SOME REQUIREMENTS OF BIOLOGICAL NITROGEN FIXATION

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Clues to the mechanism of biological nitrogen fixation have been sought through experiments aimed at defining the substances specifically required by organisms to effect the process. The nitrogen-fixing sequence in bacteria is known to proceed through ammonia (Zelitch et al., 1951; Newton et al., 1953; Wilson and Burris, 1953; Virtanen, 1953). Thus, the effect of adding ammonium salts to a fixing culture should be to reduce the requirement for those substances that are needed by the microorganism to convert elemental nitrogen to ammonia. Accordingly, a search for chemical factors involved in nitrogen fixation was made by comparing the nutritional requirements of bacteria and algae during growth on molecular nitrogen and on ammonia.

Substantially higher requirements for biotin and for iron were found in the anaerobic bacterium, Clostridium pasteurianum strain W-5, when fixing nitrogen than when assimilating ammonia. An increased requirement for iron during nitrogen fixation was also found in the blue-green alga, Nostoc muscorum. G. The chemical functions of these substances remain to be identified.

EXPERIMENTAL METHODS

C. pasteurianum. This anaerobic, nitrogen-fixing bacterium was obtained from the American Type Culture Collection (Catalog 6013), Washington, D. C. The initial inoculum was grown in the potato medium of Jensen and Spencer (1947), and, thereafter, cultures were maintained by serial transfer through a synthetic medium adapted from Rosenblum and Wilson (1950). A 1 per cent inoculum was employed, and the cultures flourished through hundreds of transfers. Growth was measured by optical density determinations and by population counts. Optical density was measured in a "Lumetron" colorimeter (650 m\(\mu\) filter) using 10-ml Klett tubes to hold the sample, while cell counts were made in a Petroff-Hausser chamber. Nitrogen fixation was measured by the micro-Kjeldahl method. A typical culture grew to an optical density of 0.75 (at 650 m\(\mu\)), corresponding to a population density of 5 \times 10^8 cells per ml in 18 to 24 hr. The generation time was 3 hr, and it could be reduced to 2 hr by a fourfold increase in biotin when the concentration of FeSO_4 employed was 100 mg per L. The amount of nitrogen fixed was 0.16 to 0.17 mg per ml, and the cell yield was 1.5 to 1.7 mg per ml (dry weight). Plots of growth and fixation against time were closely parallel, and reliable estimates of population and fixation could be made from optical density measurements alone.

Cultures were grown in 500 ml of medium at 30 C in tubes through which nitrogen containing less than 10 ppm oxygen was bubbled continuously at 10 to 15 L per hr. The culture medium comprised the following quantities of materials per 500 ml of distilled water: sucrose, 10.0 g; MgSO_4 \cdot 7H_2O, 0.05 g; NaCl, 0.05 g; Na_2MoO_4 \cdot 2H_2O, 0.005 g; KHPO_4, 0.25 g; K_2HPO_4, 0.25 g; FeSO_4, 0.005 g; CaCO_3, 5.0 g; biotin, 0.5 \mu g; p-aminobenzoic acid, 0.5 \mu g. For study of ammonia assimilation, the medium was supplemented with 0.4 g (NH_4)_2SO_4. The medium was sterilized in an autoclave except for the ferrous sulfate which was added in 5 ml of water through a Seitz syringe filter. For best results, the phosphates were autoclaved separately and added when cool. In addition to the elements intentionally included, traces of manganese, copper, aluminum, and silicon detected by emission spectroscopy were also present as impurities. For convenience, the medium was prepared routinely from stock solutions. Fresh solutions of the vitamins were prepared each week to avoid variations due to the biotin-deterioration effect (Lichstein, 1955).

Nostoc muscorum. G. This nitrogen-fixing, blue-
green alga was obtained from Dr. Jack Meyers, University of Texas, Austin, Texas. The growth medium was essentially the same as employed by Allen and Arnon (1955) except that the concentration of ferric ion was increased to 10 mg per L. Cultures were grown in 100 ml of medium at 35 C in a cooled water bath that was illuminated by four 100-watt tungsten lamps. Agitation to maintain a uniform suspension was afforded by a stream of 5 per cent CO2-in-air or 5 per cent CO2-in-nitrogen bubbled through the medium (Kratz and Meyers, 1954 and 1955). Results were the same whether the alga was grown under aerobic or anaerobic conditions. Growth measurements were made as has already described. The generation time was 12 to 14 hr, and a full-grown culture corresponded to the fixation of 0.25 mg of nitrogen per ml. The cell yield was about 1.5 mg per ml (dry weight). Optical densities exceeded 2.0 (at 650 mμ), and growth was usually logarithmic for over 80 per cent of this maximum value. For growth on fixed nitrogen, the culture medium was supplemented with either 1.0 g KNO3 or 0.20 g of urea per L.

RESULTS

Systematic variations in the components of the growth medium showed that *C. pasteurianum* was much more sensitive to biotin concentration when fixing nitrogen than when assimilating ammonia. The effect was especially noticeable in the duration of the lag phase and in the total extent of growth (figure 1). A biotin concentration of approximately 1 μg per L appeared to be the minimum requirement for maximum growth under nitrogen-fixing conditions while one-third this amount was adequate in the ammonia-supple-

![Figure 1. Stimulation of *Clostridium pasteurianum* by ammonia at various biotin concentrations.](http://jb.asm.org/)

mented medium. Another manifestation of this effect was that biotin-deficient cultures could be stimulated with ammonia while ammonia was not stimulatory to cultures that were fixing nitrogen in the presence of adequate biotin (figure 1). Accordingly, the poor growth in biotin-deficient cultures was due to a restricted nitrogen-fixing capacity.

Desthiobiotin, but not pimelic acid, could be utilized in place of biotin though not on an equimolar basis since 10 times the concentration of desthiobiotin produced only 70 per cent of normal growth. Biotin-sparing action in nitrogen-fixing cultures was detected with stearic acid but not with oxalacetic, aspartic, or ureidoglutaric acids.

Molybdenum has long been recognized as a requirement of nitrogen-fixing organisms (Jensen and Spencer, 1947). Among the other metals present in the growth media for *C. pasteurianum* and *N. muscorum*, iron has now been found essential to nitrogen fixation. *C. pasteurianum* apparently has several metabolic processes that are dependent upon iron, but the major requirement proved to be for nitrogen fixation (figure 2). Maximal growth in nitrogen-fixing cultures occurred with 100 mg FeSO4 per L, while in ammonia assimilating cultures, 5 mg per L was sufficient. At concentrations below 10 mg per L, nitrogen-fixing cultures were sharply limited in both rate and extent of growth, while at higher concentrations, the beneficial effect was chiefly evident in improved rates. An upper limit to the beneficial iron concentrations was not found in *C. pasteuria-

![Figure 2. The iron requirement of *Clostridium pasteurianum* on N2 vs. NH3. The optical densities were taken from the peaks of growth curves in individual experiments. At high concentrations of FeSO4 (100 mg per L) cultures grown on either N2 or NH3 attained maximum optical densities of about 0.75.](http://jb.asm.org/)
num. Either ferrous or ferric salts could be used. A strong chelating agent, ethylenediaminetetraacetic acid, impaired the rate though not the extent of growth. Another chelate, ferric acetylacetonate, was equivalent to the inorganic salts as an iron source. Recently, a requirement for iron in nitrogen fixation in Azotobacter vinelandii also has been reported (Esposito and Wilson, 1956).

In contrast to C. pasteurianum, N. muscorum was sensitive to too much as well as too little iron during nitrogen-fixing growth. Maximal fixation occurred with 10 mg ferric ion per L, whereas maximal growth occurred throughout the range 1 to 20 mg ferric ion per L. At higher and lower concentrations growth and fixation fell off sharply (figure 3). That iron was required specifically for nitrogen fixation was demonstrated by the fact that at the suboptimal (for fixation) concentration of 0.06 mg ferric ion per L, N. muscorum exhibited normal growth on nitrate or urea but little growth and no detectable fixation on molecular nitrogen. With optimal iron (10 mg per L) N. muscorum yielded cultures of equal density whether growing on molecular nitrogen or nitrate or urea.

The growth rate of N. muscorum on elemental nitrogen never quite equaled the growth rate during assimilation of urea or nitrate. Consequently, a restriction in some factor essential to fixation was suspected, but the condition was not relieved by supplementation with any of a large number of potential growth substances.

**DISCUSSION**

The role of biotin in nitrogen fixation in C. pasteurianum remains to be identified. From the types of biotin-dependent reactions that have been reported in the literature, it would seem that biotin might mediate in the synthesis of enzymes involved in nitrogen fixation just as it appears to influence formation of important enzymes in several other systems (Lardy and Peanasky, 1953; Hofmann and Panos, 1954; Sexton, 1953; Sundaram et al., 1954; Lichstein and Boyd, 1955; Lichstein, 1951). The biotin required by ammonia-assimilating cultures may be largely devoted to amination reactions such as the conversion of fumarate to aspartate (Lichstein, 1951).

The finding that iron is involved in biological nitrogen fixation has prompted speculation on the mechanism of the fixation process based on the knowledge that iron performs a key function in other oxidation-reduction systems, e.g., the cytochromes. The net effect of biological fixation is the reduction of elemental nitrogen to ammonia (Zelitch et al., 1951; Newton et al., 1953; Wilson and Burris, 1953; Virtanen, 1953). The only other familiar example of a reaction that results in the reduction of elemental nitrogen under mild conditions is the combination of lithium metal with nitrogen to form lithium nitride. In water, lithium nitride hydrolyzes spontaneously to ammonia and lithium hydroxide. Accordingly, our thought is that the active site in the nitrogen-fixing enzyme could be a chelated form of a variable-valent metal ion that has the power to transfer electrons to elemental nitrogen and thereby effect its reduction to ammonia by what is essentially the nitride reaction. The metal in question may be iron, although molybdenum cannot be excluded as a possibility for the proposed nitride-forming ion, nor can manganese or copper, the other variable-valent metals prominent in the cells. The molybdenum required for fixation appears to play its part in the hydrogenase enzyme (Shug et al., 1956) whose function we visualize is to mediate in reducing the nitride-forming ion after each act of nitrogen fixation.

![Figure 3. Effect of iron concentration on nitrogen fixation by Nostoc muscorum.](http://jb.asm.org/)

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To our knowledge, the proposal of a nitride-forming ion as the primary site of biological nitrogen fixation is a new one; however, Winfield (1955) has suggested that fixation occurs by hydrogenation at the site of an iron complex in hydrogenase, which in *Azotobacter* spp. at least may be an iron porphyrin compound (Hyndman et al., 1953).

**summary**

*Clostridium pasteurianum* requires both iron salts and biotin for nitrogen fixation. The evidence for this conclusion is that the organism has a larger requirement for these substances when growing on elemental nitrogen than when growing on ammonia. The blue-green alga *Nostoc muscorum* similarly requires iron for nitrogen fixation.

A mechanism for nitrogen fixation is proposed in which a chelate of a variable-valent metal ion serves as key reactant in a nitride-forming type of reaction.

**references**


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