Proceedings of the

NATIONAL ACADEMY OF SCIENCES

Volume 46 · Number 6 · June 15, 1960

*PHOSPHORYLATION COUPLED TO NITRITE OXIDATION BY PARTICLES FROM THE CHEMOAUTOTROPH, NITROBACTER AGILIS**

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Communicated by Herman M. Kalckar, April 28, 1960

Members of the obligately chemoautotrophic bacterial genus *Nitrobacter* uniquely obtain their primary energy from the biological, aerobic oxidation of the specific inorganic substrate, nitrite. The energy liberated is in part utilized to assimilate carbon dioxide which serves as the sole source of carbon for growth. The chemoautotrophs as typified by *Nitrobacter* are distinctly different from photosynthetic organisms and from heterotrophs, since the former fulfill their energy requirement by capturing the energy of light whereas the latter must use organic compounds as their primary source of both energy and carbon.

While there is a good deal of information concerning the metabolism and accompanying energy transformations in the photosynthetic and heterotrophic forms, virtually nothing is known about these aspects in the chemoautotrophs. From the standpoint of comparative biochemistry it would be expected that many of the metabolic steps carried out by the chemoautotrophs are fundamentally similar to those displayed by other organisms including the formation and utilization of high-energy phosphate bonds. It therefore seems reasonable to speculate that high-energy bonds, probably in the form of adenosine triphosphate (ATP), are produced during the oxidation by the chemoautotrophs of their particular inorganic substrates. The high-energy phosphate bonds would be presumably used for the assimilation of carbon dioxide and for other energy-requiring processes. Vogler and Umbreit[1] and Umbreit[2] on the basis of their findings with intact cells of the chemoautotrophic bacterium *Thiobacillus thiooxidans* suggested the formation of high-energy phosphate bonds in the oxidation of sulfur and their subsequent utilization in carbon dioxide assimilation. On the other hand, Baalsrud and Baalsrud,[3] and Newburgh[4] working with the same organism reported essentially negative results along these lines.

The recent data of Aleem and Alexander[5] demonstrating for the first time the presence of a nitrite-oxidizing system in cell-free *Nitrobacter* extracts suggested the possible suitability of this organism for investigation of chemosynthetic mechanisms. Aleem and Nason[6] subsequently showed that the nitrite-oxidizing activity in cell-free preparations of *Nitrobacter* resides solely in a cytochrome-containing particle designated as nitrite oxidase. Their data implicated the action of the nitrite oxidase system to involve the enzymatic transfer of electrons from nitrite

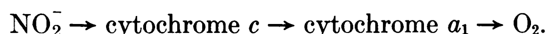
TABLE 1

FACTORS AFFECTING OXIDATIVE PHOSPHORYLATION BY PARTICULATE NITRITE OXIDASE* FROM *N. agilis*

Treatment	O_2-uptake µL per hr†	P^{32}-uptake per hr,‡ cpm	P/O (corrected) §
	Exp. I		
Complete	146	1,640	0.14
−Nitrite	7	454	...
−Hexokinase, −Glucose	154	413	0.03
$-Mg^{++}$	159	794	0.06
−Nitrite, $-Mg^{++}$	6	58	0.00
	Exp. II		
Complete	162	412	0.03
−Nitrite	12	82	...
−ADP	169	44	0.00
$-Mg^{++}$	141	274	0.01
−Nitrite, $-Mg^{++}$	1	50	0.00
	Exp. III (Mg^{++} omitted from reaction mixture)		
Complete	174	985	0.07
−Nitrite	1	58	...
−Hexokinase, −Glucose	182	99	0.004
−Nitrite, −Hexokinase, −Glucose	0	52	0.00

* Resuspended pellet collected from 27,000 × *g*-supernatant solution by centrifugation at 95,000 × *g*. The complete reaction mixture contained 10 micromoles phosphate, 25 micromoles nitrite, 0.2 micromoles ADP(PABST Co.), 15 micromoles $MgCl_2$, 0.5 mgs hexokinase (type III Sigma Co.), 50 micromoles glucose, 0.5 ml nitrite oxidase corresponding to 0.6–1.5 mg protein, P^{32} (phosphotope oral, without preservative, without carrier—E, R. Squibb and Sons, New York) approximately 3×10^5 cpm per Warburg flask.
The final volume was made up to 3.2 ml with 0.1 *M* Tris pH 7.5. The central well contained filter paper and 0.2 ml of 10% NaOH. After a 10 min preincubation period at 30°C, the reaction was started by tipping in the enzyme from the side arm and O_2-uptake determined at 10-min intervals for 1 hr.
† These values represent the consumption of O_2 by the total volume of reaction mixture (3.2 ml) contained in the Warburg flask.
‡ These values represent the actual counts per min measured in the organic phosphate fraction in an aliquot equivalent to $^1/_{48}$ of the volume of the total reaction mixture (3.2 ml) contained in the Warburg flask.
§ P/O ratios are corrected for by subtracting the endogenous O_2 and P^{32} uptakes obtained in the minus nitrite reaction mixture.
‖ Exp. I contained 6.7×10^5 CPM of initially added P^{32} orthophosphate per Warburg flask. Exp. II contained 5.2×10^5 CPM and Exp. III contains 4.3×10^5 CPM.

to molecular oxygen via cytochrome *c*- and cytochrome oxidase-like components according to the following sequence:



$$NO_2^- \rightarrow \text{cytochrome } c \rightarrow \text{cytochrome } a_1 \rightarrow O_2.$$

The present paper describes experiments demonstrating that partially purified nitrite oxidase particles catalyze the formation of high-energy phosphate bonds concomitant with the specific enzymatic oxidation of nitrite by molecular oxygen. Some of the properties of the system are described including evidence showing that ATP is formed when the corresponding nucleoside diphosphate (ADP) is used as the phosphate acceptor. Certain nucleoside diphosphates appeared to be effective when added in place of ADP whereas others were not. The electron transport system mediating nitrite oxidation is sensitive to relatively high concentrations of such respiratory chain inhibitors as antimycin A and 2*n*-heptyl-4-hydroxy-quinoline-N-oxide. Dinitrophenol, thyroxine, and dicumarol, however, failed to uncouple the nitrite-specific oxidative phosphorylation.

Methods and Materials.—Cells of *Nitrobacter agilis* (American type Culture Collection No. 9482) grown in a clear inorganic culture solution and disrupted by sonic oscillation as described by Aleem and Alexander[5,7] were centrifuged at 10,000 × *g* for 30 min. The resulting opalescent cell-free supernatant solution (S-10,000) containing nitrite oxidase activity was then fractioned by successive recentrifugation for 30 min intervals at 27,000 × *g*, 58,000 × *g*, 95,000 × *g*, and 144,000

$\times$ *g*, respectively. The reddish-brown pellets (designated as P-27,000, P-58,000, and so forth) attained by each of the centrifugations were separately suspended in 0.1 *M* tris(hydroxymethyl)aminomethane (Tris) buffer, pH 7.5, usually equal in volume to one third of that of the S-10,000 fraction from which they were derived. All of the above particulate fractions possessed nitrite oxidase activity as measured by nitrite disappearance[6] as well as by oxygen uptake. The latter was determined by means of conventional micromanometric Warburg constant-volume respirometers as detailed in the accompanying tables. One or more of the above particulate enzyme fractions containing 2 to 4 mgs of protein per ml, determined according to the procedure of Lowry *et al.*,[8] were used in the present experiments as indicated. Organic P^{32} phosphate of the reaction mixtures resulting from the transformation of added inorganic P^{32} orthophosphate was measured in a Model D-47 gas flow counter (Nuclear Chicago). This was preceded by a trichloroacetic acid precipitation of the reaction mixture and successive extraction of the phospho-molybdate complex from the supernatant solution, first with a 50/50 v/v mixture of isobutanol/benzene and then with ether (anhydrous diethyl) according to a modified procedure of Rose and Ochoa.[9] This method routinely extracted 99.95 per cent of the inorganic orthophosphate as determined by residual P^{32}.

Results and Discussion.—Factors affecting oxidative phosphorylation: The data of Table 1 indicate that a striking increase in the incorporation of inorganic p^{32} phosphate into an organic fraction accompanies nitrite oxidation. The results also show that the addition of magnesium ions and a phosphate acceptor such as ADP markedly stimulate this apparent phosphate esterification which is coupled to nitrite oxidation. In the above experiments employing catalytic quantities of ADP (0.2 μM), the dependence of phosphorylation upon the presence of added hexokinase and glucose as a "trapping system" in order to accumulate organic phosphate and provide ADP (glucose + ATP $\xrightarrow{\text{hexokinase}}$ glucose-6-PO_4 + ADP) is evident. When substrate quantities (5 micromoles) instead of catalytic quantities of ADP are used, however, appreciable phosphate esterification occurs without the addition of hexokinase and glucose to the reaction mixture, as shown in Table 2.

TABLE 2

NUCLEOSIDE DIPHOSPHATE SPECIFICITY OF *N. agilis* NITRITE OXIDASE* IN OXIDATIVE PHOSPHORYLATION

Treatment	O_2-uptake µL per hr†	P^{32}-uptake per hr,‡ cpm	P/O (corrected) §
+Nitrite, −nucleoside diphosphate	83	60	0.00
−Nitrite, +ADP	−8	43	...
+Nitrite, +ADP	81	455	0.05
+Nitrite, +IDP	142	510	0.04
+Nitrite, +GDP	96	336	0.04
+Nitrite, +UDP	70	80	0.003
+Nitrite, +CDP	130	70	0.002

* Reaction mixture contained 5 micromoles of the indicated nucleotide and lacked the hexokinase "trapping system." Pellet collected from 27,000 $\times$ *g* supernatant solution by centrifugation at 144,000 $\times$ *g*.
†, ‡, § See corresponding footnotes of Table 1. 5.3 $\times$ 10^5 CPM of initially added P^{32} orthophosphate per Warburg Flask.

ATP as the phosphorylation product: The above stimulation of phosphate esterification by both added ADP and the oxidizable substrate, nitrite, as well as by the hexokinase system when small quantities of the nucleoside diphosphate are employed, implicate the phosphorylation product of the nitrite oxidase system

to be ATP. This is borne out by experiments involving the separation of ADP and ATP from a complete nitrite oxidase reaction mixture (containing substrate quantities of ADP and no hexokinase system) according to the ion exchange column chromatography method described by Cohn[10] and by the paper chromatography procedure employing an isobutyric acid-ammonium hydroxide-water solvent system.[11] A major portion of the organic p^{32} label was found to be present in the ATP with the remainder in the ADP. In most experiments about 10 to 20 per cent of the added inorganic p^{32} phosphate was incorporated into the organic fraction. Additional evidence for ATP formation was obtained by indicating the appearance of glucose-6-phosphate in a complete nitrite oxidase reaction originally containing the hexokinase "trapping" system and catalytic amounts of ADP. This was implied by the observed reduction of added triphosph-pyridine nucleotide (at 340 mμ) in the presence of glucose-6-phospeate dehydrogenase and a boiled aliquot of the above preincubated nitrite oxidase system. Corresponding controls lacking nitrite or hexokinase exhibited no such effect.

TABLE 3

SUBSTRATE SPECIFICITY FOR OXIDATIVE PHOSPHORYLATION BY PARTICULATE NITRITE OXIDASE* FROM *N. agilis*

Treatment	O_2-uptake μL per hr†	P^{32}-uptake per hr‡ (cpm)	P/O (corrected) §
	Exp. I		
None	2	79	...
KNO_2	168	1,410	0.2
Succinate	21	292	0.1
DPNH	25	195	0.05
Fe^{++}	7	17	0.00
	Exp. II		
None	2	105	...
KNO_2	170	1,861	0.12
Ascorbic acid	90	224	0.03

* Pellet collected from 27,000 × g-supernatant solution by centrifugation at 144,000 × g. Same conditions as in Table 1 but with 25 micromoles of added substrate as indicated.
†, ‡, § See corresponding footnotes of Table 1. Exp. I contained 3.4 × 10^5 CPM of initially added P^{32} orthophosphate per Warburg flask; and Exp. II contained 4.8 × 10^5 CPM.

Nucleoside diphosphate specificity: The effects of substrate quantities of other nucleoside diphosphates in place of ADP on oxygen uptake and accompanying phosphorylation as measured by P^{32} incorporation into the organic fraction is shown in Table 2. Inosine diphosphate (IDP) and to a lesser extent guanosine diphosphate (GDP) significantly stimulated phosphate esterification as compared to a control reaction mixture which lacked any added nucleoside diphosphate. The stimulation in phosphorylation by these two nucleotides was of the same order of magnitude as that caused by ADP, although the increase in oxygen uptake induced by IDP was considerably greater. In contrast, the addition of uridine diphosphate (UDP) and cytidine diphosphate (CDP) had only a slight positive effect on P^{32} incorporation, although the latter caused a marked stimulation in oxygen uptake as compared to that produced by ADP. Other control reaction mixtures (not shown) containing each of the added nucleotides indicated in Table 2, but without added nitrite, experienced no increase in phosphorylation or oxygen uptake.

It has not yet been determined whether one or more of the above three effective nucleotides (ADP, IDP, and GDP) act directly as initial phosphate acceptors;

or alternatively, receive phosphate groups from one of the formed nucleoside triphosphates through the action of a nucleoside diphosphokinase.

Substrate specificity: Table 3 demonstrates that of various substrates tested, nitrite proved to be the most effective in producing maximal oxygen uptake and phosphate esterification. The addition of succinate or reduced diphosphopyridine nucleotide (DPNH) in place of nitrite in concentrations ranging from 10 micromoles to 25 micromeles per reaction mixture was 10 to 15 per cent as effective as nitrite in stimulating oxidation and phosphorylation. Although added ascorbic acid in place of nitrite was responsible for a substantial increase in oxygen consumption, it did not cause a corresponding increase in phosphate esterification. The addition of succinate, DPNH, or ascorbic acid, however, did seem to produce a small but definite rise in P^{32} uptake. Ferrous ions appeared not to serve as a substrate in place of nitrite, and, in fact, inhibited endogeneous P^{32} phosphate uptake.

Inhibitors and uncoupling reagents: Thus far, all attempts to uncouple phosphorylation from nitrite oxidation in the nitrite oxidase system have been unsuccessful. The separate addition to different reaction mixtures of 2,4-dinitrophenol, L-thyroxine and dicumarol in various final concentrations ranging from 10^{-6} M to 5×10^{-5} M failed to exert an uncoupling effect. In fact dinitrophenol at a final concentration of 5×10^{-4} M and thyroxine and dicumarol each at 5×10^{-5} M completely inhibited nitrite oxidation and, of course, accompanying phosphorylation. Aged nitrite oxidase preparations can be obtained which display unchanged rates of nitrite oxidation but decreased rates of phosphorylation (Table 1, Experiment II). It is of interest that antimycin A which acts as a potent inhibitor of the mammalian terminal respiratory chain at a presumed site between cytochrome *b* and *c* also caused significant inhibition (50 to 100 per cent) of the nitrite oxidase at final concentrations of 15–20 μg per ml. This concentration, however, is approximately 100 to 1,000 times greater than that required to cause a similar inhibition of mammalian succino-oxidase and DPNH oxidase. The use of 2*n*-heptyl-4-hydroxyquinoline-N-oxide in final concentrations of 50 μg per ml caused a 70 per cent inhibition of the nitrite oxidase system.

The presently reported experiments employing cytochrome-containing particles from *Nitrobacter agilis* provide evidence for an oxidative phosphorylation system which utilizes nitrite as the oxidizable substrate. The highest P/O ratios attained thus far with nitrite in the above experiments are about 0.2. It is quite possible that this value may be substantially increased as the system is further characterized and more optimal conditions are found. On the other hand, the few studies that have been conducted in the past with intact *Nitrobacter* cells indicating their efficiency to be of the order of 5 per cent,[12] might be a reflection of a relatively inefficient energy-coupling process in the course of oxidation of the inorganic substrate. On purely speculative grounds this could possibly mean that two or more enzymatic pathways are associated with the ultimate transfer of electrons from nitrite to molecular oxygen, and that one of these is coupled to phosphate esterification. It is not certain at this time whether or not the small stimulation in oxygen uptake and accompanying P^{32} uptake by added DPNH and succinate noted above is inherent in the nitrite oxidase system or due to a contamination by separate systems. Fractionation of the nitrite oxidase system by differential centrifugation as described under *Methods and Materials* has shown phosphorylative activity to be

distributed in all the particulate fractions ranging from P-27,000 to P-144,000. The fractions P-58,000 and P-95,000, however, tended to give the highest P/O ratios.

Studies are now in progress to elucidate further the properties and mechanisms of the nitrite oxidase system from *Nitrobacter* with respect to electron transport and concomitant phosphorylation. Whether or not the above system actually represents the primary means by which the intact organism obtains its energy will also be the subject of future investigations. Finally, the question as to how these cells obtain their reducing power, which is presumably also derived from nitrite oxidation, is still untouched. It would be expected from fundamental biochemical considerations that both useful energy and reducing power are necessary for the process of carbon dioxide assimilation, a process which is obviously carried on in its entirety by the autotrophs such as the photosynthetic organisms and the chemosynthetic bacteria.

Summary.—A partially purified, particulate cytochrome-containing nitrite oxidase from *Nitrobacter agilis* is shown to exhibit phosphorylation coupled to the specific oxidation of nitrite. The presence of the oxidizable substrate nitrite and a phosphate acceptor such as ADP markedly enhances inorganic P^{32} phosphate incorporation into an organic P^{32} fraction. Added magnesium ions also stimulate phosphorylation. ATP has been identified as the phosphorylation product when substrate quantities of ADP were used. When catalytic quantities of ADP are employed, virtually no organic P^{32} incorporation occurs unless a hexokinase "trapping system" is furnished in the reaction mixture. Other nucleotides such as IDP and GDP appear to serve as phosphate acceptors in place of ADP, whereas UDP and CDP are ineffective. Succinate and DPNH stimulate oxygen uptake at a rate which is 10 to 15 per cent of that induced by nitrite, accompanied by a correspondingly small stimulation in organic P^{32} incorporation. Dinitrophenol, thyroxine, and dicumarol in concentrations as high as 5×10^{-5} M failed to uncouple the system. Higher concentrations inhibited nitrite oxidation. Relatively high concentrations of antimycin A and 2*n*-heptyl-4-hydroxy-quinoline-N-oxide were also inhibitory.

We are indebted to Jean Z. Gadziola and Reginald H. Garrett for their excellent technical assistance.

* Contribution No. 279 of the McCollum-Pratt Institute. This investigation was supported in part by grants from the National Science Foundation and the United States Public Health Service (No. RG-2332).

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THE FIRST STEP IN PHOTOSYNTHESIS: EVIDENCE FOR ITS ELECTRONIC NATURE

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Communicated by C. B. van Niel, April 27, 1960

Emerson and Arnold[1] nearly thirty years ago showed that the act of photosynthesis is not carried out by one chlorophyll molecule but by the cooperation of several hundred. These results were verified and extended by Gaffron and Wohl,[2] by Kohn,[3] by Tamiya,[4] and others. Szent-Györgyi[5] suggested in 1941 that the cooperation was by way of electronic conduction bands in protein, but Evans and Gergely[6] pointed out that any band in protein would be too high on the energy scale to play a part in photosynthesis. Katz,[7] in 1949, suggested that chlorophyll in lamellae formed a two-dimensional crystal with a two-dimensional band system for the conduction of electrons and holes. The idea that semiconduction plays a part in photosynthesis was discussed further by Bassham and Calvin[8] and by a number of others. Arnold and Sherwood[9] demonstrated that dried chloroplasts act as semiconductors.

In the present paper we give new experimental evidence that in the purple bacteria the first step in photosynthesis appears to be purely *electronic* in nature. We have found in chromatophores a new class of reversible spectral changes that are the same from 300°K down to 1°K. At 1°K no ordinary chemical reaction can take place, and the fact that the spectral changes are the same over such a wide range of temperatures shows that no energy of activation is involved. We have evidence that the process indicated by these spectral changes immediately precedes the oxidation-reduction of the cytochromes that has been studied by a number of workers since Duysens' initial observation.[10] And finally we have an electrical experiment on dried chromatophores, showing that upon illumination the positive and negative electric charges are spatially separated.

Materials and Methods.—*Rhodopseudomonas spheroides*, wild type strain 2.4.1 (from Prof. C. B. van Niel's laboratory) and the carotenoidless[11] mutant strain UV-33 (obtained from Dr. W. R. Sistrom) were cultivated anaerobically in the light in a medium described by Cohen-Bazire *et al.*[12] Films of dried chromatophores were prepared as follows: The cells were suspended in distilled water and disrupted sonically; the sonic extract was clarified by centrifuging at 20,000 × g. Chromatophores in the extract were collected and washed twice with distilled water by successive 90-min centrifugations at 100,000 × g. The final aqueous suspension, dried on a glass plate, formed an optically clear film, stable at least for months and having the same absorption spectrum as the intact cells.

Spectral changes in these films were observed with a Beckman DK-1 spectro-