STUDIES IN THE BIOLOGICAL FIXATION OF NITROGEN

V. SOME OBSERVATIONS ON THE UPTAKE OF COMBINED NITROGEN BY *AZOTOBACTER VINELANDII*

by

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The addition of even small amounts of ammonium acetate or sodium nitrate to *Azotobacter vinelandii*, growing in a nitrogen-free medium[1], brings about a complete, albeit temporary, cessation of growth and respiration[2]. The utilization of combined nitrogen by organisms capable of fixing molecular nitrogen has been extensively studied[3–13], and it is generally agreed that the uptake of certain nitrogenous compounds results in the suppression of fixation, although this view has assumed that growth and respiration were largely unaffected. A few investigations have been inconclusive; thus poor growth has occurred (though this might be attributed to unsuspected deficiencies in the medium), and in some cases the analytical techniques employed to detect utilization have not been sufficiently sensitive; in still other cases, the data cover only very narrow ranges of concentration.

It was thought desirable to obtain more information by the detailed study of the uptake of nitrite- and ammonium-nitrogen by *A. vinelandii*. The rates of disappearance of these forms of nitrogen from bacterial cultures, from sterile medium, and from medium containing only dead bacteria, were determined. The compounds used were ammonium acetate, ammonium sulphate, ammonium nitrite (*i.e.* equivalent amounts of ammonium sulphate and sodium nitrite) and sodium nitrite, each being supplied over a wide range of concentration. The stability of these compounds, under the conditions existing in the cultures, was re-examined, since most of the available data related to simple, fairly concentrated aqueous solutions exposed to air[14–17]; whereas in our systems the solutions were complex and the atmosphere usually had a relatively high partial pressure of oxygen. This re-examination showed that:

(1) No decomposition or oxidation of nitrite occurred in solutions between 10^{-6} *M* and 10^{-1} *M*, at pH = 7.3 and at 30° C, in aqueous solution, in sterile medium, or in medium containing only dead bacteria, whether such solutions were exposed to air or to pure oxygen at 1 atmosphere pressure. This stability was observed over periods of many days.

(2) Ammonium was similarly stable, under the same sets of conditions, between 10^{-4} *M* and 10^{-1} *M*.

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EXPERIMENTAL

I. *Utilization data*

Nitrite. To 5-day-old cultures containing about 10^9 cells per 25 ml were added suitable small volumes of sodium nitrite solution in sterile medium to provide initial concentrations (c_0) from 10^{-6} M to 10^{-2} M (14 μg N/l to 140 mg N/l). The cultures were maintained at 30° C, and nitrite determined colorimetrically in aliquots withdrawn at intervals, using the modified method of GRIESS[18, 1]. Concentrations above 10^{-2} M proved toxic. The data are plotted, in Fig. 1, as $100\ (1-c_t/c_0)$ against time, when c_t is concentration at time t.

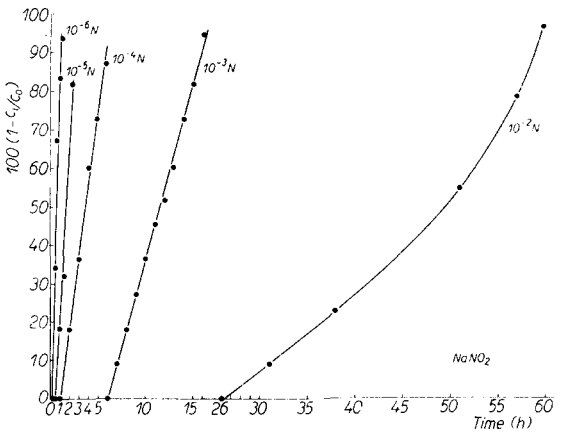

Fig. 1. Rates of disappearance of nitrite from cultures of *A. vinelandii* (~ 10^{10} cells) at 30° C and pH 7.2.

Disappearance of nitrite does not begin immediately, but only after a lapse of time the duration of which increases with increased initial concentration. The rate of uptake also increases with increased initial concentration.

Ammonium (acetate and sulphate). The initial concentrations of ammonium acetate varied from 10^{-4} M to $6 \cdot 10^{-2}$ M (1.4 mg N/l to 840 mg N/l); those of sulphate from 10^{-4} M to 10^{-2} M (2.8 mg N/l to 280 mg N/l), higher concentrations not being tolerated by the bacteria. The same procedure was followed as for nitrite, ammonium being determined by Nessler's method. The results are plotted in Fig. 2. As with nitrite, a time lag occurs before uptake begins, but this is much shorter and virtually disappears at the lowest concentrations used. Again the rate of uptake, once the process has started, increases roughly five-fold for ten-fold increases in initial concentration, but the picture is complicated by the decrease in pH of the medium resulting from removal of ammonium ions. Thus at the higher concentrations, there is a relative falling off in rate of uptake with the passage of time. This effect is already apparent in ammonium sulphate at 10^{-3} M, but is not very marked in ammonium acetate below 10^{-2} M.

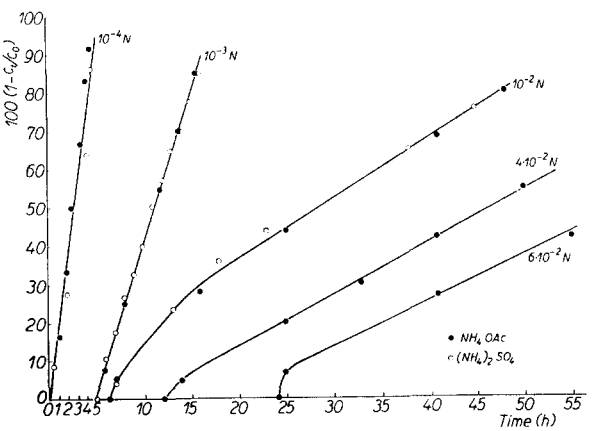

Fig. 2. Rates of disappearance of ammonium (acetate and sulphate) from cultures of *A. vinelandii* (~ 10^{10} cells) at 30° C and pH 7.2.

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Apart from this difference, the rate of uptake of nitrogen from ammonium salts is practically the same as from sodium nitrite.

Ammonium (nitrite). Equivalent amounts of ammonium sulphate and sodium nitrite were added to cultures to provide concentrations of ammonium nitrite of 10^{-3} *M* (14 mg NH_4-N and 14 mg NO_2-N per l) and 10^{-4} *M*. Nitrite and ammonium were determined in separate aliquots withdrawn simultaneously. The data are presented in Fig. 3. At both concentrations ammonium begins to disappear first, but does so more slowly than nitrite once the uptake of the latter begins. This could be due to the production of ammonium from nitrite similar to its production from nitrate[19]. The actual rate of disappearance of nitrite agrees closely with that observed in sodium nitrite of the same molar concentration, but ammonium disappears only at about one-third of this rate. On the basis of results shown in Figs. 1 and 2, the overall rate of total uptake of nitrogen should be about 1.5 times the rate shown by the corresponding nitrite or ammonium concentration; when both forms are supplied, there is therefore a relative drop in the rate of utilization. There is practically no mutual effect on the time of adaptation.

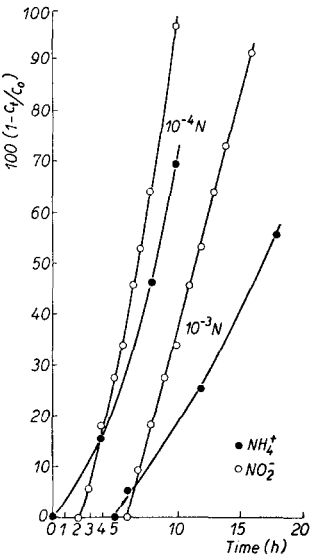

Fig. 3. Rates of disappearance of ammonium and nitrite from cultures of *A. vinelandii* (~ 10^{10} cells) at 30° C and pH 7.2.

II. *Effect of change in concentration of combined nitrogen on trained bacteria*

The dependence of the period of adaptation, and the rate of utilization, on the concentration of combined nitrogen suggested the following experiments. Cultures of known population were made 10^{-5} *M* (140 μg N/l) with respect to sodium nitrite, and the rate of uptake observed for two hours. After this time, the concentration was abruptly increased to 10^{-4} *M*, and the rate of uptake measured. This process was repeated until the nitrite concentration was $5 \cdot 10^{-1}$ *M* (7 g N/l), beyond which concentration no uptake occurred.

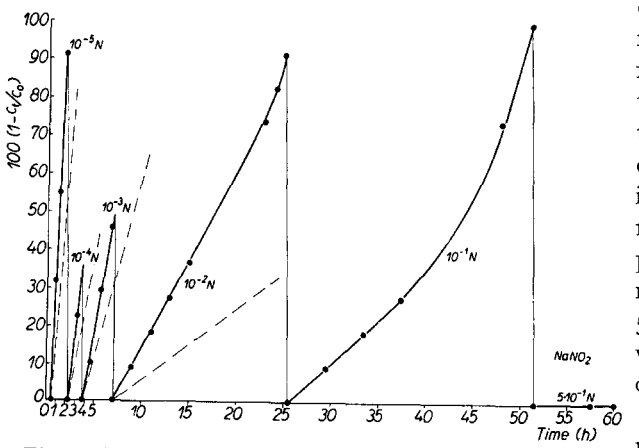

Fig. 4. Effect, on rate of uptake of nitrite, of change in concentration of combined nitrogen.

Similar experiments were made with ammonium acetate and ammonium sulphate, starting in each case with 10^{-3} N solutions. Again utilization was observed in concentrations of ammonium much greater than those tolerated by untrained bacteria.

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The results are plotted in Fig. 4 and Fig. 5, in which the data of Figs. 1 and 2, are shown, for comparison, by broken lines. In addition to being able to utilize much higher concentrations of combined nitrogen, the trained cells utilise lower concentrations at slightly greater rates than untrained cells in similar solutions. Moreover, the abrupt increase in concentration of the nitrogenous compound is not followed by any cessation in uptake, even though the total time elapsed may be less than that required by untrained cells for direct adaptation to the higher concentration.

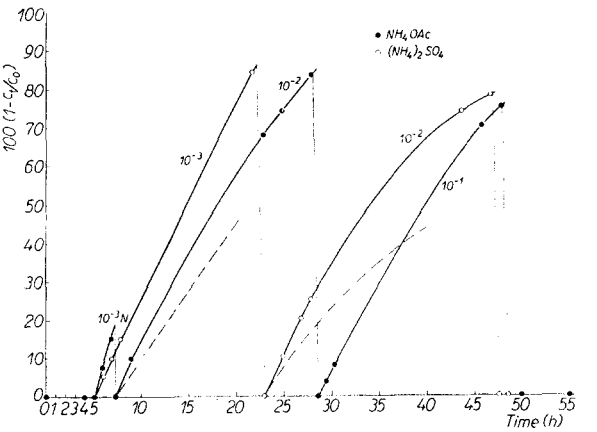

Fig. 5. Effect, on rate of uptake of ammonium, of change of concentration of combined nitrogen.

III. *Test for fixation of nitrogen in the presence of a supply of combined nitrogen*

The vigour with which cultures took up combined nitrogen, even after a long period of adaptation, suggested that they had suffered little damage or reduction during this period. The possibility that they might fix molecular nitrogen during this period was investigated. An atmosphere of oxygen (0.4 atm) and nitrogen (0.4 atm) containing a known weight of nitrogen (~100 mg) was circulated through cultures to which sufficient nitrite or ammonium had been added to require an adaptation time of 24 h. In each experiment about 60 ml of culture, containing $\sim 10^{10}$ cells, were used. The tension of carbon dioxide was kept low by a filter partly immersed in 20% potassium hydroxide solution. After a suitable time the cells were killed with acid, and uptake of nitrogen measured by analysis of the residual atmosphere; the method was sufficiently sensitive to detect the removal of less than 1% of the nitrogen available.

TABLE I

Showing mg N fixed per 100 mg N available per 10^{10} cells.

Medium	*Time of circulation*	*mg N fixed*
N-free	24 h	9.5
N-free	24 h	9.3
N-free + 10^{-2} *M* $NaNO_2$	24 h	0.2
N-free + 10^{-2} *M* $NaNO_2$	65 h	0.6
N-free + $6 \cdot 10^{-2}$ *M* NH_4OAc	24 h	nil.
N-free + $6 \cdot 10^{-2}$ *M* NH_4OAc	65 h	nil.

The results shown in Table I indicate that no significant amounts of molecular nitrogen were taken up either during or after the period during which adaptation was occurring.

SUMMARY OF RESULTS

1. At concentrations from 10^{-6} *M* upwards, nitrite is utilized by *A. vinelandii* after a period of adaptation, the duration of which depends on the initial concen-

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tration of nitrite. Up to 10^{-3} *M* nitrite, the period is roughly doubled for each ten-fold increase in concentration, but increases more rapidly at higher concentrations.

Ammonium behaves similarly, though the period is shorter, and disappears at a concentration of 10^{-4} *M*.

2. When uptake begins, three facts are observed:

(a) The rate of uptake depends on initial concentration; $\log_{10}$ rate varies linearly with $\log_{10}$ concentration, the rate increasing roughly five times for each ten-fold increase in concentration.

(b) The characteristic rate of uptake for a given concentration is reached almost instantaneously.

(c) For a given concentration of combined nitrogen, the rate is practically the same, whether the source be nitrite or ammonium. If both nitrite and ammonium be supplied, the nitrite disappears at its characteristic rate, but ammonium disappears more slowly and at a less constant rate.

3. Cultures trained to grow in low concentrations of combined nitrogen continue to take up the source when its concentration is abruptly increased. No further adaptation is necessary. Moreover, the trained cells continue to utilize sources some fifty times more concentrated than untrained cells can be adapted to use, and trained cells take up the source at a given concentration slightly more rapidly than untrained cells.

4. Both nitrite and ammonium bring about virtually complete cessation of fixation, even during the period before uptake of the combined nitrogen begins.

The following additional information, details of which will be presented at a later date, has recently been obtained by one of us (M.A.A.). During the utilization of nitrite, ammonium appears in the medium; during the utilization of nitrate, both nitrite and ammonium appear in the medium. In each case, the ammonium disappears only after the complete removal of the oxidised forms of nitrogen. These observations are in accordance with those of BURRIS AND WILSON[19], though they are not easy to correlate with the results of VIRTANEN AND LUNDBOM[20] which show that the ammonium-assimilating system is differently affected by nitrous oxide than the systems concerned in nitrate-assimilation and nitrogen fixation.

DISCUSSION

Theories suggested to account for the occurrence of adaptation lead in all cases to a common consequence, namely that the onset of utilization of the new substrate must be a relatively gradual process. This is true if the individual cells initially contain the appropriate system to assimilate the substrate, but only in sufficient amount to deal with very low concentrations of an intermediate product in the normal assimilation process, since time must be taken to build up the system to the capacity needed to handle relatively high concentrations. It seems reasonable to expect that in such a case, the existing system would utilize *some* of the new substrate, and that during the period of adaptation a very slow uptake would occur. Similarly, if a system of sufficient capacity pre-existed in the cells, but required activation (presumably under the stimulus of the new substrate), one would expect such activation to occur rapidly at least to a fraction of the total capacity; so that again the onset of uptake would be gradual, and accelerate as activation proceeded. The consequence is even more

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obviously true if adaptation reflects the preferential proliferation of a type of cell normally present in only negligible proportions.

In all the experiments reported here, with the possible exception of the uptake of ammonium in one or two cases, the uptake rates have very rapidly attained a constant value characteristic of the concentration of the substrate (combined nitrogen). The fact that part of the nitrite-nitrogen may reappear in the medium as ammonium-nitrogen does not affect the argument, since the development of a system to reduce nitrite to ammonium would still be expected to come into operation gradually and not almost instantaneously.

Thus there is an immediate qualitative difficulty in correlating these observations with the usual hypotheses regarding adaptation. Attempts to apply a quantitative treatment reveal a further difficulty, namely the different rates of uptake observed for different concentrations of substrate. The data recorded in Fig. 1 may be quoted; when the initial concentration of nitrite-nitrogen is 14 μg/l (10^{-6} M), the observed rate of disappearance is about 12–15 μg/l/h; at 10^{-5} M, it is 50–60 μg/l/h and so on. Expressed alternatively, a culture taking up nitrite from 10^{-3} M solution (14 mg N/l) does so about 120 times as rapidly as one growing in 10^{-6} M nitrite. This difference in rate is greater than would be expected if it depended solely on the immediate availability of nitrite to the cells. Moreover, it is difficult to understand the sudden change from a system incapable of taking up any nitrite to one that very rapidly does so. Because of this difficulty, one of us (M.A.A.) has recently re-examined the problem to see whether very small amounts of nitrite might be taken up during the period of adaptation, and has shown unequivocally that none occurs.

Although the data presented here are clearly insufficient to warrant the formulation of an alternative hypothesis to account for adaptation, it was thought desirable that they should be published in the hope that further work might be stimulated.

SUMMARY

The rates of disappearance of nitrate and ammonium from cultures of *A. vinelandii* have been measured for wide ranges of initial concentration and for both trained and untrained cells. In all cases, when uptake begins (after a period of adaptation during which fixation is practically completely suppressed) the rate very rapidly reaches a constant value characteristic of the initial concentration of the source of combined nitrogen.

Trained cells are able to continue to grow in concentrations of combined nitrogen some fifty times greater than the maximum concentration to which untrained cells can be directly adapted.

The difficulties of correlating these observations with current explanations of adaptation are pointed out.

RÉSUMÉ

Les vitesses de disparition des nitrites et de l'ammoniaque dans des cultures d'*A. vinelandii* ont été mesurées pour un domaine étendu de concentrations initiales et avec des cellules adaptées ou non. Dans tous les cas, quand la consommation commence (après une période d'adaptation pendant laquelle la fixation est pratiquement complètement supprimée) la vitesse atteint très rapidement une valeur constante caractéristique de la concentration initiale de la source d'azote combiné.

Les cellules entraînées peuvent continuer à croître en présence de concentrations en azote combiné environ cinquante fois plus élevées que la concentration maximum à laquelle des cellules non entraînées peuvent être directement adaptées.

Les difficultés qu'il y a à relier ces observations aux théories courantes sur l'adaptation sont mises en évidence.

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ZUSAMMENFASSUNG

Die Geschwindigkeiten des Verschwindens von Nitrit und Ammonium aus *A. vinelandii*-Kulturen wurden im Falle von vorher adaptierten und unadaptierten Zellen mit den verschiedensten Anfangskonzentrationen gemessen. Nach einer Adaptationsperiode, während der praktisch keine Aufnahme stattfindet, erreicht die Geschwindigkeit in allen Fällen einen, für die Anfangskonzentration der Stickstoffverbindung der Quelle charakteristischen, beständigen Wert.

Präadaptierte Zellen sind imstande, in Stickstoffmedienkonzentrationen fortzuwachsen, welche 50 mal höher sind als die Höchstkonzentration, welcher vorher unadaptierte Zellen unmittelbar angeglichen werden können.

Es werden die Schwierigkeiten hervorgehoben welche entstehen, wenn man diese Beobachtungen mit den gewöhnlichen Erklärungen der Adaptation in Übereinstimmung bringen will.

REFERENCES

1 B. A. PETHICA, E. R. ROBERTS AND E. R. S. WINTER, *Biochim. Biophys. Acta*, 14 (1954) 85.
2 M. T. CHAUDHARY, T. G. G. WILSON AND E. R. ROBERTS, *ibid.*, 14 (1954) 507.
3 J. G. LIPMAN, *New Jersey Agric. Exp. Sta. Ann. Rep.*, 25 (1904) 217.
4 I. G. MCBETH, *U.S. Dep. Agr. Bur. Plant Indust.*, Circular 131 (1913) 25.
5 J. STOKLASA, *Zentr. Bakteriol.* II, 21 (1908) 484.
6 S. WINOGRADSKY, *ibid.*, II, 21 (1908) 620.
7 P. R. LEWIS AND C. N. HINSHELWOOD, *J. Chem. Soc.* (1948) 824.
8 K. HORNER AND F. E. ALLISON, *J. Bacteriol.*, 47 (1944) 1.
9 E. D. ROSENBLUM AND P. W. WILSON, *ibid.*, 61 (1951) 475.
10 H. S. REED AND B. WILLIAMS, *Va. Agric. Expt. Sta. Tech. Bull.*, 4 (1915) 81.
11 J. E. FULLER AND L. F. RETTGER, *Soil Sci.*, 31 (1931) 219.
12 A. ZOOND, *Brit. J. Exptl. Biol.*, 4 (1926) 105.
13 M. V. FEDEROV, *Compt. rend. acad. sci. U.R.S.S.*, 52 (1946) 81.
14 E. DIVERS, *Trans. Chem. Soc.*, 75 (1899) 86.
15 P. C. RAY, *ibid.*, 81 (1905) 177.
16 A. S. CORBET, *Biochem. J.*, 28 (1934) 1578.
17 N. W. BARRITT, *Ann. Appl. Biol.*, 20 (1933) 165.
18 P. GRIESS, *Ber.*, 11 (1878) 624.
19 R. H. BURRIS AND P. W. WILSON, *J. Bacteriol.*, 52 (1946) 505.
20 A. I. VIRTANEN AND S. LUNDBOM, *Acta Chem. Scand.*, 7 (1953) 1223.
21 M. V. FEDEROV, *Compt. rend. acad. sci. U.R.S.S.*, 49 (1945) 675.

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