NITRATE REDUCTION BY THE SPECIES AGROBACTERIUM RADIOBACTER, CONN.

by

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ABSTRACT

Nitrate reduction by Agrobacterium radiobacter under laboratory conditions of growth was shown to be an adaptive response. Evidence is presented indicating that assimilatory and respiratory nitrate reduction is markedly influenced by environmental aeration conditions. In general, the reduction of nitrite is influenced in a similar though not identical way by changes in cultural aeration conditions.

Evidence is presented that the enzyme nitrate reductase is in a particulate form associated probably with components of the cytoplasmic membrane-cell wall complex. The nitrite reductase system, on the other hand, seems to be in a soluble form associated with cell cytoplasm.

Protoplast induction in the culture was examined as an approach to enzyme localization studies. Our results indicate that the culture undergoing an adaptation to nitrate reduction does not respond favourably to the lysozyme-EDTA protoplast induction technique. Possible reasons for this finding are discussed.

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INTRODUCTION

The role of microorganisms involved in changes occurring in inorganic soil nitrogen has been under active investigation for a number of years and from these studies several salient facts have emerged. Firstly, while various forms of inorganic nitrogen including gaseous nitrogen are acceptable, many soil microorganisms prefer ammonium salts as their nitrogen source. In at least one instance, as in the case of the chemoautotroph Nitrosomonas, ammonium constitutes the only energy substrate acceptable as well as being the nitrogen source. Second, when available ammonium supplies are depleted nitrate and nitrite nitrogen are utilized. These activities contribute to the gradual decrease of non-protein nitrogen available in the soil. In addition to assimilatory use, nitrate normally available for plant nutrition may further be depleted through utilization for respiratory activities by soil bacteria. In this respect, nitrate is used as a terminal electron acceptor in place of oxygen.

In recent years attention has been directed to studies of the enzymic steps involved in nitrogen assimilation,

particularly the pathways of nitrate and nitrite reduction. From these studies, again, several salient facts have emerged. First, the enzyme formed for the initial step in nitrate utilization seems to be adaptive rather than constitutive. The latter point has been reported by a number of investigators including Pollock (1946) and Silver (1957). Second, nitrate reductase systems studied thus far can be classified into two main types: assimilatory nitrate reductase, and dissimilatory nitrate reductase. In the assimilatory process which occurs primarily under aerobic growth conditions, nitrate is reduced through a series of enzymic steps usually to ammonium before incorporation into cell protein structures. On the other hand, in the dissimilatory process occurring mainly under anaerobic or micro-aerophilic conditions of growth, nitrate acts as stated previously as the terminal electron acceptor in place of oxygen. A common step in both these processes is the reduction of nitrate to nitrite mediated by the enzyme nitrate reductase.

Quastel *et al* (1925) were probably the first to demonstrate bacterial nitrate respiration when they found that *E. coli* failed to grow anaerobically on lactate unless

a suitable electron acceptor such as nitrate was supplied in the medium. Since then, a number of workers have confirmed the role of nitrate in assimilation and dissimilation in various bacteria.

In contrast to the relatively abundant data published on the nitrate reductase system, considerably less information is available on the subsequent step involving reduction of nitrite in the assimilation process.

The investigation reported herein was designed to examine the question of localization of two enzymes participating in the nitrate reduction sequence in Agrobacterium radiobacter. In this respect, a survey of nitrate reduction under varying cultural conditions was made and concomittant localization studies were carried out.

The use of protoplast induction techniques for study of enzyme localization is widely accepted since it provides a rapid means of separating the intact cytoplasmic membrane and its contents from the outer wall of the cell. The subsequent lysis of protoplast yields fractions which can be readily separated by differential centrifugation. The induction of protoplast forms in the genus Agrobacterium has been reported by Weiss (1962). No studies, however, have

been carried out on protoplast induction in Agrobacterium radiobacter cultures undergoing adaptation to strong nitrate reduction activity. Before proceeding with localization studies based in part on the protoplast induction technique, an evaluation of Weiss's method as it applies to this particular experimental system, was made.

HISTORICAL

Studies on the biological reduction of nitrate was observed as early as 1888 by Schimper, and somewhat later by Eckerson (1924) and Quastel et al (1925). The enzyme that catalysed this reduction was not isolated in cell-free extract till 1934 when Green, Stickland, and Tarr demonstrated the presence of nitrate reductase in E. coli.

At a glance there would appear to be a variety of types of nitrate reduction in a wide range of micro-organisms. Many classification schemes have been proposed for the various types of nitrate reductases but none has been entirely satisfactory. One of the earliest attempts to categorize nitrate reductase in micro-organisms was that reported by Jenson (1904), who proposed five categories according to the products of reaction. More recently, Verhoeven (1956) differentiated three types of nitrate reduction in bacteria. One, assimilatory nitrate reductase, in which nitrate is reduced only for the building of cell material. Two, incidental dissimilatory nitrate reductase in which nitrate acts as a non-essential hydrogen acceptor. Three, true dissimilation in which nitrate acts at least

under certain conditions as the essential hydrogen acceptor which enables the organisms to grow. He further suggests that organisms that carry out incidental dissimilation are those that have a fermentative respiration when both oxygen and nitrate are limiting factors. On the other hand, Japanese workers have classified nitrate reducing organisms on the basis of the behaviour of cytochromes towards nitrate, but this classification has not proven satisfactory. More recently, Fewson and Nicholas (1961) proposed that nitrate reduction be considered under two headings. One, nitrate assimilation in which the end products are nitrogenous cell constituents. Two, nitrate respiration or nitrate dissimilation in which nitrate is used in place of oxygen as the terminal hydrogen acceptor. This usually occurs under anaerobic conditions. Walker and Nicholas (1961) reported that Neurospora grown at a low oxygen tension exhibited the enzymatic and other metabolic properties characteristic of nitrate respiration in contrast to nitrate assimilation when grown under a more aerobic environment. These results have essentially confirmed the findings of Lenhoff et al (1956) and of Higashi (1960).

Studies on the localization of bacterial enzymes were

initiated by Stephenson (1928) and since then a wealth of literature has accumulated on this topic.

The most commonly practiced techniques for the localization of enzymes are those based on cell-free extracts, and as well, those based on protoplast formation followed by lysis and differential centrifugation. Cell-free extracts may be prepared by grinding with alumina or sand (Nicholas and Nason, 1955) or by the use of sonic or ultrasonic treatments to disrupt the cells (Taniguchi et al, 1957). These disruption techniques have been extensively employed. Hewitt and Nicholas (1963) showed that enzyme associated with dissimilation of nitrate is usually particulate while the assimilatory enzyme is in the soluble fraction. Murray and Sanwal (1963) using grinding techniques for cells grown under microaerophilic conditions found that nitrate reductase in this case was particulate. From an E. coli culture, grown anaerobically in a medium containing 0.1% KNO_3, Taniguchi and Itagaki (1959, 1960) obtained a particulate nitrate reductase system possessing a 20-30 fold greater specific activity in contrast to particulate system of aerobically grown cells.

With the continued elucidation of the enzymatic mechanism for the conversion of nitrate reduction, attention

has been turning increasingly to the subsequent reduction of nitrite. Yamagata (1957) was the first to demonstrate the presence of nitrite reductase by using cell-free preparations of Bacillus pyogenes. Subsequently Taniguchi et al (1952) observed the enzyme in Bacillus pumilus. Since then a number of workers e.g. Roussos and Nason (1960) have purified the enzyme from non-denitrifying organisms. Nicholas et al (1960) purified the enzyme 50 fold from Neurospora but failed to yield substantially more conclusive information about its properties. Working with denitrifying organisms Nason and Takahashi (1958) characterized the enzyme nitrite reductase.

The development of protoplast technique began with the work of Fischer (1900) who probably was the first to observe the formation of protoplasts in Bacillus anthracis and Vibrio proteus. Induced protoplast formation was obtained by low osmotic shock with the protoplast being ejected from the cell and assuming a spherical form. To this phenomenon he gave the name plasmoptysis. Garbowski (1907) and Raichel (1928) confirmed in part the findings of Fischer but reported that spherical forms following the treatment were not always evident. Modern methods using lysozyme for the formation of

protoplast were introduced independently by Tomscik and Guex-Holzer (1952) and Weibull (1953). Similar studies were made by McQuillen (1955), Mitchell and Moyle (1956), and Burrous and Wood (1962) and all have confirmed the effectiveness of lysozyme treatment in dissolving the bacterial cell wall of the species treated. Lysozyme treatment, however, is more effective for gram positive than gram negative bacteria. Repaske (1956) reported that the addition of EDTA supplement to the lysozyme produced lysis in suspensions of certain gram negative bacteria. As an improvement, Mahler and Frazer (1956) suggested the addition of 0.5 M sucrose to stabilize the protoplast and to prevent lysis. Warren et al (1957) and later Tuttle and Gest (1959) have reported the use of polymyxin in place of lysozyme for the formation of protoplast.

Lederberg (1956) advocated a new concept of protoplast induction by using penicillin in culture media for E. coli. Since penicillin interferes with the cell wall formation or its synthesis the result is that spherical cell forms are produced. Rubio-Huertos and Desjardins (1956) and recently Robrish and Marr (1962) have prevented the development of cell wall in gram negative bacteria by the addition of

glycine to the medium and in so doing produced spherical cells similar to protoplasts.

One of the difficulties associated with successful protoplast induction is the problem of maintaining the stability of protoplasts. If precautions are not taken, protoplasts tend to undergo lysis. Burrous and Wood (1962) used sucrose or disodium phosphate for stabilization and reported that with less than 0.2 M concentration of either solute, protoplast formation was rapidly followed by complete lysis. Conversely, above 0.6 M concentration of either sucrose or phosphate, protoplasts were stable although their conversion was slow and incomplete.

MATERIALS AND METHODS

A strain of Agrobacterium radiobacter obtained from the N.R.C. Prairie Regional Laboratory at Saskatoon was used throughout this investigation. As reported in Bergey's Manual of Determinative Bacteriology, 7th Ed., A. radiobacter may not give a positive test for nitrite when first cultured in a nitrate-containing medium. Prolonged incubation in and repeated transfer to media with nitrate as the sole nitrogen source, however, results in a gradual adaptive response culminating in a high level of nitrate reduction activity.

For stock culture and propagation purposes this species was routinely cultured in a mannitol mineral salts medium of the following composition: K_2HPO_4, 0.5 gram; $MgSO_4.7H_2O$, 0.3 g; NaCl, 0.2 g; mannitol, 1.0 g; yeast extract (Difco), 0.75 g and distilled water, 1000 ml. In addition, since Nicholas (1957) has reported that trace amounts of molybdenum and iron stimulated nitrate reductase activity, a trace element mixture was included in the medium at the rate of 0.1 ml/litre; the composition of the trace element stock solution prepared was as follows: Fe, 5.0 g; B, 1.0 g; Zn, 1.0 g; Co, 0.5 g; Cu, 0.05 g; Mn, 0.05 g and

Mo, 0.001 g made up to 1000 ml with distilled deionized water.

A. Cell Preparation

Cells cultured at 28° C under the three different conditions described below for time intervals of 12, 24, 36, 48, 60 and 72 hours were collected by centrifugation at 10,000 x g for 15 mins at 4° C. Culture aeration conditions were as follows: Series one; flasks of 1000 ml size were filled to the extent of 500 ml with medium. Series two; higher levels of aeration were provided by a flask/medium volume ratio of 200/1000 ml with glass beads to increase the shaking turbulance and gas exchange surface. All flask cultures in series one and two were incubated at 28° C on a Brunswick rotary shaker (180 cycles/min). Series three; consisted of 2000 ml flasks filled to the extent of 1900 ml with the medium and incubated without shaking at 28° C. These three series referred to hereafter as "aerobic", "aerobic with beads" and "microaerophilic" respectively.

Residual nitrite and other cultural medium residues were removed from the collected cells at the time intervals previously referred to by repeated centrifugation and washing

with 1% NaCl till the final wash gave a negative test for nitrite. The test for residual nitrite was as follows: to 0.1 - 0.2 ml of the washed supernatant, 5.0 ml of deionized water, 0.5 ml of 1% (w/v) sulphanilic acid and 0.5 ml n-naphthylethylenediamine dihydrochloride (0.2% w/v) were added in that order. After 10 minutes at room temperature to permit maximum development of colour the reaction mixture was examined for the pink colour characteristic of the presence of nitrite. When the washed supernatant gave no colour, this was considered a negative test for nitrite. Portions of the washed pellet collected at the various time intervals stated earlier were tested for nitrate and nitrite reduction activity.

B. Determination of Nitrate and Nitrite Reduction Activities in Whole Cells

Approximately 1.0 g of cells (wet weight) were suspended in 10.0 ml of 0.1 M TRIS, pH 7.5. This suspension was used for the determination of nitrate and nitrite reduction activities.

1. Nitrate reduction

To 0.1 ml of cell suspension, 0.15 ml of KNO_3 (10^{-3} M), 0.1 ml hydroxylamine hydrochloride (10^{-2} M), 0.65 ml TRIS (0.1 M) pH 7.5 was added in that order. This reaction mixture was then incubated for a period of 30 min. To stop the reaction, 0.5 ml of sulfanilic acid was added followed by 5.0 ml of deionized water. Colour was developed by the addition of 0.5 ml of n-naphthylethylenediamine-dihydrochloride (0.2% w/v) and subsequently incubated at room temperature. The colour intensity was then determined with a Klett-Summerson colorimeter using a green filter (540 mμ).

Three controls were used: one, reaction mixture without enzyme; two, reaction mixture without hydroxylamine hydrochloride; three, complete reaction mixture with boiled enzyme.

2. Nitrite reduction

To 0.1 ml of cell suspension, 0.1 ml of KNO_2 (10^{-3} M) and 0.7 ml of TRIS (0.1 M, pH 7.5) was added in that order. This reaction mixture was then incubated for a period of 30 min. To stop the reaction, 0.5 ml of sulfanilic acid was added followed by 5.0 ml of deionized water. Colour was developed by the addition of 0.5 ml of n-naphthylethylene-

diamine-dihydrochloride (0.2% w/v) and subsequently incubated at room temperature. Again, the colour intensity was determined with a Klett-Summerson colorimeter using a green filter (540 mμ). Two controls were used: one, reaction mixture without enzyme; two, complete reaction mixture with boiled enzyme.

C. Preparation of Cell-free Extracts and Localization of Nitrate and Nitrite Reductases

Cells collected and washed as described previously were ground in a mortar with twice their weight of acid washed levigated alumina and five volumes of 0.1 M phosphate buffer, pH 7.2. To protect the sulfhydryl groups, L-cysteine-HCl neutralized just before use was incorporated into extraction buffer at a final concentration of 10^{-4} M. The resulting slurry was centrifuged at 2000 x g for 20 min to precipitate the alumina and any remaining intact cells. The crude extract thus prepared from cells grown under micro-aerophilic conditions were centrifuged at 35,000 x g for 45 min. The pellet was resuspended in one half the original volume of 0.1 M phosphate buffer, pH 7.2, containing 10^{-4} M cysteine.

The nitrate and nitrite reductase activities were tested in both parts, i.e., pellet and supernatant.

Nitrate Reductase Assay

The assay mixture used for determination of nitrate reductase activity was as follows:

0.15 ml KNO_3 (10^{-3} M)

0.1 ml Hydroxylamine hydrochloride (10^{-2} M)

0.1 ml DPNH (10^{-3} M)

0.1 ml FAD (10^{-4} M)

0.45 ml Phosphate buffer (0.1 M, pH 7.2)

0.1 ml enzyme

The assay for nitrate reductase was conducted anaerobically using Thunberg tubes. The side-arm of the Thunberg tube contained the enzyme preparation and the remaining assay components were in the body of the tube. After evacuation of the Thunberg tube by vacuum pump, the two portions of the assay mixture were combined and allowed to react for 30 min. Colorimetric determination of nitrite formed in the reaction was the same as that described for nitrate reduction in the whole cells.

Nitrite Reductase Assay

The assay mixture used for determination of nitrite

reductase activity was as follows:

0.1 ml KNO_2 (10^{-3} M)

0.1 ml DPNH (10^{-3} M)

0.1 ml FAD (10^{-4} M)

0.6 ml Phosphate buffer (0.1 M, pH 7.2)

0.1 ml Enzyme

Assay procedures were the same as those reported for nitrite reduction in whole cells.

D. Protoplast Production and Localization Studies

Cells cultured in mannitol nitrate broth were transferred to a medium comprised of three parts, (a) distilled water with glucose, (b) K_2HPO_4 and KNO_3, (c) $MgSO_4 \cdot 7H_2O$ in a volume of 200 ml. Each portion was separately autoclaved and these were then combined aseptically to yield a medium of the following composition: glucose, 2.0%; K_2HPO_4, 0.02%; KNO_3, 0.5%; $MgSO_4.7H_2O$, 0.3%. The pH was adjusted to 6.6. The cells were subcultured repeatedly under microaerophilic conditions, i.e., 500 ml flasks containing 400 ml of the inoculated medium were incubated unshaken at 28° C for about 24 hours, or until the population density for the interval of incubation was equivalent to that

obtained in yeast extract medium. The cells were then collected by centrifugation at 10,000 x g for 15 min. The cell pellet was washed repeatedly until the final wash gave a negative test for nitrite as described before.

For the purpose of protoplast induction for nitrate adapted cells, a method similar to that described by Weiss (1962) was adopted. Washed cell pellet was suspended in 15 ml of TRIS buffer (Sigma Chemical Co.) containing 10% sucrose, pH 8.0. This suspension was shaken for 15 min on a rotary shaker at 28° C. Following this treatment, 0.3% $MgSO_4.7H_2O$, 0.5 mg/ml EDTA, and 3 mg/ml lysozyme was added in rapid succession and shaking was continued for another 30 min. One ml of the sample was diluted 1:4, 1:10 and 1:20 with distilled water and after about one hour the per cent conversion to protoplast was estimated microscopically by the hanging drop preparation method described by Weiss (1962).

Preliminary experiments on protoplast induction of Agrobacterium radiobacter during culture adaptation to a strong nitrate reduction activity had suggested that a study of the effects of various energy sources on protoplast yield might be profitable. Accordingly, a survey of

protoplast yield by our A. radiobacter culture was carried out on the basis of Weiss's medium using three different carbohydrate sources.

A. radiobacter which had been routinely cultured in mannitol mineral salts medium was transferred for the purpose of protoplast induction to a series of media each consisting of three parts: A, distilled water with (1) glucose (2) sucrose (3) mannitol; B, K_2HPO_4 and $(NH_2)_4HPO_4$; C, $MgSO_4.7H_2O$. The total medium volume was 200 ml. Each portion was separately autoclaved and then combined together aseptically to yield the respective carbohydrate media whose final composition was (a) specific carbohydrate, 2%; (b) K_2HPO_4, 0.02%; (c) $(NH_2)_4HPO_4$, 0.6%; (d) $MgSO_4.7H_2O$, 0.25%. The final pH of the medium was adjusted at 6.6. The cells were cultured in these carbohydrate media on a rotary shaker at 28° C for 24 hours. From each culture, 20 ml of the actively grown cells were transferred to 200 ml of the fresh medium of the same composition and again incubated for 18 hours on a rotary shaker at 28° C. Finally, the cells were collected by centrifugation at 10,000 x g for 15 min and washed twice with distilled water. Further treatment for protoplast induction with lysozyme and subsequent enumeration

was carried out as described before.

After having studied the effects of different carbohydrates on the protoplast yield, different concentrations of lysozyme were tried to evaluate their effects as a function of protoplast yield. A. radiobacter again routinely cultured in mannitol mineral salt medium was transferred to the glucose medium for protoplast induction. Lysozyme concentrations used were as follows: 1.5 mg/ml, 6.0 mg/ml and 9.0 mg/ml. These concentrations represent one-half, twice, and three times the concentration of lysozyme proposed in the Weiss method.

RESULTS

A. Adaptation Studies

Transfer of the culture inoculum from mannitol yeast extract medium to the mannitol mineral salts broth with nitrate as the nitrogen source resulted initially on incubation in a marked increase in length of generation time. Repeated transfer of cells in the log phase to fresh nitrate medium, however, produced a gradual increase in growth rate as the culture became adapted to the new nutritional environment. By the end of the fourth transfer to the new medium, cell population densities for a 24 hour interval were roughly equivalent to those originally obtained in the mannitol yeast extract medium. Further, it was noted that the addition of the trace element complex to the nitrate medium stimulated the adapted response by Agrobacterium radiobacter in a manner similar to that reported by Stachow (1962) for Agrobacterium rubi.

B. Studies on Nitrate and Nitrite Reduction under Varying Aeration Conditions

1. Nitrate reduction

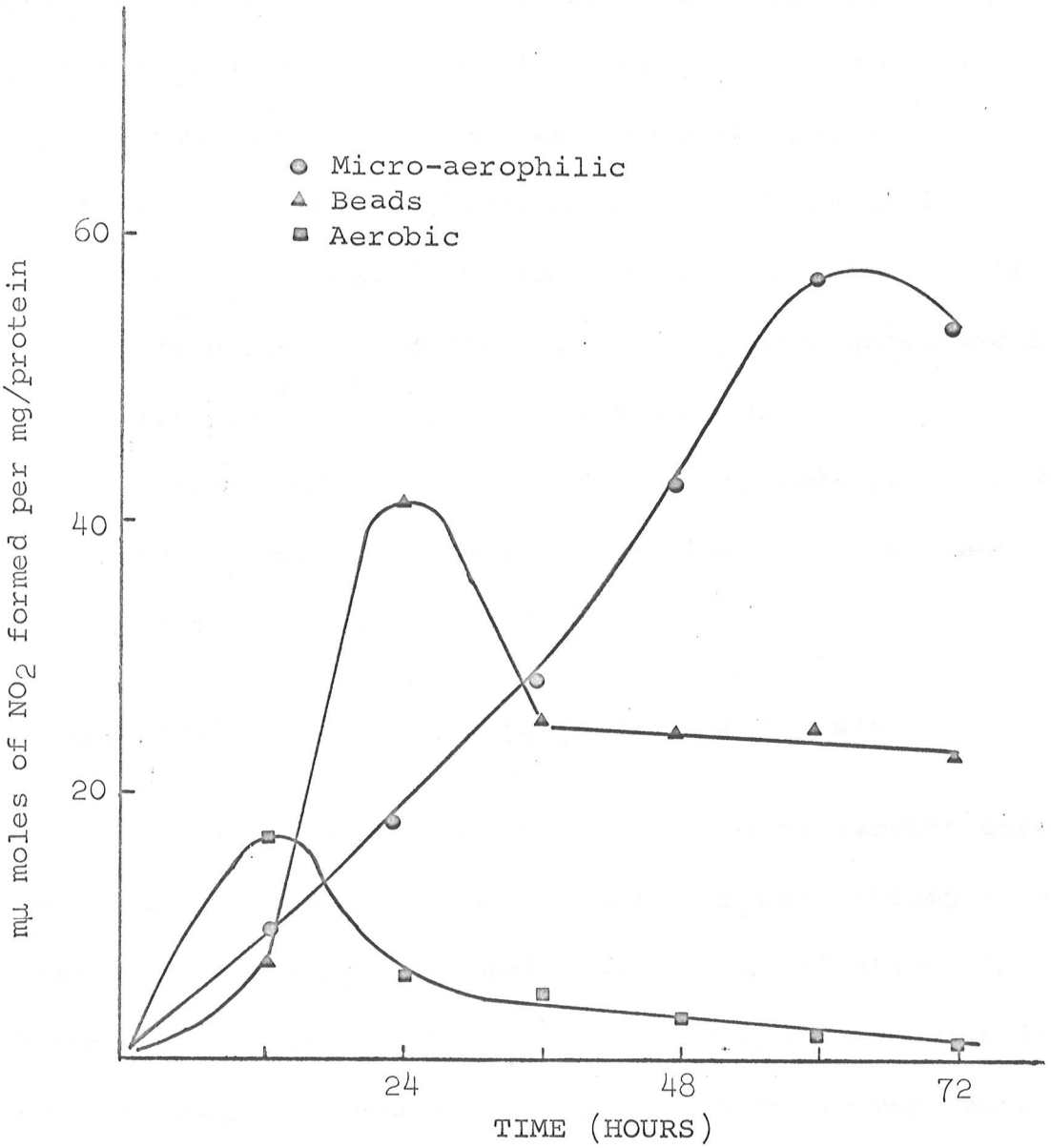
The response by the adapted culture to varying aeration conditions is shown in Fig. 1. The greatest total nitrate reduction activity was evident in the culture grown under micro-aerophilic conditions although for the first thirty six hours growth response and nitrite accumulation was very slow.

As opposed to this the performance by the highly aerated culture (in the presence of glass beads) presents a quite different picture. An initial rapid increase of nitrite occurred within the first 24 hours of incubation. Subsequently, nitrite levels decreased steadily until the 36th hour of incubation. Beyond this point nitrite levels remained nearly constant at 20 to 25 mμM nitrite/mg cell protein. Under "normal" shaking conditions, without glass beads, nitrite levels introduced with the inoculum gradually decreased until they approached zero concentration in 72 hours.

2. Nitrite reduction

The capacity of the culture to reduce to nitrite is

Fig. 1. Study on the pattern of nitrate reduction in whole cells under three different aeration conditions.



shown in Figure 2. In general, the performance under micro-aerophilic conditions closely parallels that shown for nitrate reduction. Peak nitrite reduction activity was obtained at 60 hours followed by a significant drop in activity within the next 24 hours. Under "normal" shaking conditions, the peak nitrite reduction was obtained in 36 hours followed by a rapid decrease to approximately zero by 72 hours. In contrast very little nitrite reduction was evident in the cultures grown under highly aerobic conditions with glass beads.

Comparisons of nitrate and nitrite reduction activity in cultures grown under varying aeration conditions are presented in Figures 3, 4 and 5.

C. Localization of Nitrate and Nitrite Reductases

After having studied the influence of varying aeration conditions on the induction of these enzymes, attempts were then made to obtain information on the localization of nitrate and nitrite reductases. From Figs. 1 and 2 it is evident that the induction of these enzymes is much more marked under micro-aerophilic conditions and since these figures also show that cells harvested at 60 hours of growth

Fig. 2. Study on the pattern of nitrite reduction in whole cells under three different aeration conditions.

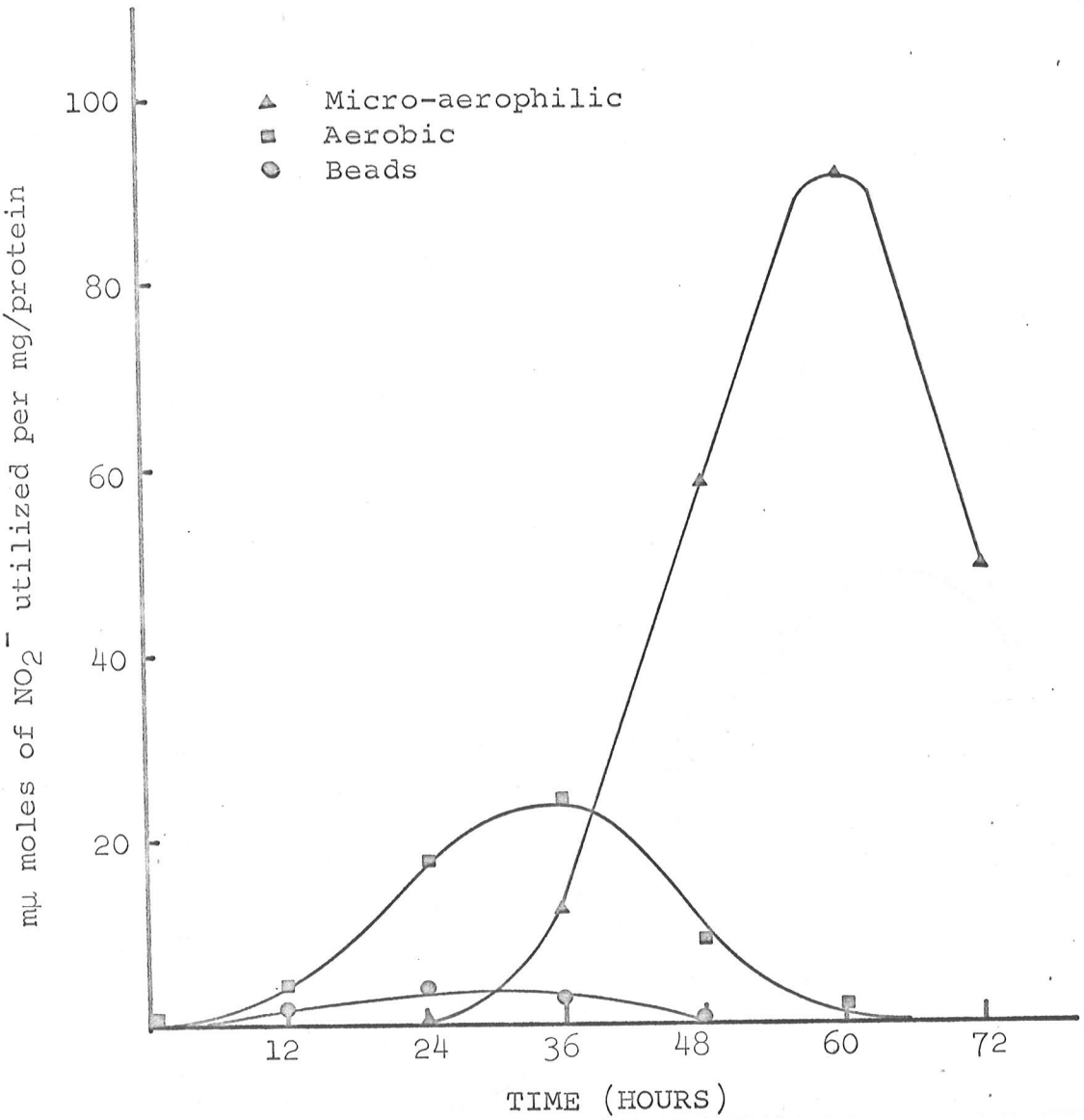


Fig. 3. Study on the pattern of nitrate and nitrite reduction in whole cells under micro-aerophilic conditions of growth.

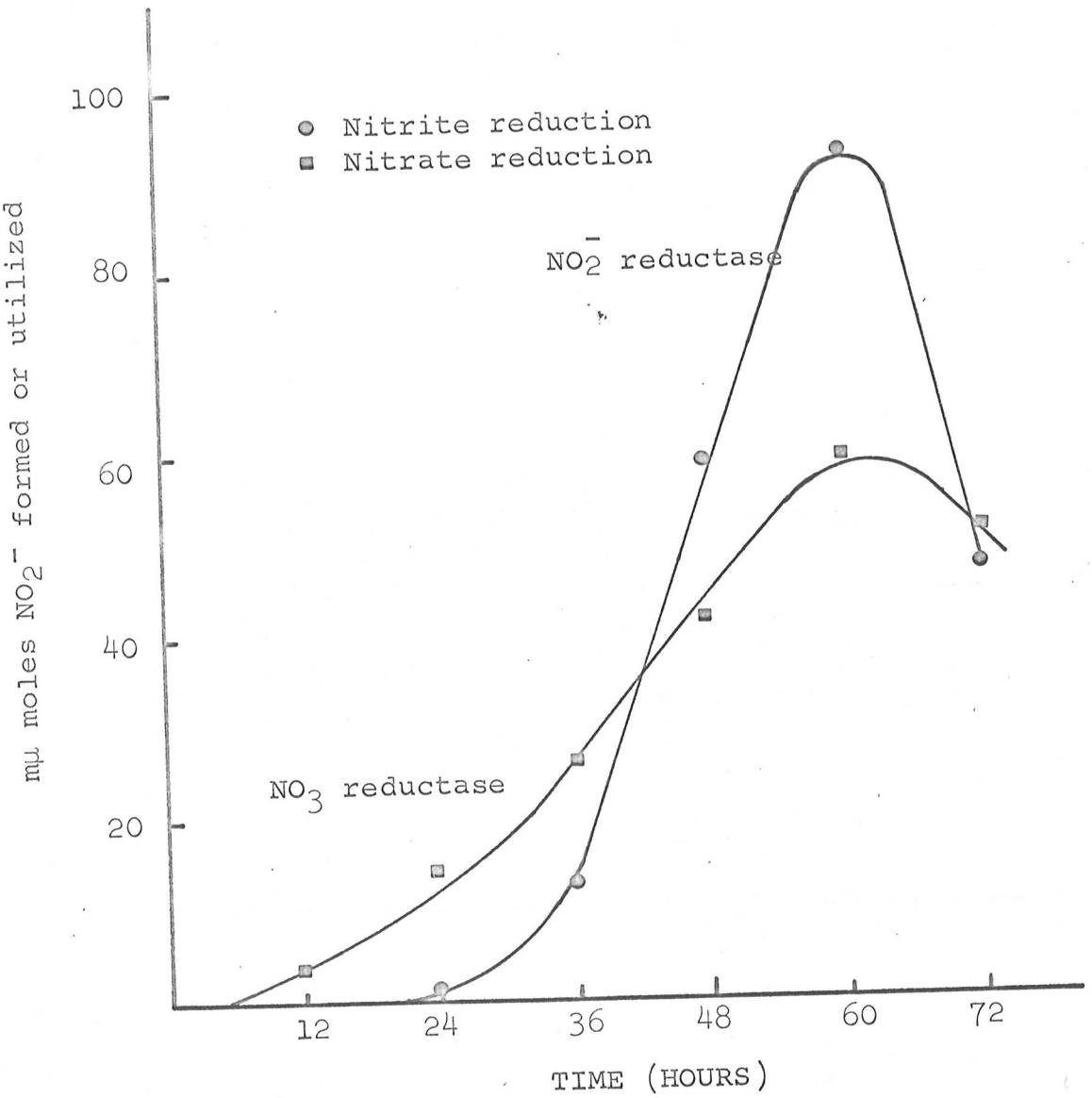


Fig. 4. Comparison of studies on NO_3^- and NO_2^- reduction under aerobic conditions of growth.

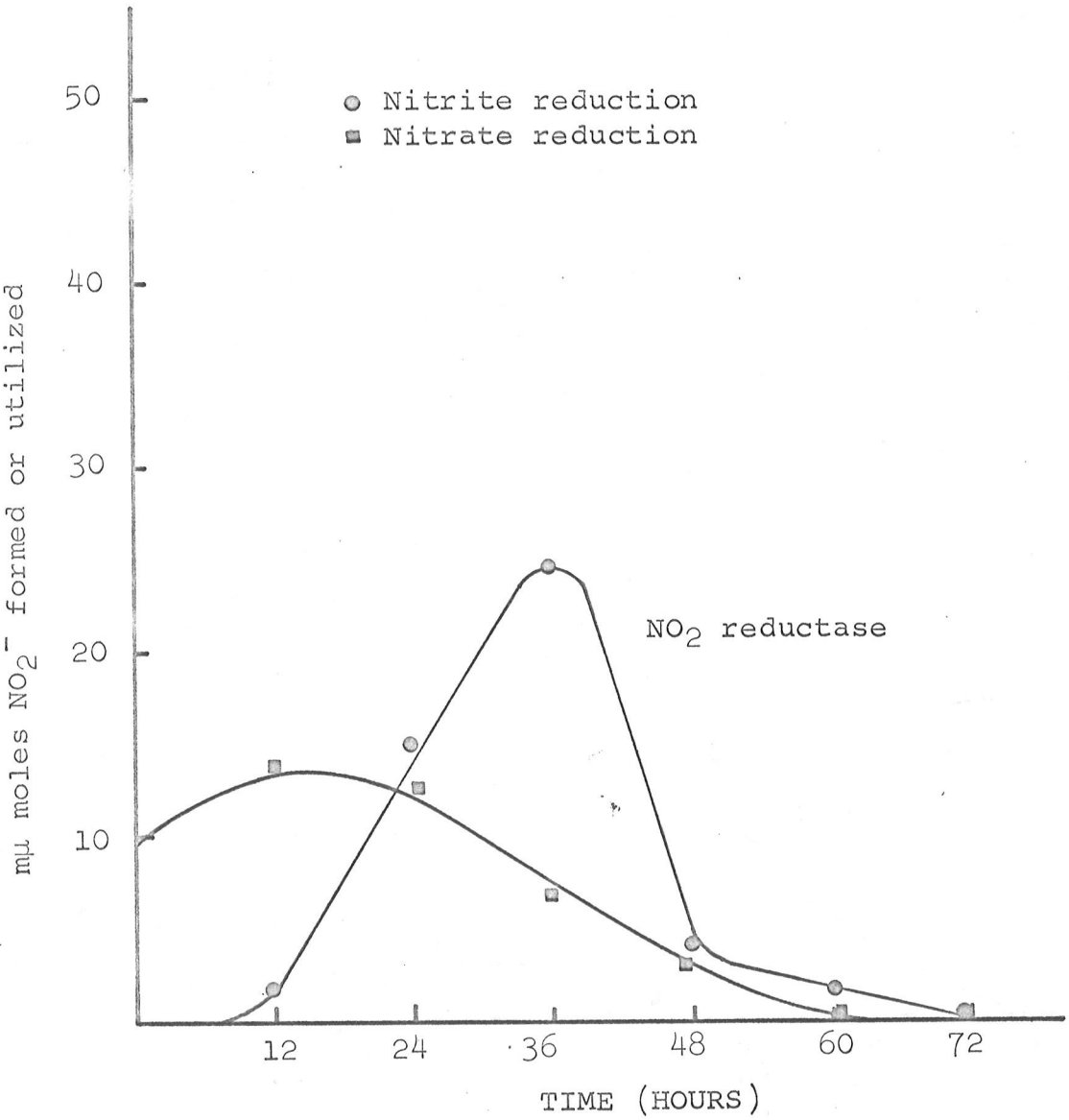
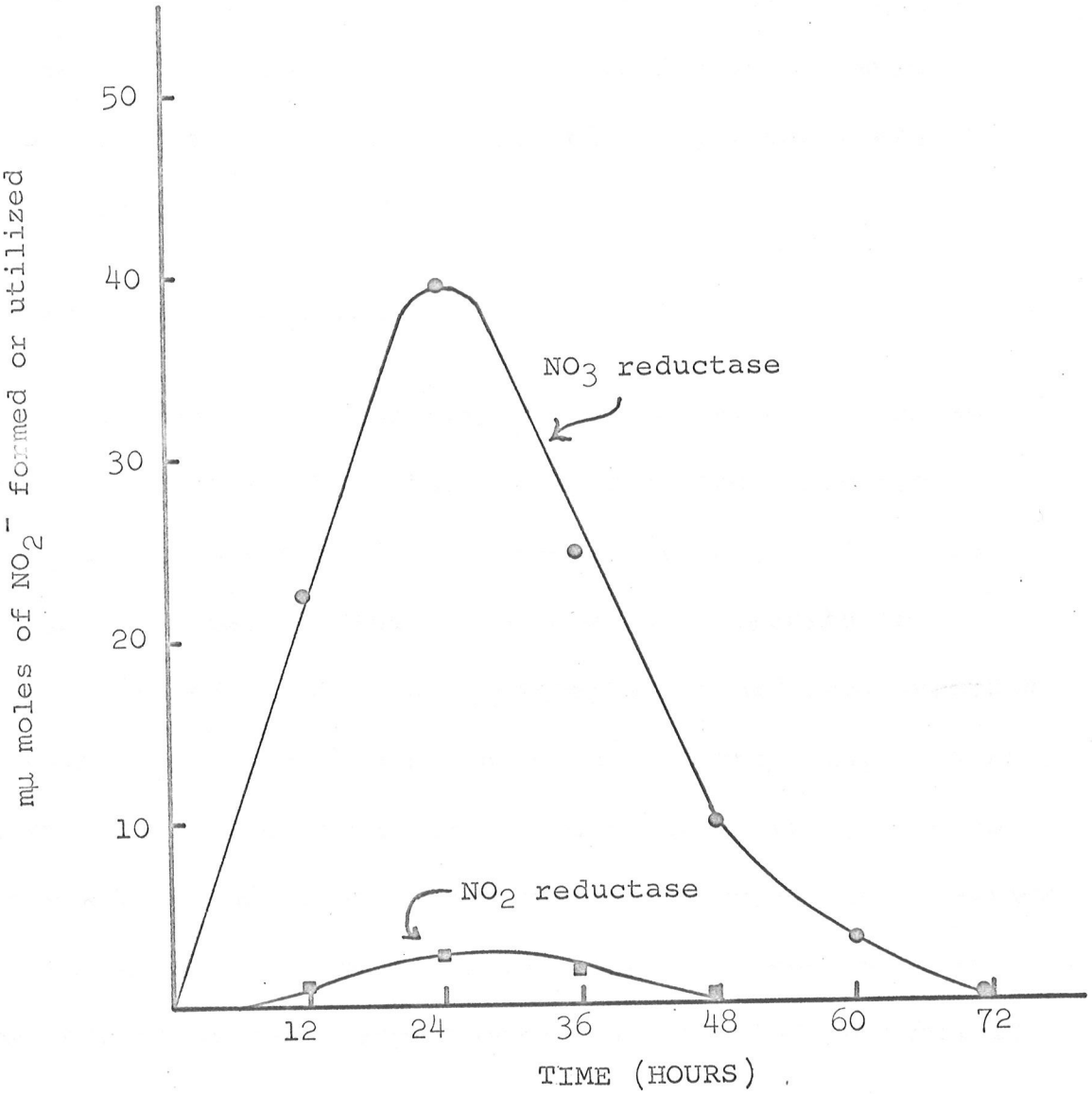


Fig. 5. Comparison of studies on NO_3^- and NO_2^- reductions under highly aerobic (beads) conditions of growth.



displayed highest activity for both nitrate and nitrite reductions, cells grown under micro-aerophilic conditions for this time interval were harvested for localization studies. The results of localization experiments are presented in Table I.

D. Protoplast Induction

In the early experiments a medium with glucose as a source of energy was employed for protoplast induction according to the methods outlined by Weiss (1962). This medium, as noted previously, gave as good results for protoplast induction in A. radiobacter as had been reported by Weiss (1962) for other species of Agrobacterium. In an attempt to determine whether other carbohydrates could be successfully substituted for glucose, an experimental series was conducted using mannitol and sucrose as energy sources. The results of these experiments are presented in Table II. It is evident that glucose is still the preferred carbohydrate and, accordingly, all subsequent experiments were carried out using glucose in the medium.

One characteristic of members of this genus in terms of protoplast induction and yield is the need for post-

TABLE I. Localization studies data on nitrate and nitrite reductases produced under microaerophilic conditions of growth.

Enzyme	mμmoles of NO_2^- formed or utilized per mg protein/30 minutes in: Pellet	Supernatant
NO_3^- reductase	51.6	9.5
NO_2^- reductase	22.8	73.2

TABLE II. Effect of different carbohydrate sources on protoplast formation with A. radiobacter. Percent conversion estimated by direct microscopic count.

Carbohydrate source	Number of trials	Percent conversion without dilution	Dilution for optimum conversion	Percent* conversion	Average conversion
Glucose	5	negligible	1:9	95-97-99	97
Mannitol	5	negligible	1:9	40-50-55	50
Sucrose	3	negligible	1:9	20-30-35	30

*Low, average and high conversions.

lysozyme shock treatments by dilution with distilled water. Without this osmotic shock treatment, very little protoplast conversion is observed. The results of an experimental series to determine the optimal dilution factor for maximum protoplast yield are presented in Table III. It should be noted that any dilution in excess of 1:9, although giving good conversion, results in a marked lysis and subsequent loss of protoplasts.

As previously noted, the induction of nitrate and nitrite reductases requires the presence of nitrate as the inducing substrate. Accordingly nitrate (as KNO_3) was incorporated into the glucose medium in place of ammonium phosphate. Quite unexpectedly, this substitution resulted in a considerable decrease in protoplast yields ranging from the previous high of 97 per cent (with ammonium phosphate) down to approximately 30 per cent with nitrate.

The results of experiments on varying lysozyme concentrations in protoplast induction with A. radiobacter are presented in Table IV. It seems obvious that increase or decrease in lysozyme concentration over the "normal" (3 mg/ml) do not give better results. At the lower concentration protoplast yield is markedly decreased while

TABLE III. Effect of varying dilution-shock treatments on protoplast formation* with A. radiobacter. Per cent conversion estimated by direct microscopic count.

Number of trials	per cent conversion			
	without dilution	in 1:4 dilution	in 1:9 dilution	in 1:20 dilution
5	nil	55-60	95-99	95-99 with some considerable lysis of protoplasts observed

*Protoplast induction carried out in optimal glucose-ammonium phosphate medium.

TABLE IV. Effects of varying concentrations of lysozyme on protoplast yield.

Lysozyme concentration	Effect on conversion as compared to optimum*	Remarks
6 mg/ml	reduced	Some clumping of whole cells, lower conversion
9 mg/ml	reduced	Much increased clumping of whole cells.
1.5 mg/ml	reduced	No clumping, but per cent conversion to protoplasts greatly reduced.

*On basis of previous experience (Weiss, 1962), 3 mg/ml of lysozyme was considered as optimum for protoplast yields in other members of the genus Agrobacterium.

at higher concentrations cell clumping becomes progressively greater.

DISCUSSION

The results presented in our investigation indicate that A. radiobacter can form an adaptive nitrate reductase enzyme system in the presence of an inducer, KNO_3. This finding supports the contention by Pollock (1946) working with E. coli that generally nitrate reductase is adaptive rather than constitutive and further that the adaptive process is slow to develop. He also showed the necessity for the presence of inducing substrate. Similar observations have been made by Sacks and Baker (1952), and Kluyver and Verhoeven (1954). Verhoeven (1956) concludes that the probable behaviour of most species of microorganisms toward nitrate and its reduction products would be adaptive.

Increase in nitrate accumulation that occurred when iron and molybdenum were added to the medium suggests that one or both of these metals increased the nitrate reductase activity or were necessary for greater enzyme production. Taniguchi and Itagaki (1960) prepared a protein containing Mo and Fe from Neurospora. They claim that molybdenum functions as an electron carrier as suggested earlier by Nicholas and Stevens (1955) for the Neurospora nitrate

reductase. In addition to molybdenum, iron may also be required for the Neurospora enzyme at early stages of growth particularly when the culture is grown under conditions of low oxygen tension with nitrate serving as a terminal electron acceptor in oxidation (Walker and Nicholas, 1961). The need for iron may be related to the increased cytochrome production when low oxygen tensions prevail.

The respiratory or dissimilatory nitrate reductase is usually more active when bacteria are grown under anaerobic conditions when nitrate is the obligatory terminal acceptor (Hewitt and Nicholas, 1963). This fact is well illustrated for nitrate reductase and nitrite reductase in cultures of Pseudomonas aerugenosa (Fewson and Nicholas, 1961), and Walker and Nicholas (1961). The activities of both these enzymes were reduced by increasing oxygen tension in the medium, and this is the usual pattern for denitrifying enzymes as well as the dissimilatory one from Pseudomonas aerugenosa. The assimilatory nitrate reductase system, however, is not as markedly affected by changes in oxygen tension in the medium. The findings in our investigation are quite comparable in this respect except that the bacterial cells in our study were cultured under micro-aerophilic conditions rather than

anaerobically.

Pewson and Nicholas (1961) observed that in dissimilation of nitrate, the rate of reduction of nitrate will be much greater than that obtained during assimilation of nitrate, since in dissimilation the nitrate has a respiratory function. Comparing the nitrate and nitrite reductions under various aeration conditions, our results show that nitrate and nitrite reductases were most active when cells were cultured in a micro-aerophilic environment where, presumably, the enzyme is mainly used in a dissimilatory sense. It is of interest to note from our results that the biosynthesis of nitrite reductase under micro-aerophilic conditions as reflected by its specific activity is more pronounced as compared to that of the nitrate reductase. From our results it is quite apparent that the nitrite reductase was most active under micro-aerophilic conditions as mentioned before, followed by a decrease in activity when under normal aerobic conditions. The least active system developed under highly aerated conditions with beads. In the case of nitrate reductase, as indicated before, highest activity was under micro-aerophilic conditions followed by a reduction in activity under highly aerated conditions with beads and least under

aerobic conditions. These studies suggest that oxygen tensions prevailing in cultural media markedly influence the nitrate reduction activities of these enzymes.

It was found essential to add hydroxylamine hydrochloride to the assay mixture when determining the nitrate reductase activity. This may be due to the fact that since nitrate reductase and nitrite reductase run sequentially, hydroxylamine changes the equilibrium of the NO_2 to NH_2OH reaction to provide accumulation of the substrate, nitrite, thus effecting something similar to a feed-back mechanism.

There are conflicting reports available in literature concerning the location of nitrate and nitrite reductases in microorganisms. This confusion arises primarily because the different investigators reporting on localization studies did not all use the same aeration-environment conditions. Some reported on the basis of aerobic conditions, others on the basis of anaerobic or micro-aerophilic conditions of growth. Murray and Sanwal (1963) reported a dissimilatory pattern in cultures of E. coli, strain B, under micro-aerophilic conditions of growth, and further reported that this nitrate reductase is particulate. The findings in our investigation have produced similar results and suggest that nitrate reductase is

particulate in A. radiobacter. Growth conditions have also influenced the findings concerning the location of nitrite reductase. It is evident from our investigation that nitrite reductase is soluble and is present in the supernatant. Similar observations have been made by Spencer et al. (1957) working with Azotobacter agile.

In any study of protoplast induction by lysozyme treatment, consideration should be given to the nature and extent of extra-cellular slime production since the lysozyme-cell wall contact would presumably be directly influenced by such extra-cellular material. The nature of extra-cellular polysaccharides of Aerobacter aerogenes and related organisms and the factor affecting their production have been reviewed by Duguid and Wilkinson (1953) and Wilkinson et al. (1954). The polysaccharide of these organisms may occur as loose slime or as distinct capsule. In our investigation, in an attempt to influence the adherence character of extra-cellular polysaccharide, three different carbohydrate sources, i.e., glucose, sucrose, and mannitol were incorporated with the media. It is apparent from the results that, when glucose was the source of carbohydrate, then there was a better production of protoplasts. This may be possibly attributed

to the fact that in medium containing glucose, although there was great amount of slime material observed in the culture, it appeared to be readily removed when the cells are washed and centrifuged repeatedly. On the other hand, when sucrose or mannitol were the sources of carbohydrate the slime layer material was not removed by repeated washing and centrifugation. If the mannitol and sucrose media encouraged the production of a more tightly adhering slime, then the subsequent action of lysozyme may have been impaired in a mechanical sense. As stated previously, slime production under conditions where glucose formed the energy source, although more extensive, was loosely bound and readily removed by washing. The "clean" cells thus produced gave much higher protoplast yields presumably because the lysozyme came in more intimate contact with the cell wall material.

Incorporation of KNO_3 as an inducer of nitrate reductase in the medium containing glucose, also resulted in the marked decrease in the production of protoplasts as is evident from the results. We can offer no clear explanation for this observation except to suggest that the nitrate reductase induction may alter, in a biosynthetic sense, the formation of cell components which subsequently are incorporated

in cell wall structure. Any alteration in cell wall make-up presumably would influence the likelihood of successful lysozyme-cell wall binding and subsequent lyses to yield protoplasts. An investigation of this curious phenomenon was not within the scope of our investigation and awaits further study.

Varying concentrations of lysozyme were employed to whether greater yields of protoplasts could be obtained. The amount prescribed by Weiss (1962) was found to be optimum for maximum protoplast yield with this species. Any marked increase or decrease in lysozyme concentration gave lower protoplast yields. Moreover, with increase in lysozyme concentration above the optimal level a much greater tendency for cell clumping was observed in the cell treatment mixtures. Whether this clumping could result in poorer lysozyme-cell wall contact can only be evaluated by further investigation.

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