CARBON DIOXIDE FIXATION AND PHOSPHORYLATION BY NITROBACTER AGILIS

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During the autotrophic oxidation of a gram mole of nitrite by Nitrobacter approximately 17.5 kg. cal. of energy become available for CO_2 fixation. It is reasonable to assume that at least part of this energy is mediated to other reactions as high energy phosphate bonds of adenosine triphosate. Work done with other autotrophic microorganisms such as Thiobacillus denitrificans (Aubert et al., 1957) makes it attractive to envisage the carbon dioxide fixation by Nitrobacter as following the pathway that is now known to occur in higher plants (Bassham et al., 1954).

Nitrobacter agilis as obtained from the American Type Culture Collection or isolated from a garden soil was grown in 8 liters of the medium described by Aleem and Alexander (1958), harvested with the aid of a Sharples centrifuge and after two washings with cold distilled water suspended in 25 ml. of pH 7.8 tris buffer. The yield expressed as dry weight of the cells usually varied from 110 - 150 mg.

In order to study the fixation of CO_2, 10 ml. of cell suspension were incubated with 1 ml. of 0.1 M KNO_2, 2 ml. of 0.01 M $Na_2\ C^{14}O_3$ and 2 ml. of water in a stoppered conical flask for 3 hours. The cells were then centrifuged, suspended in 5 ml. of water and poured into boiling ethanol. After centrifugation and separation of the lipids the extract was concentrated under vacuum and chromatographed first in phenol and then in butanol: propionic acid: water (Benson et al., 1950).

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During the incubation period all the nitrite disappeared from the system and approximately one micromole of CO_2 was fixed. It was found that nearly 75 percent of the radiocarbon was in the alcoholic extract, with that remaining appearing in the insoluble residue. Very little radioactivity could be detected in the lipid fraction.

Radioautographs made from the bidimensional chromatograms showed a pattern of spots quite similar to those found in previously mentioned studies of higher plants and microorganisms. By spraying the paper with the molybdic acid reagent of Hanes and Isherwood (1949) several phosphorylated compounds were found to be present in the chromatograms. After elution and cochromatography in the solvents used by Bandski and Axelrod (1951) the following substances could be identified: hexose mono and diphosphates, phosphoglyceric acid and probably phosphopyruvic acid. A total of 11 spots were detected on the film; Fig. 1, however, shows only those which made a darker imprint on the x-ray film. Sixty per cent of the activity applied originally to the paper was recovered in phosphoglyceric acid.

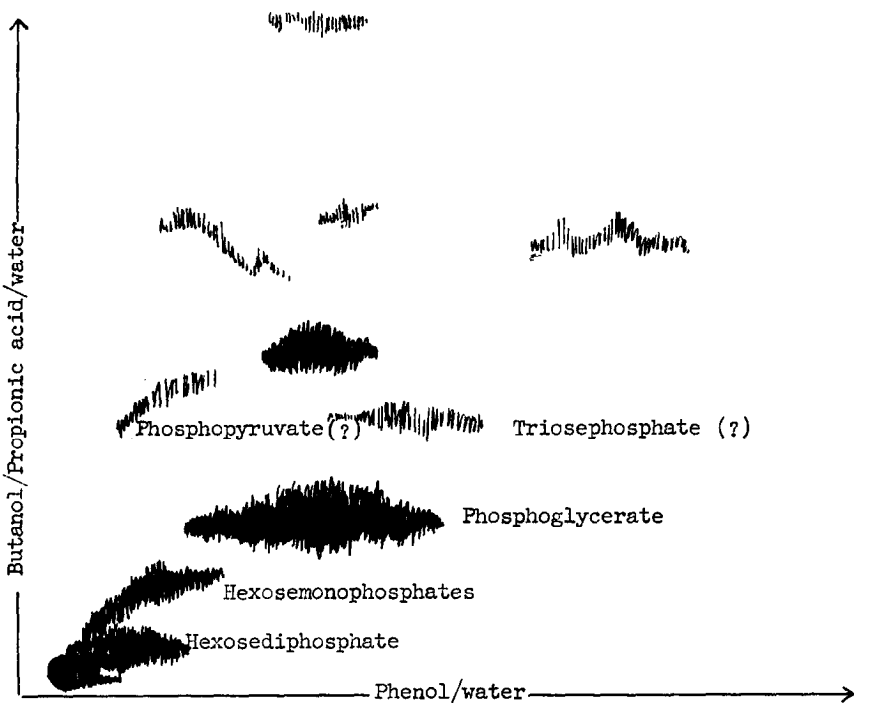

Fig. 1. Products of CO_2 assimilation by Nitrobacter agilis; the number of bars indicate the relative darkness of the spots.

The formation of organic phosphoric compounds by N. agilis was observed both with intact cells and with sonic extracts. In the first case the reaction mixture contained 10 ml. of cell suspension, 0.5 ml. of 0.1 M KNO_2, 0.5 ml. of $P^{32}O_4$ ion, 0.2 ml. of 0.075 KF and 0.2 ml. of 0.05 M $MgCl_2$. For a control treatment nitrite was omitted from the reaction mixture. After incubating 90 minutes the cells were killed with cold 35 per cent trichloracetic acid, centrifuged and the supernatant solution was chromatographed with the same solvents mentioned above. Radioautography and chemical tests (Burrows et al., 1952) on the paper chromatograms ascertained the presence of adenosine triphosphate (ATP) only in the complete system. Those radioautographs corresponding to the minus nitrite treatment showed only the inorganic P^{32} spot. The cells, during the incubating period, oxidized all the nitrite which was added. Since this was a purely qualitative trial designed simply to verify the ability of Nitrobacter to produce ATP, no data can be given with respect to the amount of P^{32} incorporated in relation to nitrite oxidized.

Cell-free extracts were prepared by the sonic disintegration of cells in a 10 KC Raytheon oscillator for 20 minutes and separation of cell debris centrifugally at 3500x g for 30 minutes (Aleem and Alexander, 1958). In experiments with this cell-free extract suspended in hydroxymethylaminomethane (tris) buffer at pH 7.8 the systems shown in Table 1 were used.

Table 1.
Phosphorylation by Nitrobacter

Treatment	µmoles NO_2^- oxidized	µmoles PO_4 esterified
Complete	4.75	2.37
Complete, boiled	0.12	0.00
Minus NO_2^-	0.10	0.00
Plus DNP	4.50	0.10

The complete reaction mixture contained 2.5 ml. of the cell-free preparation (0.8 mg. N per ml.), 1 ml. 0.005 M $K_4P_2O_4$, 1 ml. 0.005 M KNO_2, 0.1 ml. 0.05 M ADP, 0.2 ml. 0.05 M MgCl2, 0.2 ml. 0.075 M KF and 0.1 ml. 0.5 per cent cytochrome c in a total volume of 10 ml. 1 ml. of 0.001 dinitrophenol(DNP) was added where indicated.

After incubating 90 minutes, 0.5 ml. cold 35 per cent trichloracetic acid was add and the deproteinized solution was analyzed for inorganic phosphate by the method of Gomori (1942). In another aliquot withdrawn before the addition of the acid, nitrite was determined (Rider and Mellon, 1946). As shown in Table 1, for each micromole of nitrite oxidized 0.5 micromoles of inorganic phosphate disappeared. It is probable therefore that at least one high energy phosphate bond is yielded by the oxidation of a molecule of nitrite. A consideration of the overall energy yield of the reaction suggests that at most two high energy phosphates might be expected. This would also be consistent with the mechanism for electron transport in the oxidation of nitrite proposed by Aleem and Nason (1949). An ATPase was found to be active in Nitrobacter; and no means was provided in the experimental procedure to trap the ATP formed. In another experiment the same system was used but carrier-free $P^{32}O_4$ ion was added instead of unlabeled phosphate. The radiochromatograms showed tagged ATP to be present that was identifiable by its Rf and by chem cal tests on the paper. Although the tracer experiments with intact cells showed only tagged ATP to have been produced, with the cell-free preparation other organ phosphorus compounds were found whose identification has not yet been attempted (see Fig. 2).

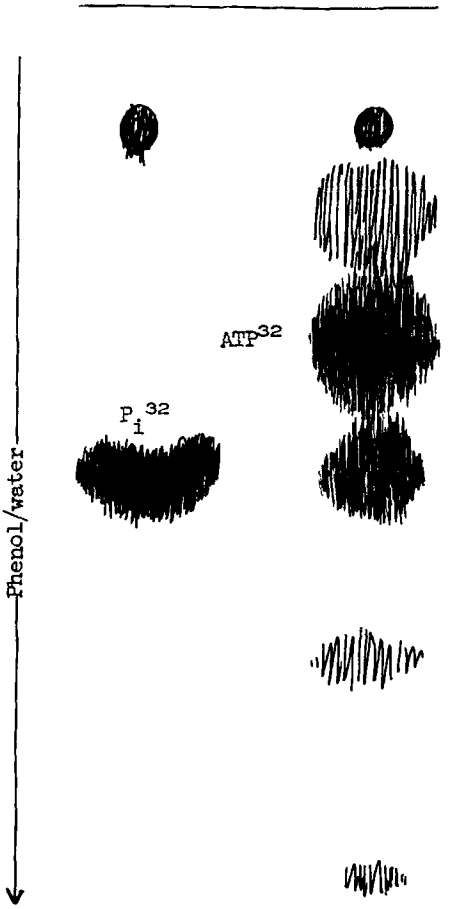

Fig. 2. Products of P^{32} incorporation by cell-free preparation of Nitrobacter agilis as appear in radiochromatogram; inorganic phosphate was used as a reference.

It seems licit to conclude that in Nitrobacter mechanisms exist for the syn-

thesis of high energy phosphate and for carbon dioxide fixation not unlike those of other organisms. A more detailed account of these and other experiments in progress will be published elsewhere.

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