FORMATION OF A BACTERIAL ADAPTIVE ENZYME SYSTEM IN THE ABSENCE OF SUBSTRATE: PRODUCTION OF NITRATASE BY *BACT. COLI* WITHOUT NITRATE.

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PREVIOUS studies have shown that incubation of washed suspensions of the coliform organism " 1433 " with nitrate, in the absence of an exogenous N-source, results in a 20- to 30-fold increase in the ability of the cells to catalyse the reduction of nitrate to nitrite (Pollock, 1946). The presence of amino-acids stimulates this adaptation process (Pollock and Wainwright, 1948).

When nitrate-adapted cells of this organism were grown in glucose-ammonia media not containing nitrate there was no loss of preformed nitratase activity, and the enzymic activity per unit of cell mass decreased towards that of control (unadapted) cells in a manner consistent with a " dilution out " of the enzyme throughout the new cell protoplasm. However, some " basal " nitratase (i.e. nitratase produced in the absence of detectable nitrate) was produced in these media ; i.e. the rate of reduction of nitrate by the whole culture progressively increased throughout the experimental period. In the case of cells not previously adapted to nitrate this production of " basal " activity resulted in a transient increase of nitratase activity per unit of cell mass in the early stages of growth (Wainwright and Pollock, 1949).

Similar experiments with broth and casein hydrolysate media have shown that under certain circumstances there is a very marked production of nitratase activity in the absence of detectable nitrate. An extension of previous studies (Pollock and Wainwright, 1948) on the influence of an exogenous N-source upon the production of nitratase activity with nitrate has also been made, in the hope of obtaining some indication of the role of nitrate in the adaptation process.

As formerly, the production of nitratase activity has been considered purely functionally, as a specific increase in the ability of a given cell mass to catalyse the reduction of nitrate to nitrite under defined conditions. No attempt has been made to determine the exact nature of the increase in activity.

METHODS.

Organism.

The organism used was a strain of *Bacterium coli* (Intermediate Type I) labelled " 1433 " (Pollock, 1946). Cells were harvested from the 16 to 18 hours' aërobic growth on tryptic meat agar, as in former studies (Pollock and Wainwright, 1948).

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Stock suspensions of adapted cells were prepared as previously (Wainwright and Pollock, 1949) by incubation of a 1 mg./ml. concentration of cells for 3 to $3\frac{1}{2}$ hours at 35° in M/100 glucose, M/50 Na nitrate, M/5 phosphate buffer (pH 7·2) and M/600 $MgSO_4$. Control unadapted cells were prepared by similar treatment but without nitrate.

Anaërobic experiments.

Except where otherwise stated, all anaërobic experiments were done in an atmosphere of argon in precision-bore stoppered tubes as previously described (Pollock and Wainwright, 1948). Samples were treated with oxine, to arrest growth and further adaptation, as described by Wainwright and Pollock (1949).

Estimation of nitratase activity.

Nitratase activities were determined as previously (Wainwright and Pollock, 1949), with the further inclusion of M/2000 oxine in the reactants, to minimize any possible further adaptation during the course of the estimation. This concentration of oxine has been shown to have no significant effect upon the nitratase enzyme system.

Cells were added from the stoppers of evacuated Thunberg tubes to a solution containing M/5 phosphate buffer (pH 7·2), M/50 Na formate (as H-donor), M/100 Na nitrate, M/800 $FeSO_4$ and M/2000 oxine, at 35°.

Nitratase activity is defined as μ mol. of nitrite produced by 1 mg. dry weight of cells per hour. *Total nitratase activity* of a culture is defined as the rate of nitrite production by the cells of the culture; i.e. μ mol. of nitrite produced per hour.

Media.

The stock casein hydrolysate medium was a 4 per cent solution of "Ashe" "vitamin-free" casein hydrolysate of pH 7·2 (adjusted with N NaOH), supplemented with M/1560 dl. (synth.) tryptophan and M/1250 l. cystine. The medium was sterilized by Seitz-filtration. The total N content of the medium was 4·0 mg./ml. It contained no detectable nitrate or nitrite.

Where casein hydrolysate is referred to in the text, the concentrations used were obtained by suitable dilution of this medium. In addition to varying amounts of exogenous N-source, except where otherwise stated, all media contained M/5 phosphate buffer (pH 7·2), M/50 glucose, M/1200 $MgSO_4$ and 1 in 2000 stock Fe solution (1·56 g./l. of ferrous ammonium sulphate in M/125 citric acid).

Estimation of nitrate.

Methods tested were the diphenyl benzidine reaction (B.D.H. 1947), the phenoldisulphonic acid, 2 : 4 xylen-l-ol, and zinc-copper couple reduction methods (Allport, 1945), and the reduction method of Lees and Quastel (1946). The first-named method is not sufficiently quantitative for sensitive determinations (I am indebted to Mr. F. W. Edwards for this information), whilst the other methods were not sufficiently sensitive for estimation of very small concentrations of nitrate, or the presence of high concentrations of casein hydrolysate interfered with the estimation.

Accordingly a biological assay was used. For this purpose the test medium was incubated with a very thick suspension (5 to 10 mg./ml.) of nitrate-adapted cells, and samples tested for nitrite with the Griess-Ilosvay reagent. Amino-acids and casein hydrolysate did not interfere with the development of colour.

RESULTS.

Preliminary experiments.

A marked increase in nitratase activity was observed when either nitrate-adapted or unadapted cells were grown anaërobically in 90 per cent tryptic meat broth (total N 7 to 8 mg./ml.) containing no detectable nitrate or nitrite (Fig. 1). Similar increases of nitratase activity were observed with casein hydrolysate media.

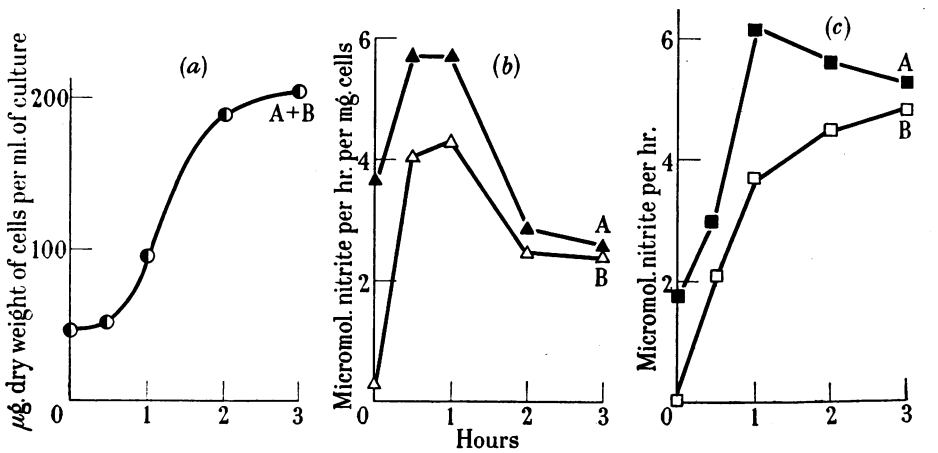

FIG. 1.—Variation of nitratase activity with age of anaërobic cultures of " 1433 " in tryptic meat broth.

(*a*) Growth curves. (*b*) Nitratase activity. (*c*) Total nitratase activity of cultures. (A) Adapted cells. (B) Unadapted cells.

The data presented in Table I show the influence of cell concentration and presence of 0·1 per cent casein hydrolysate upon the production of nitratase activity by unadapted cells with and without nitrate. With a heavy inoculum there was no significant production of nitratase in the absence of nitrate, with or without casein hydrolysate. On the other hand, small inocula produced considerable nitratase with the casein hydrolysate alone.

It thus appeared possible that the extent of nitratase production without nitrate by unadapted cells might be dependent on the *amino-acid concentration per cell*, and further experiments were designed to test this possibility.

Influence of amino-acid concentration per cell on the production of nitratase without nitrate.

A series of media were prepared containing varying concentrations of casein hydrolysate. These media were inoculated with varying concentrations of

TABLE I.—*The Influence of Inoculum Size, Casein Hydrolysate and Nitrate upon the Production of Nitratase Activity.*

Thunberg Tube No.	Concentration of cells μg./ml.	Tube containing: 0·1% casein hydrolysate.	Tube containing: M/50 Na nitrate.	Nitratase activity μ mols. nitrite formed/mgm. dry wt./hr.
1	1000	—	—	0·5
2	1000	+	—	0·6
3	1000	—	+	2·3
4	1000	+	+	4·6
5	50	—	—	0·6
6	50	+	—	1·5
7	50	—	+	0·9
8	50	+	+	4·8
Initial nitratase activity:				0·5

All tubes contained M/5 phosphate buffer (pH 7·2), M/50 glucose, and M/600 $MgSO_4$. Tubes were incubated anaërobically at 35° C. for 1½ hours.

unadapted cells, ranging from 50 μg./ml. to 2000 μg./ml., and incubated anaërobically at 35° for a standard period of one hour.

The extent of growth varied considerably throughout the series of cultures. With inocula of 50 μg./ml. growth occurred in all tubes except those containing no added N-source. The increase of cell mass varied with the concentration of casein hydrolysate, the maximum observed resulting in a two-to-three-fold increase in opacity at the highest concentration of casein hydrolysate. With a given medium the extent of growth decreased with increase of inoculum size. Increases of opacity from inocula of 1000 μg./ml. were rarely observed, whilst only on one occasion was an increase of opacity found with an inoculum of 2000 μg./ml.; a decrease in opacity was usually observed in all media receiving these large inocula.

The results of a typical experiment are illustrated in Fig. 2. Whilst there was some variation in results between several series, all showed the same characteristic dependence of the extent of nitratase production without nitrate upon both the concentration of casein hydrolysate in the medium and the size of the inoculum. From the nature of the curves, it appears possible that with higher concentrations of casein hydrolysate than those employed, even the largest inocula used would attain a maximal nitratase activity of the same order as that found for inocula of 50 μg./ml. Maximal nitratase activities, in the range of 5·5–7·5, were consistently obtained from inocula of 50 μg./ml., with concentrations of casein hydrolysate in the range of 1 to 1·5 per cent.

It has been shown that washed suspensions of *B. cereus* " B 569 " produce considerable adaptive penicillinase in response to the presence of concentrations of penicillin as low as 2×10^{-7} M (Pollock, unpublished experiments). It therefore appeared possible that the marked production of nitratase without added nitrate might be due to undetectable concentrations of nitrate in the medium. Fig. 3 and 4 illustrate experiments in which inocula of 1000 μg./ml. were incubated in washed suspensions and casein hydrolysate medium respectively, with varying concentrations of nitrate. In both cases very small concentrations of added nitrate caused considerable stimulation of nitratase production. Conclusions

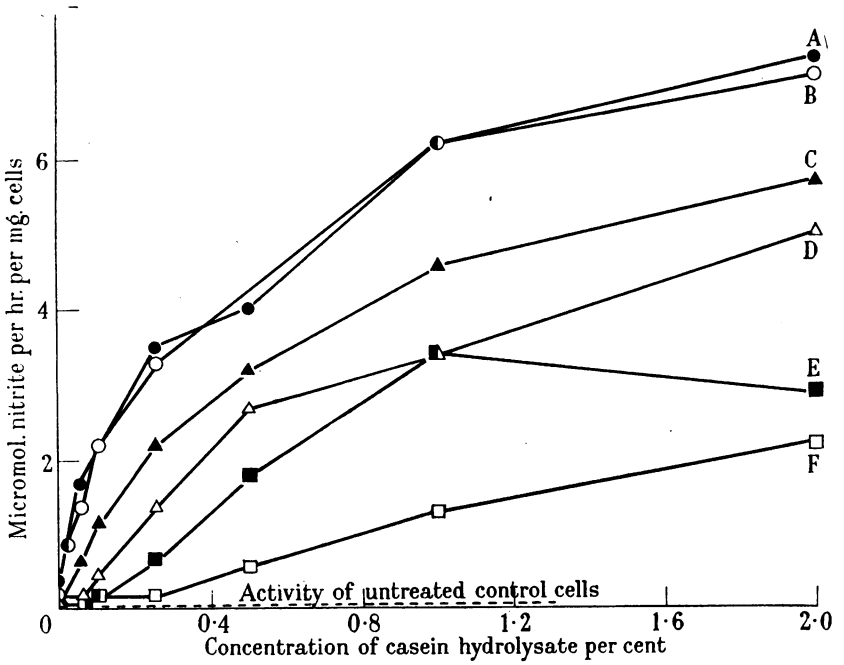

FIG. 2.—Nitratase activity developed by unadapted cells without nitrate with concentration of casein hydrolysate and inoculum size.

(A) Inoculum 50 μg./ml. (B) Inoculum 100 μg./ml. (C) Inoculum 200 μg./ml.
(D) Inoculum 500 μg./ml. (E) Inoculum 1000 μg./ml. (F) Inoculum 2000 μg./ml.

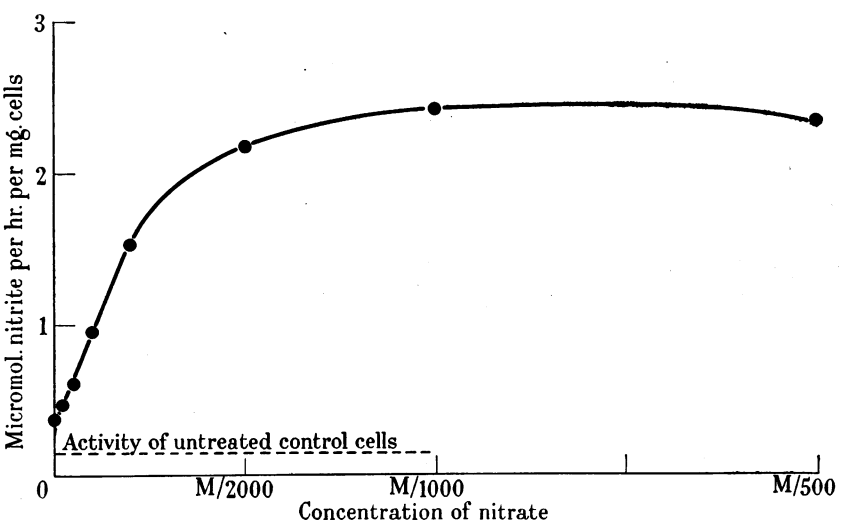

FIG. 3.—Influence of nitrate concentration upon nitratase production in washed suspension. 1 mg./ml. suspensions in M/5 phosphate buffer (pH 7·2), M/50 glucose, M/1200 $MgSO_4$, and 1 in 2000 stock Fe solution were incubated anaërobically at 35° for 1 hour.

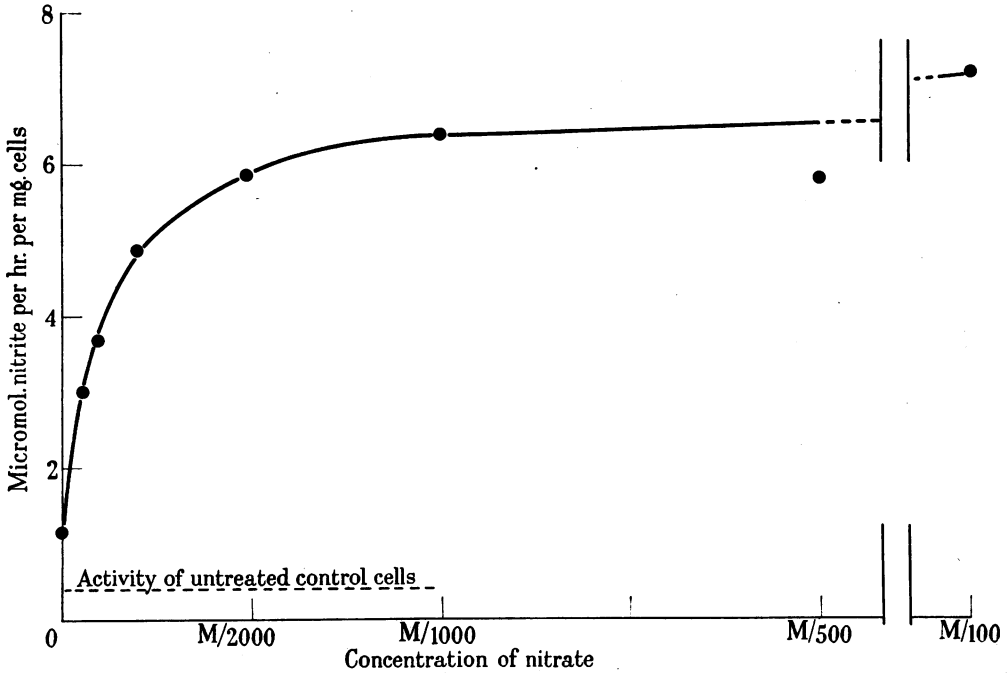

FIG. 4.—Influence of nitrate concentration upon nitratase production in 0·4 per cent casein hydrolysate medium. Time of incubation, 1 hour.

drawn from the experiments done to determine the influence of amino-acid concentration per cell upon " basal " nitratase production are, therefore, open to the possible objection that the observed variations of nitratase activity were due to nitrate present in the media. However, assays of the stock casein hydrolysate medium were completely negative, indicating a possible *maximum* content of 2×10^{-6} M nitrate (plus nitrite). From the nature of the curves of Fig. 3 and 4 it is highly improbable that the marked variations of " basal " nitratase production observed were due to such a low nitrate concentration.

Further, experiments were performed with a mixture of six highly purified amino-acids (for composition see appendix) to show that the activity of the casein hydrolysate medium was due to the amino-acids contained therein, rather than traces of other factors. Making no allowance for possible biological activity of the D isomers, series of media were prepared to contain approximately the same amounts of total available N as in the casein hydrolysate media. These media were inoculated with 50, 200 and 500 μg./ml. respectively of unadapted cells, and the nitratase activities attained after anaërobic incubation at 35° for one hour determined. The nature of the curves obtained (Fig. 5) is very similar to that of curves with casein hydrolysate, although higher concentrations of the amino-acids appear to have been inhibitory to nitratase production. The maximal nitratase activities attained were in the range of 50 to 80 per cent of those observed with the casein hydrolysate media, and it appeared likely that the whole of the activity of the latter could be attributed to the amino-acid content. A mixture of 16 accessory growth factors (at half the concentrations previously employed

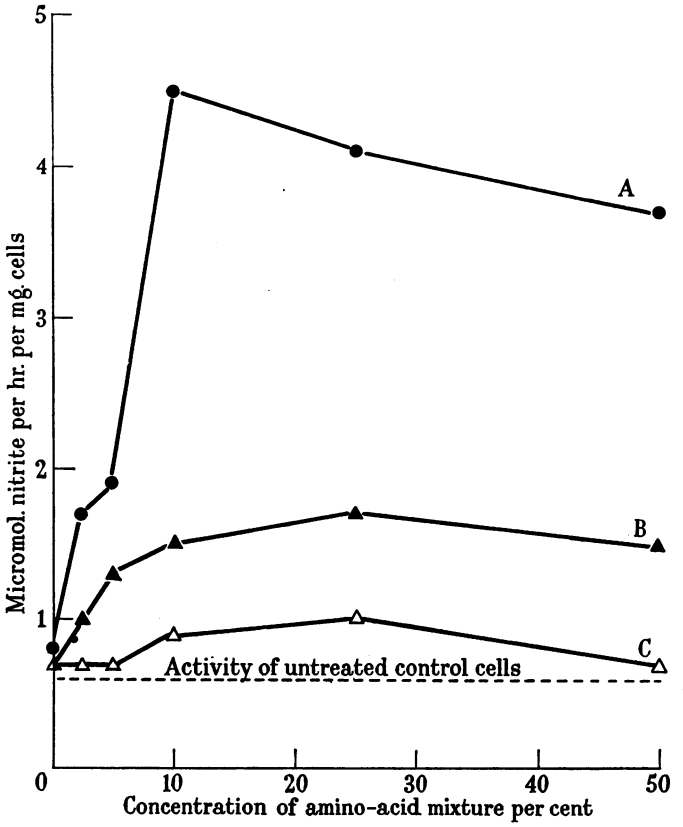

FIG. 5.—Nitratase activity developed without nitrate by unadapted cells in amino-acid media. (A) Inoculum 50 μg./ml. (B) Inoculum 200 μg./ml. (C) Inoculum 500 μg./ml.

(Pollock and Wainwright, 1948)) had no significant effect upon the development of nitratase activity without nitrate under limiting conditions, i.e. with large inocula and low casein hydrolysate concentrations.

Influence of amino-acid concentration per cell upon the development of nitratase in the presence of nitrate.

Previous studies (Pollock and Wainwright, 1948) had shown that broth, or mixtures of amino-acids, both stimulated the production of nitratase with nitrate, and prevented a marked decrease in the rate of adaptation with dilution of the cell concentration. Comparative experiments were therefore made, in the hope of obtaining some indication of the role of nitrate in the adaptation process.

A series of media containing M/50 Na nitrate and varying concentrations of casein hydrolysate were inoculated with unadapted cells, and the nitratase activities attained after anaërobic growth at 35° for one hour determined. Results from a typical experiment are summarized in Fig. 6.

Comparison of these results with those given in Fig. 2 shows the pronounced influence of the presence of nitrate upon the development of nitratase activity

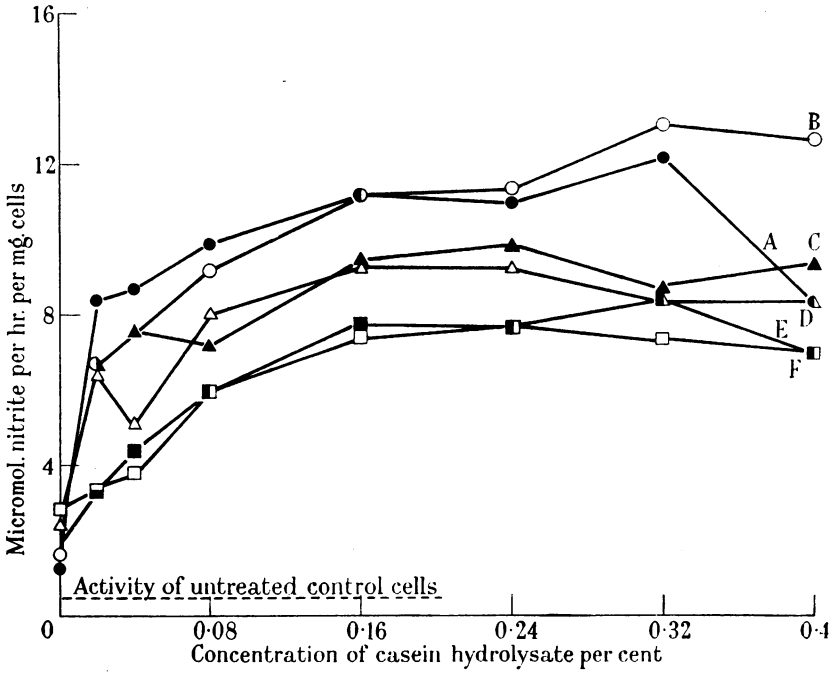

FIG. 6.—Nitratase activity developed by unadapted cells with concentration of casein hydrolysate and inoculum size in the presence of nitrate.

(A) Inoculum 50 μg./ml. (B) Inoculum 100 μg./ml. (C) Inoculum 200 μg./ml.
(D) Inoculum 500 μg./ml. (E) Inoculum 1000 μg./ml. (F) Inoculum 2000 μg./ml.

by unadapted cells, although nitrate had no significant effect upon the extent of growth during the experimental period with all levels of inocula tested.

Although the maximal levels of nitratase activity attained were influenced to some extent by inoculum size, the concentrations of casein hydrolysate required for development of these levels of maximal activity were largely independent of the size of the inoculum. Furthermore, considerably lower concentrations of casein hydrolysate medium were required for development of the maximal nitratase activities with nitrate than without. Thus, using an inoculum of 50 μg./ml. of cells, in the presence of nitrate maximal nitratase activities were produced with concentrations of 0·08 to 0·15 per cent of casein hydrolysate, whilst without nitrate, concentrations of 1 to 1·5 per cent were required ; with higher cell concentrations the difference was more marked.

The extent of production of nitratase activity with a given inoculum was always higher in the presence of nitrate than in its absence, at all levels of casein hydrolysate concentration tested. Thus, with inocula of 50 μg./ml. of cells maximal nitratase activities produced with nitrate were in the range of values 9 to 12, whilst corresponding values without nitrate ranged from 5·5 to 7·5.

Influence of the chemical nature of the exogenous N-source.

Media were prepared containing concentrations of ammonium chloride to correspond to the final levels of total N in the casein hydrolysate media. Inocula

of 50, 200 and 500 μg./ml. respectively of unadapted cells were incubated anaërobically at 35° for one hour.

A decrease in opacity was observed in all cultures, due probably to the high salt content of the media. Fig. 7 illustrates the nitratase activities attained in

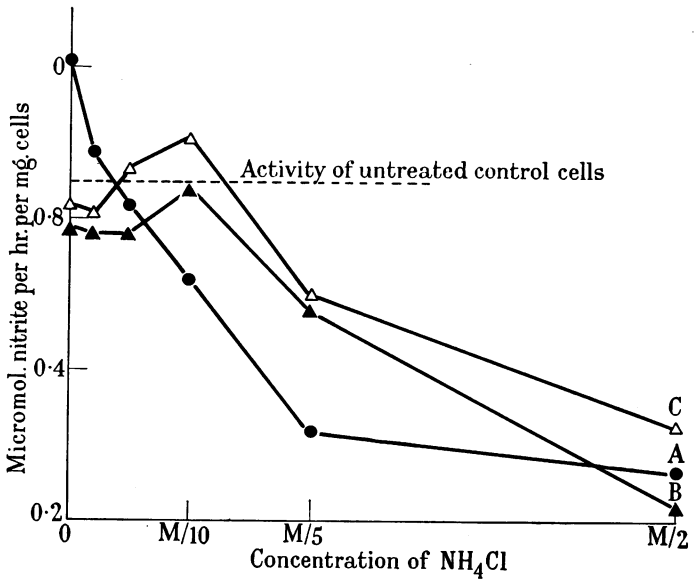

FIG. 7.—Production of nitratase activity without nitrate with ammonium chloride concentration. (A) Inoculum 50 μg./ml. (B) Inoculum 200 μg./ml. (C) Inoculum 500 μg./ml.

these cultures. Ammonium chloride could not replace preformed amino-acid in the production of nitratase without nitrate, and appeared to be toxic in high concentrations to the enzyme.

Influence of other factors.

It appeared possible that the influence of nitrate upon the development of nitratase activity could be ascribed to its known properties as an H-acceptor (Stickland, 1931). However, other known H-acceptors (sodium tetrathionate and sodium fumarate) were found to have no significant influence upon the development of nitratase activity, with or without nitrate, by inocula of 1000 μg./ml. of cells. Fig. 8 illustrates an experiment in which the nitratase activities attained after anaërobic incubation for 1 hour in casein hydrolysate supplemented with M/50 Na nitrate and/or M/50 Na fumarate (pH 7·2) were compared.

The influence of glucose concentration upon the development of nitratase activity, with and without nitrate, is illustrated by Fig. 9. The concentrations of casein hydrolysate medium used were those for which near-maximal nitratase activities were developed with M/50 glucose and inocula of 50 μg./ml. of cells. With nitrate in lower casein hydrolysate concentrations stimulation by glucose was, as expected, marked (Fig. 9*a*). However, in the high concentrations of casein hydrolysate the stimulatory effect of glucose was slight (Fig. 9*b*). Further,

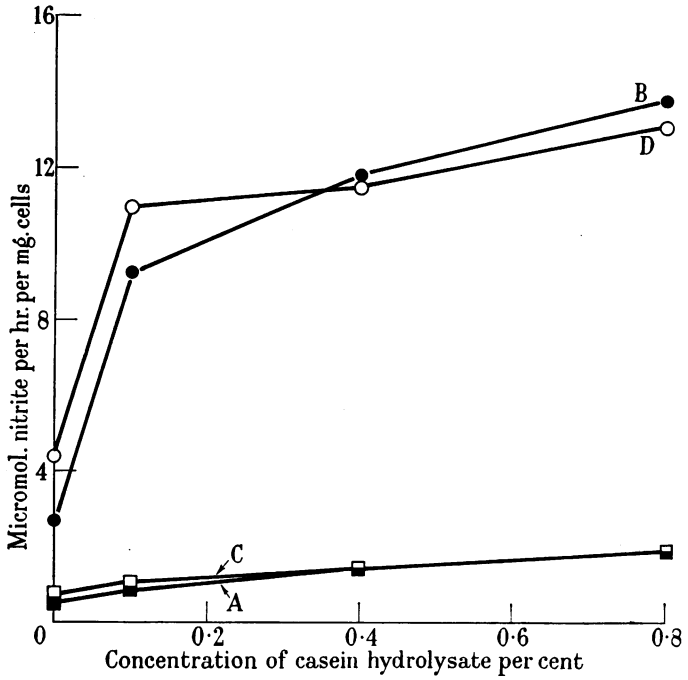

FIG. 8.—Influence of nitrate and fumarate on development of nitrate activity with 0·8 per cent casein hydrolysate.

(A) No added H-acceptor. (B) With M/50 Na nitrate.
(C) With M/50 Na fumarate (pH 7·2). (D) With M/50 nitrate in M/50 fumarate (pH 7·2)
Inoculum 1000 μg./ml.

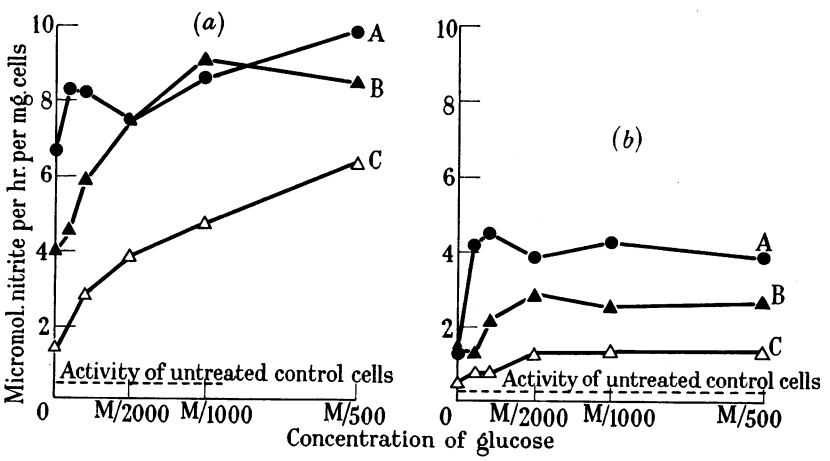

FIG. 9.—Influence of glucose concentration on development of nitratase activity :
(*a*) 0·08 per cent casein hydrolysate with M/50 Na nitrate.
(*b*) 0·5 per cent casein hydrolysate without nitrate.

(A) Inoculum 50 μg./ml. (B) Inoculum 200 μg./ml. (C) Inoculum 500 μg./ml.

the concentration of glucose causing maximal stimulation of nitratase production without nitrate was independent of inoculum size.

Omission of the mixture of phosphate buffer, $MgSO_4$ and stock Fe had no consistent significant effect upon the production of nitratase activity without nitrate from inocula of 200 μg./ml. of cells, or less, with 0·5 per cent of casein hydrolysate.

DISCUSSION.

These results show that under certain conditions *Bact. coli* " 1433 " can produce considerable nitratase activity, in the absence of detectable nitrate. Although it has not been possible to show the " basal " enzyme to be identical with that formed in the presence of nitrate, there is no evidence suggesting differences between them.

It would appear that the prime factor determining the extent of " basal " nitratase production is the *amino-acid concentration per cell* (Fig. 2), and that when this concentration is sufficiently high, almost as much nitratase may be formed without nitrate as in its presence. With nitrate, however, it appears to be the absolute concentration of amino-acids which largely determines the extent of nitratase production (Fig. 6). The decrease in maximal activity attained with increase in inoculum size does not appear to be influenced by amino-acid concentration, as increase of the latter over the range of 0·15 to 1·5 per cent of casein hydrolysate has no significant effect, and may be due to limiting concentrations of glucose per cell (Fig. 9*a*) or to the accumulation of toxic metabolites—in particular nitrite.

Short growth periods (1 hour) are of considerable importance in the demonstration of extensive formation of nitratase without nitrate. Maximal nitratase activity is attained approximately at the end of the lag phase, and the activity subsequently falls fairly rapidly, whilst there is no significant further increase in the total nitratase activity of the culture (Fig. 1). Further, the nitratase activity of older cells (15 to 18 hours) from cultures without nitrate is relatively low whatever the growth conditions (Pollock, 1946). In this connection it is of interest that data presented by Hegarty (1939) suggest that young cells (2 hours) of *Streptococcus lactis* from nutrient peptone media containing no added sucrose possess significant saccharase activity, whilst older cells (12 hours) require a period of adaptation before showing detectable activity in fermenting this sugar.

Amongst other factors involved in the formation of adaptive enzymes, it appears that the nature and amount of N available for protein synthesis is of great importance. When the supply of amino-acids per cell is reduced to limiting amounts, through growth and metabolism, increase of inoculum size, or the necessity of synthesis from ammonium salts, the extent of " basal " nitratase production is markedly reduced.

It also appears that the presence of substrate alters the " nitratase-forming system " such that (*a*) limiting amounts of amino-acids are more efficiently utilized in the formation of specific adaptive enzymes, and (*b*) the amount of nitratase produced is determined by the absolute concentration of amino-acids rather than the concentration per cell. Further, the results obtained with the penicillinase of *B. cereus* (Pollock, unpublished experiments) and the nitratase system (Fig. 3 and 4) show that this " directive " influence upon the amino-acid metabolism of the cell can be effected by very small concentrations of substrate,

although it is highly improbable that the observed variations of " basal " nitratase can be attributed to the presence of traces of nitrate. Monod (1949) has postulated that the function of the substrate in the adaptation process is to stabilize unstable enzyme normally formed in the absence of specific substrate, but in the case of the nitratase of *Bact. coli* at least this is highly improbable. Almost as much nitratase may be formed in the absence of detectable nitrate as in its presence, and, once formed, the enzyme is apparently completely stable during growth in nitrate-free media (Wainwright and Pollock, 1949).

SUMMARY.

Considerable increases of activity of the adaptive nitratase system of *Bact. coli* " 1433 " were produced in casein hydrolysate media containing no detectable nitrate or nitrite. The effect of the media could be almost entirely ascribed to the amino-acid content.

The extent of development of nitratase activity without nitrate was dependent on the *amino-acid concentration per cell.*

Maximal nitratase activities produced with nitrate were higher than those developed in the absence of nitrate, whilst the concentrations of casein hydrolysate necessary for development of maximal nitratase activity were very considerably reduced by the presence of nitrate.

Small concentrations of nitrate were sufficient to stimulate greatly the production of nitratase activity.

Ammonium chloride at the same concentration of total N could not replace preformed amino-acids in the marked production of " basal " nitratase.

I am greatly indebted to Dr. M. R. Pollock for his valuable advice and criticism, and for permission to quote unpublished work. Thanks are also due to Mr. F. W. Edwards for helpful advice.

APPENDIX.

Amino-Acid Mixture Tested for Activity in the Development of Nitratase Activity without Nitrate.

	Concentration.
Glycine (synth.) . . .	M/18
dl. Alanine (synth.) . .	M/9
dl. Aspartic acid (synth.) . .	M/9
dl. Glutamic acid (synth.) .	M/9
dl. Leucine (synth.) . .	M/9
l. Arginine monohydrochloride .	M/18

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