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# Energy-Conserving Reactions in Phosphorylating Electron-Transport Particles from *Nitrobacter winogradskyi*

## ACTIVATION OF NITRITE OXIDATION BY THE ELECTRICAL COMPONENT OF THE PROTONMOTIVE FORCE

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1. In electron-transport particles (ET particles) prepared from *Nitrobacter winogradskyi*, the uncoupling agent carbonyl cyanide phenylhydrazone increased the rate of NADH oxidation but decreased the rate of oxidation of $NO_2^-$. Its effectiveness in stimulating NADH oxidation closely paralleled its effectiveness in inhibiting $NO_2^-$ oxidation. 2. In the presence of ADP and phosphate the oxidation of NADH was stimulated, whereas the oxidation of $NO_2^-$ was inhibited. In the presence of excess of $P_i$ the concentration-dependence with respect to ADP was the same for acceleration of NADH oxidation and inhibition of $NO_2^-$ oxidation. 3. Oligomycin inhibited NADH oxidation and stimulated the oxidation of $NO_2^-$. The concentration of oligomycin required to produce half-maximal effect in both systems was the same. 4. The apparent $K_m$ for $NO_2^-$ was not affected by ADP together with $P_i$, by uncoupling agent or by oligomycin. 5. With NADH as substrate, classical respiratory control was observed. With $NO_2^-$ as substrate the respiratory-control ratio was less than unity. 6. A reversible uptake of $H^+$ accompanied the oxidation of $NO_2^-$ by ET particles. 7. In the presence of $NH_4Cl$ or cyclohexylamine hydrochloride, $H^+$ uptake was abolished and increased rates of $NO_2^-$ oxidation were observed. When valinomycin was present in the reaction medium, low concentrations of $NH_4Cl$ inhibited $NO_2^-$ oxidation. 8. Pretreatment of ET particles with oligomycin enhanced the stimulation of $NO_2^-$ oxidation induced by $NH_4Cl$ or by cyclohexylamine hydrochloride. Pretreatment with the uncoupler carbonyl cyanide phenylhydrazone prevented these stimulations. 9. In the presence of dianemycin together with $K^+$, the uptake of $H^+$ was abolished and the rate of $NO_2^-$ oxidation was increased. In contrast, in the presence of valinomycin together with $K^+$, the uptake of $H^+$ was increased, and the rate of $NO_2^-$ oxidation decreased. 10. Sodium tetraphenylboron was found to be an inhibitor of $NO_2^-$ oxidation, but caused a stimulation of NADH oxidation which was dependent on the presence of $NH_4Cl$ or cyclohexylamine hydrochloride. 11. It is concluded that the enhanced rate of $NO_2^-$ oxidation observed in the absence of energy-dissipating processes clearly relates to some state before the involvement of adenine nucleotides, and it is suggested that the oxidation of $NO_2^-$ generates a protonmotive force, the electrical component of which controls the rate of $NO_2^-$ oxidation.

Chemolithotrophs of the genus *Nitrobacter* obtain their energy from the oxidation of the weak reductant $NO_2^-$ with $O_2$ ($\Delta G_0' = -74.8$ kJ/mol; $-17.8$ kcal/mol) and assimilate $CO_2$ by using a Calvin cycle (Aleem, 1965). The NADH required for $CO_2$ assimilation is generated by energy-dependent reversed electron transport from $NO_2^-$ to $NAD^+$ ($\Delta G_0' = +149.5$ kJ/mol; $+35.6$ kcal/mol) (Kiesow, 1963). It is known that substituted phenols, which uncouple respiratory-chain-linked phosphorylation, inhibit the oxidation of $NO_2^-$ by intact cells of *Nitrobacter* and by electron-transporting particles derived from them (Simpson, 1955; Aleem & Alexander, 1958; Butt & Lees, 1960). Aleem & Nason (1960) have demonstrated that ET particles can couple the oxidation of $NO_2^-$ to the phosphorylation of ADP, but concluded that since these substituted phenols inhibit both oxidation and phosphorylation, true uncoupling does not occur. The oxidation of NADH by ET particles from *Nitrobacter winogradskyi* is also coupled to ATP synthesis (Kiesow, 1964).

The present paper describes experiments on the energy-coupling processes in ET particles from *N. winogradskyi*, and a translocation of $H^+$ is shown to accompany $NO_2^-$ oxidation. Ionophorous anti-

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biotics and lipid-soluble compounds have been used as tools to investigate the relationship between the rate of $NO_2^-$ oxidation and the $\Delta pH$ and $\Delta\psi$ components of the protonmotive force (Mitchell, 1966).

## Experimental

### *Methods*

*Growth conditions.* The strain of *N. winogradskyi* used was that isolated by Professor H. Engel (Botanischer Garten, Hamburg, West Germany) and was kindly supplied by Dr. N. Walker (Department of Microbiology, Rothamsted Experimental Station, Harpenden, Herts., U.K.). A stock culture was maintained in a medium containing 50 $\mu$M-$MgSO_4$, 5mM-$K_2HPO_4$, 10mM-$NaNO_2$, 4$\mu$g of biotin/l, 1.7ml of heavy-metal solution/l, 5$\mu$M-$CaCl_2$ and 1$\mu$l of 'dialysed iron' (a colloidal suspension of $Fe_2O_3$, supplied by BDH Chemicals, Poole, Dorset, U.K.)/l. The components were added in the order listed to a sufficient quantity of singly distilled water, and the pH was adjusted to 7.8 with NaOH and HCl. The heavy-metal solution contained: 40mM-EDTA (sodium salt), 7.2mM-$FeSO_4$, 3.5mM-$ZnSO_4$, 1mM-$MnCl_2$, 1mM-$H_3BO_3$, 100$\mu$M-$Co(NO_3)_2$, 30$\mu$M-$(NH_4)_6Mo_7O_{24}$ and 12$\mu$M-$CuSO_4$. A 1-litre round-bottomed flask containing 100ml of the growth medium was autoclaved for 15min at approx. 100kPa (15lb/in$^2$), cooled and inoculated (1%, v/v). The inoculated medium was incubated at 28°C in a reciprocating shaker. Portions of the culture were transferred to fresh medium at weekly intervals.

Cells in amounts sufficient for experimental purposes were grown in continuous culture in the medium described above, except that the concentration of $NaNO_2$ was increased to 70mM and $K_2HPO_4$ was replaced by 5mM-$H_3PO_4$ and 10mM-$KHCO_3$. The latter two solutions were autoclaved separately, the $KHCO_3$ in a sealed thick-walled Pyrex tube. The bulk of the medium (18 litres) was autoclaved for 45min at approx. 100kPa (15lb/in$^2$). A 20-litre Pyrex aspirator served as the growth vessel (working volume 16 litres) and was painted black on the outside (to protect the culture from the deleterious effect of light). Both the medium (dilution rate 0.02h$^{-1}$) and air (flow rate 15 litres/min) entered the growth vessel at the top through a common nylon tube which extended almost to the bottom of the vessel. The effluent, composed of air and bacterial suspension, passed through a second nylon tube which extended from the surface of the culture to a collecting reservoir via the port at the base of the aspirator. The culture was maintained at 28°C by means of an i.r. lamp, controlled by a thermistor immersed in the culture. Since the dilution rate was low, adequate mixing resulted from the turbulence created by the air flow. The effluent was collected daily and kept in a cold-room at 4°C. After 3–4 days the cells were harvested.

*Preparation of ET particles.* Effluent from the chemostat at 4°C was passed through a Sharples centrifuge (25000rev./min with a flow rate of 15 litres/h) to harvest the cells. The cell paste (approx. 3g wet wt.) was washed twice by suspension in 40ml of a medium containing 300mM-sucrose, 1mM-GSH (reduced glutathione), 1mM-EDTA (sodium salt) and 10mM-Tricine [*N*-tris(hydroxymethyl)methylglycine] adjusted to pH7.8 with tetramethylammonium hydroxide, followed by centrifugation at 10000*g* for 15min. The cells were suspended after this in 20ml of medium with 35g of glass beads (100 mesh; BDH) in a special 60ml Pyrex bottle and the cells broken by a Braun MSK mechanical homogenizer (Shandon Southern Instruments, Camberley, Surrey GU16 5ET, U.K.) running for 1min at the higher setting. A jet of pressurized $CO_2$ served to maintain the cell suspension at 4–0°C while the homogenizer was running. The cell homogenate was decanted from the beads, which were washed with 3×20ml of the medium used for homogenization. The homogenate and washings were pooled and centrifuged at 15000*g* for 10min; the pellet was discarded and the supernatant centrifuged at 100000*g* for 60min. The pellet was resuspended in the same medium to give a final protein concentration of approx. 40mg/ml and the suspension was stored at 0°C. This method of preparation of ET particles is similar to that described by Kiesow (1964).

*Measurement of phosphorylation coupled to $NO_2^-$ oxidation.* The phosphorylation of ADP was followed continuously by monitoring the pH change consequent on the synthesis of ATP (Chance & Nishimura, 1967). This technique is particularly useful for measuring changes caused by phosphorylation when $NO_2^-$ is being oxidized, since the alkaline pH change due to ADP phosphorylation is at least three orders of magnitude greater than that due to the oxidation of $NO_2^-$ ($pK = 3.35$). The stoicheiometry of ADP phosphorylated to $H^+$ consumed was taken from Alberty (1969). Changes in pH were followed by using an E.I.L. electrode (type GMF 23/B micro glass electrode; E.I.L., Richmond, Surrey, U.K.) with a remote calomel electrode. The pH-meter (model 292, Pye Unicam, Cambridge, U.K.) was matched to a pen recorder (Rikadenki Kogyo; agents TEM Sales Ltd., Crawley, Surrey, U.K.) by a suitable resistance network. The sealed reaction vessel held 2ml of medium which was maintained at 25°C by means of water circulating through a glass jacket, and stirred by the rotation of a Teflon-coated metal bar moving in the field of an external rotating magnet.

*Measurement of $H^+$ translocation.* pH changes resulting from the uptake and release of $H^+$ by the ET particles were measured with a glass electrode in the apparatus described above. Attempts to follow $NH_4^+$ changes by using a '$K^+$-sensitive' glass electrode

(E.I.L.; GM 23B, BH 115 glass) were also made in this apparatus.

*Other assays.* Oxygen uptake was determined polarographically as described by Chappell (1964) in an all-glass vessel of 1.25 ml working volume.

Protein was assayed by the method of Gornall *et al.* (1949), except that 1.5% (w/v) deoxycholate was included to facilitate the dissolution of membrane protein.

## Materials

*Materials*

GSH and the sodium salts of ADP and NADH were supplied by Boehringer Corp. (London) Ltd., London W5 2TZ, U.K., and biotin, oligomycin and bovine serum albumin (fraction V) by Sigma (London) Chemical Co., Norbiton Station Yard, Kingston-upon-Thames, Surrey KT2 7BH, U.K. Dialysed iron and sodium tetraphenylboron were obtained from BDH Chemicals. Carbonyl cyanide phenylhydrazone was a gift from Dr. P. G. Heytler (DuPont de Nemours, Nemours, Wilmington, DE, U.S.A.). $Ba(NO_2)_2$ was purchased from K & K Laboratories (Plainview, NY, U.S.A.). Dianemycin and valinomycin were gifts from Dr. R. L. Hamill (Eli Lilly and Co., Indianapolis, IN, U.S.A.) and Dr. E. L. Patterson (Lederle Laboratories, Pearl River, NY, U.S.A.) respectively. Other organic and inorganic chemicals were of AnalaR grade where obtainable.

Solutions of choline chloride were adjusted to pH 11 with tetramethylammonium hydroxide and gently boiled for 4h to facilitate the removal of lower amines and $NH_3$; the solution was then neutralized with HCl. Solutions of tetramethylammonium nitrite were prepared as follows: a solution of 0.5M-tetramethylammonium sulphate containing 1mM-Tricine at pH 8.5 was mixed with an equal volume of a solution of 0.5M-$Ba(NO_2)_2$ containing the same quantity of the same buffer at the same pH. The resulting precipitate of $BaSO_4$ was removed by centrifugation and the supernatant tested for any remaining $Ba^{2+}$ by addition of a drop of tetramethylammonium sulphate. The solution was standardized by using the Griess-Ilosvay reagents (BDH Chemicals). The solution was stable for months at a storage temperature of 4°C; at low pH values it was unstable.

## Results

*Effect of carbonyl cyanide phenylhydrazone on oxidation of NADH and $NO_2^-$*

In Fig. 1(*a*) the effects of varying the concentration of carbonyl cyanide phenylhydrazone on the rates of oxidation of NADH and $NO_2^-$ by ET particles from *Nitrobacter* are shown. In the presence of maximal amounts of the uncoupler the rate of NADH oxidation was almost twice that observed in its absence and the rate of $NO_2^-$ oxidation was strongly inhibited. Comparison of the two curves shows that these two effects have an identical dependence on the concentration of uncoupler added.

*Effects of ADP and $P_i$ on the oxidation of NADH and $NO_2^-$*

The stimulation of NADH oxidation and the inhibition of $NO_2^-$ oxidation by different amounts of ADP in the presence of a fixed concentration of $P_i$ are shown in Fig. 1(*b*). Both systems had an identical concentration-dependence for the effect of ADP, but these effects were considerably less marked than with carbonyl cyanide phenylhydrazone (Fig. 1*a*). In the absence of $P_i$, ADP had no effect. When the effect of varying the $P_i$ concentration was determined in the presence of an excess of ADP a mirror-image relationship was shown again (Fig. 1*c*).

*pH-dependence of oxidation of NADH and $NO_2^-$*

The pH optimum for $NO_2^-$ oxidation was measured to be pH 7.7 in the absence of ADP and pH 8.1 in the presence of ADP together with $P_i$. This change in pH optimum occurs because inhibition of $NO_2$ oxidation by ADP is only detectable at pH values less than 8.1. At pH 7.0, $NO_2^-$ oxidation was inhibited by 60% on the addition of 0.8mM-ADP. The oxidation of NADH was stimulated by ADP between pH 6.8 and 8.1.

*Effect of oligomycin on oxidation of NADH and $NO_2^-$*

Oligomycin stimulated the oxidation of $NO_2^-$ in both the presence and the absence of ADP together with $P_i$. The oxidation of NADH under conditions under which phosphorylation was occurring was inhibited by oligomycin to a greater extent than would be expected if the ADP-induced stimulation alone were being reversed. In Fig. 2 the rates of oxidation of $NO_2^-$ and NADH in the absence of ADP together with $P_i$ at different concentrations of oligomycin are compared. NADH oxidation was inhibited over the exact range of oligomycin concentrations which stimulated $NO_2^-$ oxidation. The maximum percentage stimulation of $NO_2^-$ oxidation induced by oligomycin was observed at pH 8.4; stimulation of $NO_2^-$ oxidation by oligomycin was not detectable at pH 7.2.

In the presence of 200$\mu$M-carbonyl cyanide phenylhydrazone neither ADP together with $P_i$ nor oligomycin exerted an effect on the rate of oxidation of either $NO_2^-$ or NADH. After preincubation with oligomycin (10$\mu$g/mg of protein) the rates of oxidation of NADH or $NO_2^-$ were not affected by the addition of ADP together with $P_i$.

*Kinetic parameters of $NO_2^-$ oxidation*

Double-reciprocal plots (Lineweaver & Burk,

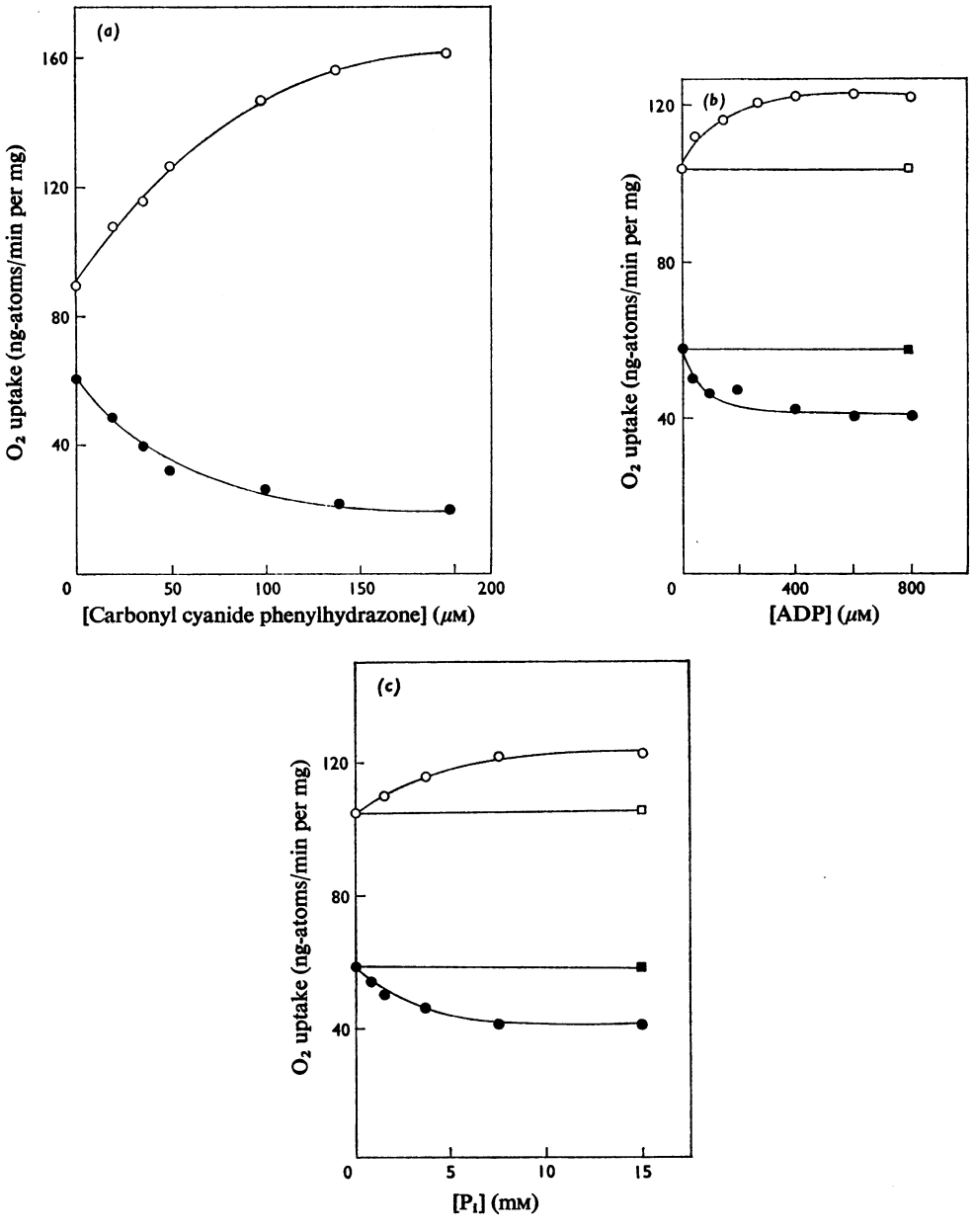


Fig. 1. *Effects of ADP, $P_i$ and carbonyl cyanide phenylhydrazone on the rates of oxidation of NADH and $NO_2^-$*

ET particles at a final concentration of 0.8mg of protein/ml were suspended in a medium containing 100mM-choline chloride, 5mM-$MgCl_2$, 10mM-tetramethylammonium phosphate and 10mM-Tricine; the final pH was 7.8 and the temperature 25°C. The reaction was started by the addition of either 1mM-NADH (○) or 3mM-$NaNO_2$ (●). (*a*) Rates of oxidation as a function of the concentration of carbonyl cyanide phenylhydrazone. (*b*) Rates of oxidation as a function of ADP concentration; 1mg of bovine serum albumin/ml of reaction medium was present initially. The rates of oxidation observed for NADH (□) and $NO_2^-$ (■) when $P_i$ was omitted are also shown. (*c*) Rates of oxidation as a function of $P_i$ concentration; 1mg of bovine plasma albumin/ml of reaction medium and 0.8mM-ADP were present initially. Also shown are the rates of oxidation of NADH (□) and of $NO_2^-$ (■) when ADP was omitted.

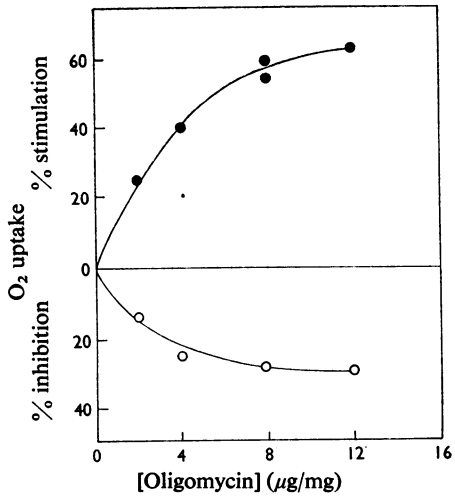


Fig. 2. *Effect of oligomycin on the rates of oxidation of NADH and $NO_2^-$ by ET particles*

The conditions were those described in the legend to Fig. 1(*a*), except that the final concentration of ET particles was 0.5mg of protein/ml. The particles were incubated for 3min with the oligomycin before the addition of either 1mM-NADH (○) or 2.4mM-$NaNO_2$ (●).

Table 1. *Apparent $K_m$ values for $NO_2^-$ and $V$ for $NO_2^-$ oxidation by ET particles under a variety of conditions*

The conditions were those given in the legend to Fig. 1(*a*), except that 1mg of bovine serum albumin/ml of reaction medium was included. Where indicated 25μM-carbonyl cyanide phenylhydrazone, 0.8mM-ADP or oligomycin (10μg/mg of protein, added 3min before $NaNO_2$) were added. Least-squares estimates of $V$ and $K_m$ for $NO_2^-$ were calculated by linear regression by using the Lineweaver & Burk (1934) equation. In the linear regression each point was weighted by the square of its velocity. The values given are least-squares estimates±S.E.M. Under each of the conditions, rates were measured at five concentrations of $NaNO_2$ (between 0.5 and 5mM).

| Conditions | $K_m$ for $NO_2^-$ (mM) | $V$ (ng-atoms of O/min per mg of protein) |
|---|---|---|
| Control | 0.89±0.08 | 75±3 |
| Carbonyl cyanide phenylhydrazone | 1.07±0.09 | 37±1 |
| ADP | 0.82±0.13 | 57±4 |
| Oligomycin | 1.08±0.08 | 124±4 |

1934) of the rate of $NO_2^-$ oxidation as a function of $NO_2^-$ concentration in the presence of ADP together with $P_i$, of carbonyl cyanide phenylhydrazone and of oligomycin revealed that the apparent $K_m$ for $NO_2^-$ did not vary significantly under these different conditions, but that $V$ did (Table 1).

*Respiratory control*

ET particles oxidizing NADH in a medium containing $P_i$ exhibited respiratory control (Chance, 1959) and ADP/O ratios approaching 2 were measured as a routine for this substrate (Fig. 3*a*). The respiratory-control ratios, although low, were reproducible from one preparation to the next and were constant within any one experiment, i.e. the ratio did not vary with the time of incubation. With $NO_2^-$ as substrate the addition of ADP in the presence of $P_i$ caused a decrease in the rate of oxygen consumption, followed by an increase when the added ADP had been consumed or partially consumed (see below); subsequent addition of ADP led to a repetition of this behaviour (Fig. 3*b*). Thus the respiratory-control ratio (defined as rate of oxygen consumption in the presence of ADP/rate of oxygen consumption when that ADP has been phosphorylated) was less than unity with $NO_2^-$ as substrate. It should be noted that the rate observed after the added ADP had attained a steady state of phosphorylation was always less than the rate observed before the addition of exogenous adenine nucleotide (Fig. 3*b*).

*Phosphorylation coupled to $NO_2^-$ oxidation*

P/O ratios for $NO_2^-$ oxidation were determined by simultaneously recording $O_2$ consumption polarographically and ADP phosphorylation by following the removal of $H^+$ consequent on ATP synthesis (see the Experimental section). Measurements were made under the conditions described in the legend to Fig. 1(*a*) except that the Tricine buffer was omitted and the concentration of ET particles was 3mg of protein/ml. The reaction was started by the addition of $NaNO_2$. No alkaline pH change was observed either in the absence of ADP or in the presence of oligomycin (10μg/ml of protein). The pH change which occurred aerobically in the presence of ADP ceased abruptly on exhaustion of oxygen; at this point there was a slow acidification of the medium, which represented a rate of ATP hydrolysis which was approx. 10% of the rate of ATP synthesis. Taking the oxygen content of the air-saturated medium to be 475ng-atoms/ml at 25°C (Chappell, 1964) and the ADP/$H^+$ stoicheiometry to be 0.86 under these conditions (Alberty, 1969), the calculated P/O ratio for $NO_2^-$ oxidation was in the range 0.2–0.35 for all the preparations tested. Values higher than 0.35 could not be obtained by varying the pH of the medium or the concentrations of ADP, $P_i$ or $NO_2^-$.

*Uptake of $H^+$ by electron-transport particles oxidizing $NO_2^-$*

The oxidation of $NO_2^-$ by ET particles was found to be accompanied by a disappearance of $H^+$ from the suspending medium (Fig. 4*a*). This $H^+$ reappeared in the suspending medium when the oxygen which had been added was consumed. The duration of the

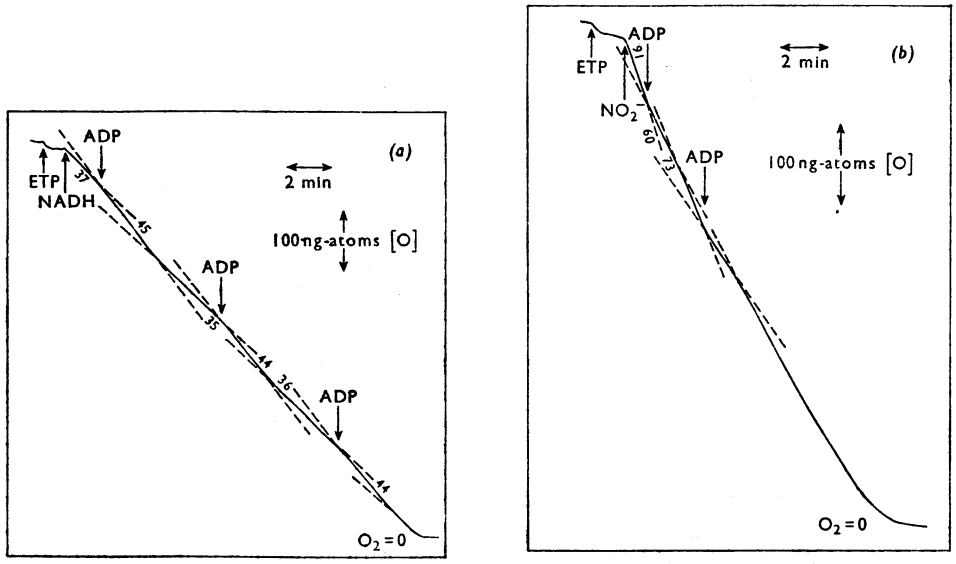


Fig. 3. *Respiratory control in ET particles*

The reaction medium was that described in the legend of Fig. 1(*a*), with the inclusion of 1 mg of bovine serum albumin/ml of reaction medium. The initial reaction volume was 1.25 ml. Subsequent additions were (*a*) 0.5 mg of ET-particle protein, 1.25 $\mu$mol of NADH and two lots of 150 nmol of ADP, and in (*b*) 2.2 mg of ET-particle protein, 1.25 $\mu$mol of $NaNO_2$ and two additions of 50 nmol of ADP. The numbers close to the traces are the rates of oxygen consumption expressed in ng-atoms of O/min. At the end of each experiment dissolved $O_2$ in the medium had been completely reduced ($O_2 = 0$). The broken lines indicate the constructions used to calculate ADP/O and respiratory control ratios for the oxidation of NADH (1.5 and 1.25 respectively) and of $NO_2^-$ (0.7 and 0.8 respectively).

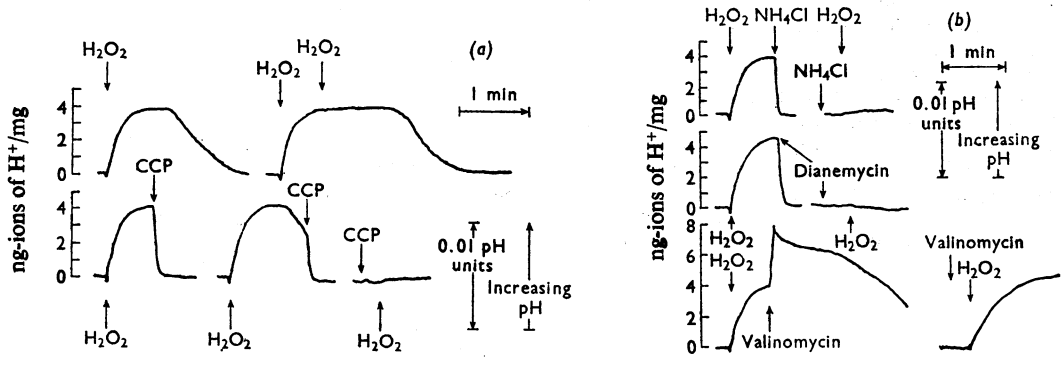


Fig. 4. *Uptake of $H^+$ associated with $NO_2^-$ oxidation by ET particles*

The pH changes were measured by using a glass pH electrode in the apparatus described under 'Methods'. ET particles (1.5 mg/ml final protein concentration) were suspended in 2 ml of a $N_2$-flushed medium containing 100 mM-choline chloride, 0.5 mM-Tricine and catalase (0.2 mg/ml) at pH 7.6 and at 25°C; 10 mM-KCl was included in those experiments where valinomycin (2.5 $\mu$g/ml) and dianemycin (2.5 $\mu$g/ml) were used. In each experiment tetramethylammonium nitrite (5 mM) was added, and 10 min allowed for equilibration. Other additions were $H_2O_2$ (5 $\mu$l of 0.2%, v/v), carbonyl cyanide phenylhydrazone (CCP; 300 nmol) and $NH_4Cl$ (10 $\mu$mol).

alkaline pH-shift was proportional to either the amount of $H_2O_2$ added in the presence of catalase to generate $O_2$ or the amount of $O_2$ added as air-saturated medium. Carbonyl cyanide phenylhydrazone (200 $\mu$M) abolished the pH changes if it was present initially or if added during the oxidation of $NO_2^-$ when a pH-gradient had been established, accelerated the release of $H^+$ (Fig. 4*a*).

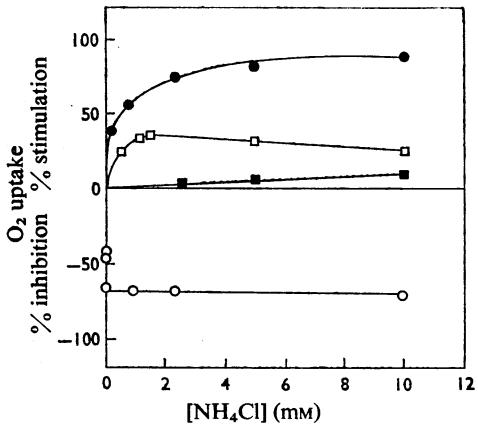


Fig. 5. *Effect of $NH_4Cl$ on the rates of oxidation of NADH and $NO_2^-$ measured in the presence and absence of valinomycin*

ET particles (0.9mg/ml final protein concentration) were suspended in 100mM-choline chloride and 20mM-Tricine at pH7.65 and at 25°C. Reactions were started with either 2.5mM-tetramethylammonium nitrite (○, □) or 2mM-NADH (●, ■) in the presence (○, ●) or in the absence (□, ■) of valinomycin (4$\mu$g/ml).

*Effect of $NH_4Cl$ and cyclohexylamine hydrochloride on $NO_2^-$ oxidation and $H^+$ uptake*

The addition of $NH_4Cl$ resulted in a stimulation of the rate of $NO_2^-$ oxidation by ET particles (Fig. 5). A maximal stimulation of 35% was observed at 2mM-$NH_4Cl$; higher concentrations of this compound caused less stimulation. Double-reciprocal plots (Lineweaver & Burk, 1934) of the rate of $NO_2^-$ oxidation as a function of $NO_2^-$ concentration reveal that $NH_4Cl$ alters the $V$ value, without substantially changing the $K_m$ for $NO_2^-$ (Table 2). When $H^+$ changes were followed, it was found that 5mM-$NH_4Cl$ abolished the pH change associated with the onset of $NO_2^-$ oxidation, and if added while $NO_2^-$ was being oxidized it collapsed the pH gradient that had been generated (Fig. 4*b*). Control experiments, in which both $O_2$ and $NH_4^+$ concentrations were monitored, showed that ET particles from *N. winogradskyi* were unable to oxidize $NH_4^+$ with either $O_2$ or $NO_2^-$ as electron acceptors.

In the presence of primary, secondary or tertiary amines $NO_2^-$ oxidation was stimulated in a manner similar to that observed in the presence of $NH_4Cl$. These stimulations were observed at pH values both above and below the pH optimum for $NO_2^-$ oxidation. This contrasts with the pH-dependence for the inhibition of $NO_2^-$ oxidation by ADP together with $P_i$, where effects of significance were only observed at pH values lower than those optimal for $NO_2^-$ oxidation. In general, although the amines stimulated $NO_2^-$ oxidation less than did $NH_4Cl$, they were more effective than $NH_4Cl$ in the stimulation of NADH oxidation (cf. Figs. 5 and 6). After stimulation by 5mM-$NH_4Cl$, $NO_2^-$ oxidation was not further stimulated by cyclohexylamine hydrochloride. Tetramethylammonium and tetrabutylammonium chlorides (quaternary amines) were without effect on the rates of oxidation of NADH or $NO_2^-$.

When valinomycin was included in the assay medium $NH_4Cl$ inhibited rather than stimulated $NO_2^-$ oxidation (Fig. 5), and the maximum degree of inhibition obtained (65–70%) was the same as that brought about by 200$\mu$M-carbonyl cyanide phenylhydrazone. In the presence of valinomycin, stimulation of NADH oxidation was observed, but the concentration of $NH_4Cl$ required to produce this stimulation was approximately 100 times higher than that required to inhibit $NO_2^-$ oxidation (Fig. 5); as low as 40$\mu$M-$NH_4Cl$ gave maximum inhibition of $NO_2^-$ oxidation in the presence of valinomycin. When 200$\mu$M-carbonyl cyanide phenylhydrazone was included in the assay medium, the rates of oxidation of NADH and $NO_2^-$ were independent of the presence of 5mM-cyclohexylamine hydrochloride and of 5mM-$NH_4Cl$, in both the presence and the absence of valinomycin.

At pH7.2 stimulation of $NO_2^-$ oxidation by oligomycin does not occur (see above). However, pretreatment of ET particles with oligomycin at this pH value resulted in a greater stimulation of $NO_2^-$ oxidation by $NH_4Cl$ or cyclohexylamine hydrochloride than was observed in untreated particles (a 70% stimulation as opposed to a 14% stimulation). This potentiation by oligomycin was also measurable at higher pH values but was less pronounced.

At pH7.7, ADP together with $P_i$ caused a 25% inhibition of $NO_2^-$ oxidation. In the presence of 5mM-

Table 2. *Influence of $NH_4Cl$ on the apparent $K_m$ value for $NO_2^-$ and $V$ for $NO_2^-$ oxidation by ET particles*

ET particles (0.75mg/ml final protein concentration) were suspended in 100mM-choline chloride, 4mM-$MgCl_2$, 4mM-tetramethylammonium phosphate and 1mg of bovine serum albumin/ml at pH7.7 and at 25°C. Rates were measured in the presence and absence of 2.5mM-$NH_4Cl$ after the reaction had been started with tetramethylammonium nitrite. Least-squares estimates were calculated as described in Table 1. The values given are least-squares estimates ±S.E.M. Under both conditions rates were measured at five concentrations of $NaNO_2$ (between 0.5 and 5mM).

| Conditions | $K_m$ for $NO_2^-$ (mM) | $V$ (ng-atoms of O/min per mg of protein) |
|---|---|---|
| Control | 0.82±0.05 | 74±2 |
| $NH_4Cl$ | 0.89±0.07 | 96±3 |

cyclohexylamine hydrochloride the inhibition caused by ADP together with $P_i$ was decreased to 13%.

*Effects of dianemycin and valinomycin on $NO_2^-$ oxidation and on the associated $H^+$ uptake*

When $NO_2^-$ oxidation was assayed in the presence of dianemycin and KCl, a 15% stimulation of $NO_2^-$ oxidation was measured, and the inhibition of $NO_2^-$ oxidation induced by ADP together with $P_i$ was abolished. After stimulation by dianemycin, no further stimulation could be induced by $NH_4Cl$ or by amines. The uptake of $H^+$ in a medium containing KCl was prevented by the inclusion of dianemycin, and the addition of this antibiotic during $NO_2^-$ oxidation resulted in the rapid release of $H^+$ (Fig. 4*b*).

In the presence of valinomycin together with KCl, a 30% inhibition of $NO_2^-$ oxidation was observed, and this inhibited rate could be further decreased by the addition of ADP or of 200 $\mu$M-carbonyl cyanide phenylhydrazone. Although the rate of $H^+$ uptake occurring at the onset of $NO_2^-$ oxidation was decreased by valinomycin together with KCl, the extent of $H^+$ uptake was slightly increased. A large increase in the extent of $H^+$ uptake occurred when valinomycin was added during the course of $NO_2^-$ oxidation, and this change took place more rapidly than even the initial $H^+$ uptake observed on adding $O_2$ in the absence of valinomycin (Fig. 4*b*). In the presence of valinomycin together with KCl, dianemycin acted as an inhibitor of $NO_2^-$ oxidation, and the maximum

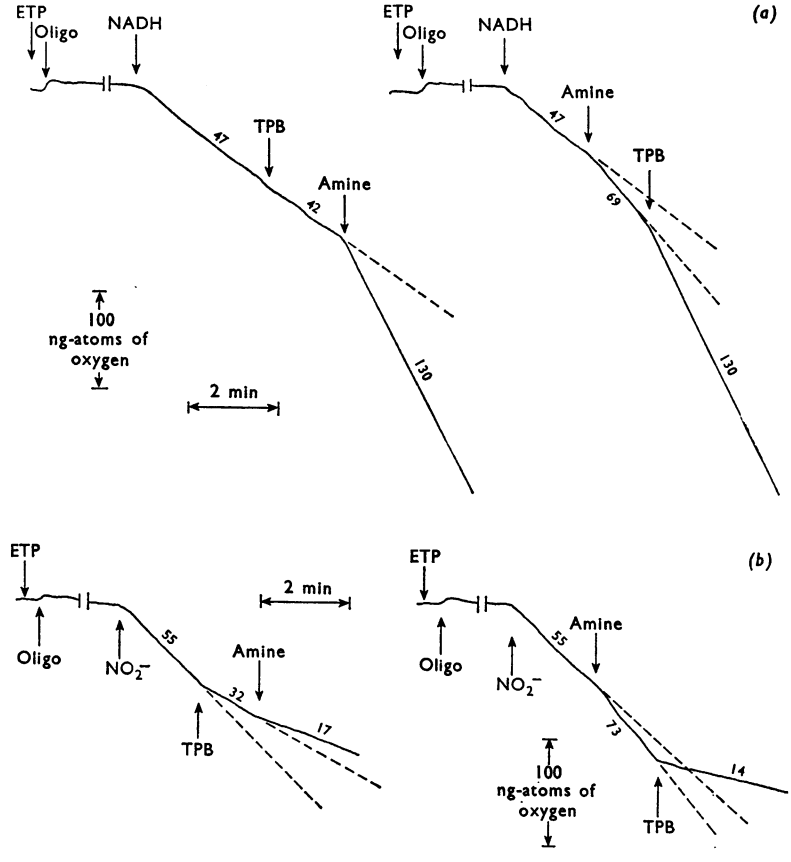


Fig. 6. *Oxygen-electrode traces showing the influence of tetraphenylboron and cyclohexylamine on the rates of oxidation of NADH and $NO_2^-$*

ET particles (ETP, 0.7 mg of protein) were added to 1.25 ml of the medium described in the legend of Fig. 5, except that 5 mM-$MgCl_2$ was included. The pH was 7.8 and the additions were 10 $\mu$g of oligomycin (Oligo), 2.5 $\mu$mol of NADH (*a*) or 1.5 $\mu$mol of $NaNO_2$ (*b*), 6.25 $\mu$mol of cyclohexylamine hydrochloride (Amine) and 25 nmol of sodium tetraphenylboron (TPB). The bars which interrupt the recordings indicate where the chart drive was turned off for a 3 min period. Numbers close to the trace are rates of oxygen uptake expressed in ng-atoms of oxygen/min per mg. Broken lines indicate the course of the reaction in control experiments.

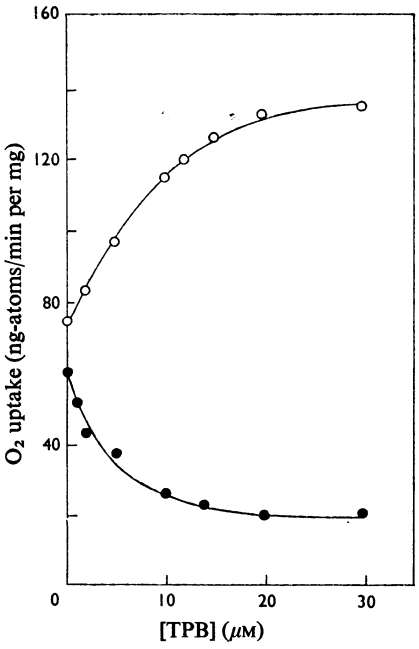


Fig. 7. *Rates of oxidation of NADH and $NO_2^-$ in the presence of cyclohexylamine plotted as a function of tetraphenylboron concentration*

Reactions were conducted in 1.25ml of the medium described in the legend of Fig. 6 except that 5mM-cyclohexylamine hydrochloride was included. The pH was 7.8 and the temperature 25°C. Reactions were started by the addition of either 2.5μmol of NADH (○) or 1.5μmol of $NaNO_2$ (●).

inhibition which could be produced by the combined action of these two antibiotics (65–70%) was the same as that measured in the presence of 200μM-carbonyl cyanide phenylhydrazone. In the presence of valinomycin, $H^+$ uptake was completely prevented by either dianemycin or carbonyl cyanide phenylhydrazone.

*Effect of sodium tetraphenylboron on the rates of NADH and $NO_2^-$ oxidation*

Sodium tetraphenylboron acted as an inhibitor of $NO_2^-$ oxidation (Fig. 6*b*) without significantly altering the rate of NADH oxidation (Fig. 6*a*). However, in the presence of $NH_4Cl$ (not shown) or cyclohexylamine hydrochloride, NADH oxidation was stimulated by tetraphenylboron. In Fig. 7 the rates of oxidation of NADH and $NO_2^-$, both measured in the presence of 5mM-cyclohexylamine hydrochloride, are plotted as a function of tetraphenylboron concentration. Under these conditions the rates of NADH and $NO_2^-$ oxidation measured at saturating concentrations of tetraphenylboron were the same as those measured in ET particles that had been fully uncoupled with 200μM-carbonyl cyanide phenylhydrazone.

## Discussion

The experiments described here lend strong support to the contention that although $NO_2^-$ is oxidized by a thermodynamically spontaneous process ($\Delta G_0' = -74.8$kJ/mol; $-17.8$kcal/mol) its rate is accelerated when the ET particle is 'energized'. Kiesow (1964) has attempted to explain this situation by postulating the existence of a number of oxidation–reduction steps, at least one of which depends on a supply of energy derived from the hydrolysis of ATP. Because $NO_2^-$ oxidation is known to lead to a net synthesis of ATP (Aleem & Nason, 1960), Kiesow (1964) was further led to postulate that the number of partial oxidation–reduction reactions coupled to ATP synthesis exceeded those utilizing ATP. Mechanistically it was considered that structurally bound adenine nucleotides mediated between ATP-producing and ATP-utilizing oxidation–reduction steps. If this were the case an inhibition of $NO_2^-$ oxidation by oligomycin would be predicted; precisely the opposite is shown to be the case in the present paper. It seems clear therefore that the enhanced rate of $NO_2^-$ oxidation observed in the absence of energy-dissipating processes relates to some state before the involvement of adenine nucleotides.

An explanation involving an effect of uncouplers and of ADP together with $P_i$ on the accumulation of $NO_2^-$ by the vesicles is rendered extremely unlikely by the finding that these agents (and oligomycin) affect the $V$ values for $NO_2^-$ oxidation, but have a small and probably not significant effect on the apparent $K_m$ for $NO_2^-$; if these agents had affected a process of $NO_2^-$ accumulation, then it would be predicted that the $K_m$ values would be markedly changed, which they are not (Table 1).

It has been claimed by Fischer & Laudelout (1965) that both the rate of $NO_2^-$ oxidation and of phosphorylation are increased by increasing the concentration of $P_i$ in the suspending medium; these findings are contrary to those reported in the present paper. However, the increases reported by Fischer & Laudelout (1965) were small, correction factors for non-specific $O_2$ uptake and non-specific incorporation of $[^{32}P]P_i$ were required, and the results of experiments using less than 2mM-$P_i$ were not reported.

In membrane suspensions capable of oxidative or photosynthetic phosphorylation, reversible pH changes characteristically accompany coupled oxidation–reduction reactions. $H^+$ uptake accompanying the oxidation of inorganic ions by ET particles from chemolithotrophic bacteria has not previously been reported. In the chemiosmotic hypothesis (Mitchell, 1966, 1968) it is considered that in the process of con-

serving energy a transmembrane gradient of $H^+$ activity is an obligatory intermediate, and that uncouplers prevent energy conservation by rendering membranes permeable to $H^+$. The concept of a $H^+$-activity gradient can be used only if ET particles can be shown to contain two phases separated by a membrane across which such a gradient can be maintained. As estimated by Tsien & Laudelout (1968), the smallest ET particle from *N. winogradskyi* capable of oxidizing $NO_2^-$ has a diameter of 22 nm, and these investigators concluded that membrane integrity is necessary for a conservation of activity. Evidence has been presented above (and see Kiesow, 1964) that $NO_2^-$ oxidation can only proceed rapidly when the ET particle is 'energized'. When these morphological and biochemical findings are considered together, it seems reasonable that it is the conservation of energy which depends on the integrity of the membrane. In this respect a requirement for chemiosmotic coupling appears to be met.

According to the chemiosmotic hypothesis (Mitchell, 1966, 1968) the protonmotive force can have two components, a pH gradient ($\Delta$pH) and an electrical potential difference ($\Delta\psi$). In conditions unfavourable for the formation of $\Delta$pH, the protonmotive force could, in a limiting case, equal $\Delta\psi$. It has been proposed that dianemycin, $NH_4^+$ ions or certain amines bring about such conditions, as they catalyse the collapse of $\Delta$pH by electroneutral mechanisms (see Walker & Crofts, 1970; Henderson, 1971). That these mechanisms provide an adequate description of the events occurring in suspensions of ET particles from *N. winogradskyi* is suggested by the similarity of the effects induced by $NH_4Cl$, by cyclohexylamine hydrochloride and by dianemycin. They each abolished $H^+$ uptake and stimulated $NO_2^-$ oxidation, but had little effect on the rate of NADH oxidation. Further, once stimulated by amines to the full extent, $NO_2^-$ oxidation was not further stimulated by dianemycin.

In the other limiting case the protonmotive force would have no $\Delta\psi$ component and would be equal to $\Delta$pH. Since the tetraphenylboron anion can pass freely through membranes (Liberman & Skulachev, 1970) and since valinomycin renders membranes permeable to $K^+$ (Mueller & Rudin, 1967) these compounds minimize the contribution of $\Delta\psi$ to the protonmotive force. It is proposed therefore that migration of either $K^+$ or the tetraphenylboron anion across the membranes of the ET particle resulted in the collapse of $\Delta\psi$, and that $\Delta$pH increased to maintain the protonmotive force (see Fig. 4*b*). Under these conditions the rate of NADH oxidation was not altered, but the rate of $NO_2^-$ oxidation decreased.

The stimulation of NADH oxidation then was only brought about by those agents or combinations of agents which, as suggested by model membrane studies, produce conditions under which both $\Delta\psi$ and $\Delta$pH are decreased, i.e. when a protonmotive force cannot be maintained. The influence of amines, ionophorous antibiotics and of tetraphenylboron on the rate of $NO_2^-$ oxidation indicate that this oxidation responds not to the magnitude of the protonmotive force but more to its quality. Either stimulation of $NO_2$ oxidation by $\Delta\psi$ or inhibition by $\Delta$pH could explain the results, but since carbonyl cyanide phenylhydrazone is an inhibitor of $NO_2^-$ oxidation and dissipates both $\Delta\psi$ and $\Delta$pH it is proposed that the rate of $NO_2^-$ oxidation is directly related to $\Delta\psi$ rather than inversely related to $\Delta$pH. Dianemycin and amines stimulate $NO_2^-$ oxidation, because only when the formation of $\Delta$pH is prevented can all the energy available from $NO_2^-$ oxidation be conserved as $\Delta\psi$.

The stimulation of $NO_2^-$ oxidation by amines was greater in the presence of oligomycin and less in the presence of ADP together with $P_i$. Since this stimulation by amines has been attributed to the collapse of $\Delta$pH it can be concluded that during the phosphorylation of ADP the $\Delta$pH component of the protonmotive force was decreased. This conclusion supports the chemiosmotic approach to coupling which requires that energy for the phosphorylation of ADP be derived directly from the protonmotive force.

According to the chemical hypothesis, classical uncoupling agents (such as 2,4-dinitrophenol) and the amines which uncouple chloroplasts (Krogmann *et al.*, 1959; Good, 1960) both catalyse the hydrolysis of chemical intermediates. However, the former class of compound inhibits $NO_2^-$ oxidation, whereas the latter class stimulates. This difference in response is compatible with the chemiosmotic hypothesis, which resolves the intermediary form of conserved energy into two components, $\Delta\psi$ and $\Delta$pH. It is difficult to see how the approach to uncoupler action used in the chemical hypothesis could provide a simple but adequate explanation of the results here described.

The biological importance of energy-dependent electron transport in the metabolism of *Nitrobacter* has been stated in the introduction. The situation appears to be that the oxidation of nitrite is rapid only under those conditions that favour the reduction of $NAD^+$ by reversed electron transport at the expense of $NO_2^-$ oxidation. A similar situation may exist in the chemolithotrophs *Nitrosomonas europaea* and in *Thiobacillus ferro-oxidans*, since the oxidations of respectively $NH_4^+$ and $Fe^{2+}$ are inhibited by 2,4-dinitrophenol (Laudelout *et al.*, 1968; Beck & Shafia, 1964).

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