytometer count for a consumption of 1 mg./l. should here be greater in the inverse of this ratio, that is it should be $1{\cdot}95 \times 5{\cdot}3/4{\cdot}6 = 2{\cdot}24$. The mean value found in various direct experiments was 2·35.

(8) *Discussion.*—The utilisation of nitrate by the coliform bacteria seems, in the light of the results described above, to depend upon the reduction of nitrate to nitrite and upon the further reduction of the latter. The relative rates of the two processes: (1) $NO_3' \longrightarrow NO_2'$ and (2) $NO_2' \longrightarrow$ further reduction, seem to vary considerably among different strains. In the least-adapted strains encountered in these experiments (1) was the slower, but rapidly increased during the training process. With all the strains (2) was the slowest after training and appeared to be susceptible to long, slow improvement by serial subculture in media which contained nitrite. In the final state the rate of nitrite removal seemed to determine the overall rate of growth, which, at least, for *Bact. lactis aerogenes*, always remained lower than that attainable in media containing ammonium salts. In so far as ammonia is assumed to be an intermediate in the utilisation of nitrite, it can therefore never attain in the medium a concentration corresponding to the optimum growth rate. This limits the degree of training that can be achieved. The reason why optimum growth in nitrite and optimum growth in ammonia are incompatible in this way is discussed in the following paper.

Training to high concentrations of nitrite seems to be a special phenomenon more related to drug-adaptation than to substrate-adaptation, since at low concentrations the growth rate in nitrite shown by most strains already approaches the optimal.

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