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# OXIDATIVE PHOSPHORYLATION IN *MICROCOCCUS DENITRIFICANS*

## II. THE PROPERTIES OF PYRIDINE NUCLEOTIDE TRANSHYDROGENASE

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SUMMARY

A pyridine nucleotide transhydrogenase activity, supported by ATP or by succinate oxidation, was demonstrated in phosphorylating membrane fragments from *Micrococcus denitrificans*. The ATP-supported reaction was inhibited by various energy-transfer inhibitors and uncouplers or by treatment with high concentrations of LiCl. $P_i$ and arsenate showed a stimulatory effect on the ATP-supported activity; half-maximal stimulation was attained by about 80 $\mu$M phosphate.

The transhydrogenase reaction dependent on succinate oxidation was not appreciably inhibited by energy-transfer inhibitors, although oleate and pentachlorophenol were almost equally effective in both reactions. $P_i$ did not stimulate the succinate-supported activity.

From the effects of thyroxine and its derivatives on the energy-dependent and -independent reductions of $NAD^+$ by NADPH, the involvement of the same transhydrogenase enzyme in both reactions was suggested.

These and other results indicated that the energy-transfer system of *M. denitrificans* was very similar to, though not identical with, that of mammalian mitochondria.

## INTRODUCTION

Studies of energy-linked processes in mitochondria have provided valuable information concerning the mechanism of oxidative phosphorylation[1]. Especially the results obtained with energy-linked transhydrogenase first noticed by KLINGENBERG AND SLENCZKA[2], have been of great use in the elucidation of mitochondrial energy-transfer pathways[3]. Although energy-linked processes have also been demonstrated in certain bacterial preparations[4–6], studies on these have been limited because of technical difficulties. The phosphorylating membrane fragments of *Micrococcus denitrificans* described in our previous paper[7] are suitable for studies on energy-linked processes in bacteria, in view of their high P:O ratios among the bacterial systems, low content of bypass enzymes, and availability of well characterized inhibitors of electron- and energy-transfer pathways in this system. As already reported

Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; NOQNO, 2-*n*-nonylhydroxyquinoline-*N*-oxide; TMPD, tetramethyl-*p*-phenylenediamine.

preliminarily[8] it was in fact possible to detect the energy-linked reduction of $NADP^+$ by NADH in these membrane fragments.

This paper reports that the reduction of $NADP^+$ by NADH in the membrane fragments of *M. denitrificans*, as in the case of submitochondrial particles, could be supported either by exogenous ATP or by succinate oxidation. The effects of oligomycin and other uncouplers and energy-transfer inhibitors were generally similar to those on the mitochondrial transhydrogenase reaction. An interesting finding was that $P_i$ and arsenate stimulated the ATP-supported reaction without affecting the process driven by succinate oxidation. Based on these results, the energy-transfer system of this organism is discussed.

## MATERIALS AND METHODS

### *Materials*

Phosphorylating membrane fragments of *M. denitrificans* were prepared as described previously[7]. Thyroxine and its derivatives were generous gifts from Dr. M. SUZUKI of Gunma University. Isocitrate dehydrogenase was purified from pig heart up to Step 3 of the procedure described by SIEBERT *et al.*[9]. Glutathione reductase was prepared by the method of VENNESLAND[10] from wheat germ which was kindly supplied by Nissin Seifun, Ltd. Other chemicals and biochemicals used were the same as described previously[7].

### *Measurements of transhydrogenase reactions*

Reduction of $NADP^+$ by NADH accelerated by the addition of ATP was measured essentially as described by DANIELSON AND ERNSTER[11] by following the reduction of $NADP^+$ under the conditions permitting the maintenance of constant level of NADH with ethanol and alcohol dehydrogenase. The reaction mixture contained, in a cuvette of 1-cm light path: Tris–HCl buffer (pH 8.2), 100 $\mu$moles; $MgCl_2$, 20 $\mu$moles; ethanol, 40 $\mu$moles; KCN, 2.5 $\mu$moles; potassium phosphate buffer (pH 8.2), 2.5 $\mu$moles; crystalline yeast alcohol dehydrogenase, 300 $\mu$g; NADH, 0.3 $\mu$mole; $NADP^+$, 0.4 $\mu$mole; washed membrane fragments, 0.5–1.5 mg protein; and water to a final volume of 2.0 ml. After establishment of the steady-state rate for energy-independent pyridine nucleotide transhydrogenase, the reaction was started by the addition of 2.5 $\mu$moles of ATP and the reaction was followed at 340 m$\mu$ in a Cary Model 14 recording spectrophotometer at 20–25°. For the assay of succinate-supported reaction, the reaction medium was modified as follows: KCN was replaced by 50 $\mu$g of rotenone and the reaction was started by the addition of 25 $\mu$moles of potassium succinate (pH 7.4), instead of ATP. Pyridine nucleotide transhydrogenase reaction in the direction from NADPH to $NAD^+$ was assayed by the method of KAPLAN[12], using isocitrate and isocitrate dehydrogenase as an NADPH-generating system.

### *Measurements of ATP hydrolysis and pyrophosphatase activity*

ATP hydrolysis under the conditions for the assay of ATP-linked pyridine nucleotide transhydrogenase was measured after termination of the reaction by the addition of 1.0 ml of 10% trichloroacetic acid. Inorganic pyrophosphatase activity was measured in the same way except that ATP was replaced by 2.5 $\mu$moles of inor-

ganic pyrophosphate (pH 8.2). When arsenate was included in the reaction mixture, phosphate determination was conducted according to MARTIN AND DOTY[13]. Zero time control with arsenate was always taken and subtracted from the experimental value.

*Other determinations*

Adenylate kinase activity was assayed as described previously[14]. Protein was determined by the biuret method modified for mitochondria[15].

## RESULTS

*Demonstration of and requirements for ATP-linked transhydrogenase activity*

As shown in Table I, the slow reduction of $NADP^+$ catalyzed by the membrane fragments of *M. denitrificans* was stimulated 4-fold by ATP. ADP was almost as effective as ATP, probably due to the presence of adenylate kinase in the preparation (18 mμmoles ATP formed per min per mg protein). Actually, removal of most of the adenylate kinase by repeated washing with a $Mg^{2+}$-free Tris–sucrose medium produced a preparation which responded only to ATP, though this treatment caused some inactivation. Neither 2'-, 3'- nor 5'-adenylic acids were effective as the energy source. Although the energy-independent transhydrogenase reaction was partially inhibited by added $Mg^{2+}$ (Table I), this divalent cation seemed to be required for the ATP-linked activity. By using the membrane fragments which had been washed almost free from endogenous $Mg^{2+}$, it was in fact possible to demonstrate clearly the $Mg^{2+}$ requirement; maximal activity was attained at 2 mM $Mg^{2+}$ in the presence of 1.25 mM ATP. Table I indicates further that the ATP-linked system required $P_i$ for maximal activity. That NADPH was actually formed by this reaction was confirmed by a rapid drop in absorbance at 340 mμ on addition of oxidized glutathione and NADPH-specific glutathione reductase.

The apparent $K_m$ values of the ATP-linked transhydrogenase system for NADH, $NADP^+$ and ATP were determined to be 7.3 μM, 200 μM and 60 μM, respec-

TABLE I

REQUIREMENTS FOR ATP-SUPPORTED PYRIDINE NUCLEOTIDE TRANSHYDROGENASE

The complete reaction medium and reaction conditions were as described in MATERIALS AND METHODS except that 2.91 mg protein of the membrane fragments were used. Indicated additions and omissions were made when necessary. Even when no $Mg^{2+}$ was added, the system contained 0.5 mM $Mg^{2+}$ because of the $Mg^{2+}$ in the suspension medium of the membrane fragments. Adenylate kinase activity of the preparation was 18.5 mμmoles ATP formed per min per mg protein.

| *Reaction medium* | *mμmoles NADPH formed per min per mg protein* |
|---|---|
| Complete | 14.2 |
| *minus* ATP | 3.6 |
| *minus* ATP *minus* $Mg^{2+}$ | 6.4 |
| *minus* ATP *plus* ADP | 11.4 |
| *minus* ATP *plus* AMP | 3.7 |
| *minus* $P_i$ | 9.2 |
| *minus* NADH | 0.3 |
| *minus* $NADP^+$ | 0.0 |

tively. These values were low enough for function of the system under physiological conditions.

*Stimulation of ATP-linked transhydrogenase by $P_i$ and arsenate*

The fact that preincubation of the membrane fragments with ATP stimulated the ATP-linked transhydrogenase reaction considerably when $P_i$ was omitted from the system, together with the requirement of $P_i$ for full activity (Table I), suggested the possibility that $P_i$ formed from ATP hydrolysis during preincubation stimulated the reaction. This possibility was confirmed by the experiments illustrated in Fig. 1. As may be seen, the addition of $P_i$ at a concentration as low as 15 $\mu$M to the non-preincubated system caused a clear stimulation of the $NADP^+$ reduction. When 1.5 mM $P_i$ was used, the reaction rate was even higher than that of the system pre-incubated with ATP. ADP, the other product of ATP hydrolysis, was not effective as an activator. It seemed that no preincubation was needed for the effect of $P_i$. The phosphate concentration giving half-maximal stimulation was estimated to be about 80 $\mu$M. Arsenate and pyrophosphate at similar concentrations could replace phosphate as stimulators. The effect of pyrophosphate could be explained by the presence of powerful pyrophosphatase (125 m$\mu$moles $P_i$ produced per min per mg protein) in the membrane fragments. Other anions tested such as $Cl^-$ and $SO_4^{2-}$ showed no stimulation, and the activation by phosphate was not dependent on partner cations.

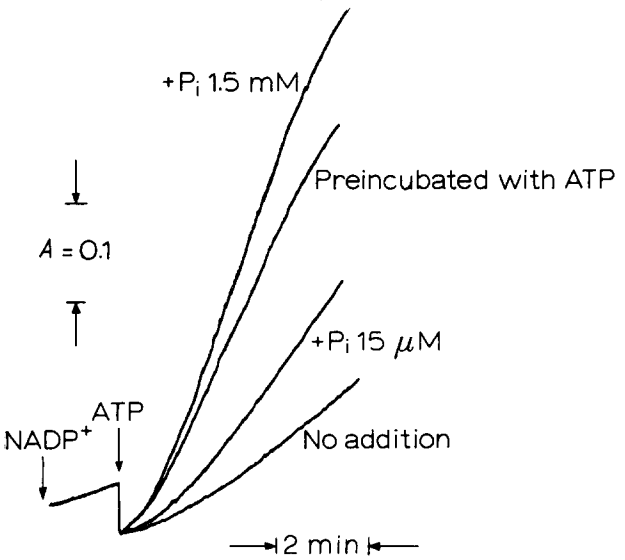


Fig. 1. Effect of $P_i$ on ATP-supported reduction of $NADP^+$ by NADH. The conditions employed were as described in MATERIALS AND METHODS except that 2.57 mg protein of the membrane fragments were used and the indicated amount of $P_i$ was added.

*ATP as energy source for transhydrogenase reaction*

To demonstrate the energetic role of ATP in stimulating the bacterial transhydrogenase activity, it was necessary to exclude the possibilities of direct phosphorylation of NADH to NADPH by ATP and of a catalytic role of ATP in stimulating the reaction. The former possibility could be ruled out, because the amount of NADPH formed exceeded the total amount of NADH *plus* $NAD^+$ present in the system,

especially when low concentrations of NADH were used. For example, more than 0.09 μmole of $NADP^+$ was reduced in the ATP-dependent reaction in a system containing 0.06 μmole of NADH *plus* $NAD^+$.

On the other hand, two types of experiments were designed to disprove the catalytic function of ATP. In the first type, the reduction of $NADP^+$ was followed in the presence of limited amounts of ATP and the total NADPH formation, corrected for the contribution due to the energy-independent reaction, was correlated with the amount of ATP added. Since the preparation contained adenylate kinase, it was assumed that the two high-energy bonds in the ATP molecule were both utilized for driving the $NADP^+$ reduction. In Table II are recorded the molar ratios of two times the amount of ATP to the amount of NADPH formed. These values were higher than 1, the value expected from the mole to mole stoichiometry between the high-energy bond utilized and the NADPH formed. It should, however, be pointed out that ATP hydrolysis unrelated to the energy-linked process would tend to increase the ratio.

The second approach was to correlate the NADPH formation and the stimulation of ATP hydrolysis caused by the energy-linked transhydrogenase reaction. The results obtained are shown in Table III, in which the amounts of $P_i$ liberated during

TABLE II

$NADP^+$ REDUCTION BY NADH IN THE PRESENCE OF LIMITED AMOUNTS OF ATP

The conditions employed were as described in MATERIALS AND METHODS except that 1.33 mg protein of the membrane fragments were used and the amount of ATP used was varied as indicated. ATPase activity of the same membrane preparation was 0.231 μmole $P_i$ produced per 10 min under similar conditions. NADPH formation reached a plateau after 8–13 min of ATP addition.

| *ATP added* (μ*M*) | *NADPH formed* (μ*M*) | $\frac{2 \times ATP\ added}{NADPH\ formed}$ |
|---|---|---|
| 20 | 31.0 | 1.28 |
| 40 | 46.5 | 1.73 |
| 60 | 68.4 | 1.75 |

TABLE III

ACTIVATION OF ATP HYDROLYSIS DURING ATP-SUPPORTED REDUCTION OF $NADP^+$ BY NADH

The conditions employed were as described in MATERIALS AND METHODS except that 1.20 mg protein of the membrane fragments were used and additions were made as indicated. Determination of phosphate was conducted according to MARTIN AND DOTY[13]. Amounts of $P_i$ shown in Columns B and C were the value obtained by subtracting time zero value from that of complete system. As, arsenate.

| *Energy source* | *Activator* | *$NADP^+$ reduced* (μ*mole*) (*A*) | *$P_i$ liberated in complete system* (μ*moles*) (*B*) | *$P_i$ liberated in control** (μ*moles*) (*C*) | $\frac{B - C}{A}$ |
|---|---|---|---|---|---|
| ATP | As (1 mM) | 0.262 | 1.03 | 0.87 | 0.61 |
| ADP | As (1 mM) | 0.149 | 0.25 | 0.16 | 0.60 |
| ATP | $P_i$ (0.25 mM) | 0.296 | 1.07 | 0.92 | 0.50 |
| ATP | As (0.5 mM) | 0.425 | 1.78 | 1.50 | 0.66 |
| ADP | As (0.5 mM) | 0.228 | 0.76 | 0.28 | 2.10 |

* In most of experiments the tubes without NADH and without $NADP^+$ (or in some cases both without $NADP^+$ and NADH) served as controls. Average of these control values was cited in Column C.

the transhydrogenase reaction are recorded in Column B and those determined in control tubes lacking either $NADP^+$ or NADH or both are listed in Column C. The difference between the two values (B–C) could therefore be taken as representing the liberation of $P_i$ coupled to the action of transhydrogenase. The last column shows the molar ratios of (B—C) to the amount of $NADP^+$ reduced during the same incubation period (Column A). These ratios, corresponding to the moles of high-energy bond utilized per mole of $NADP^+$ reduced, were 0.5–0.7 in most of the experiments. In view of technical difficulties and the possibility that ATP hydrolysis in control tubes might be partly due to the action of an energy-conserving ATPase, the true ratio was thought to be higher than the values obtained, probably approaching 1.

Thus it may be concluded that ATP acted as an energy source in the reduction of $NADP^+$ by NADH in the bacterial preparation and that 1 mole of high-energy bond of ATP was probably required to drive the reduction of 1 mole of $NADP^+$.

*Effects of uncouplers and energy-transfer inhibitors on ATP-linked transhydrogenase*

The ATP-linked transhydrogenase reaction was sensitive to such inhibitors and uncouplers of mitochondrial energy transfer as oligomycin, tributyltin chloride, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), pentachlorophenol and oleate (Table IV). The concentrations of these inhibitors required for complete inhibition of the bacterial transhydrogenase activity were, however, considerably higher than those needed to inhibit oxidative phosphorylation and ATP-linked pyridine nucleotide transhydrogenase in mitochondria[1,3]. As reported previously[7], these uncouplers and energy-transfer inhibitors at similar concentrations also inhibited oxidative phosphorylation by the bacterial membrane fragments. The bacterial ATP-linked transhydrogenase activity was further sensitive to 2-*n*-nonylhydroxyquinoline-*N*-oxide (NOQNO) and treatment by high concentrations of LiCl, and this inhibition paralleled the inhibition of the ATPase activity of the same preparation[7].

These results suggested that ATP provided energy to the transhydrogenase system *via* a partial reversal of oxidative phosphorylation.

TABLE IV

DIFFERENTIAL EFFECTS OF INHIBITORS, $P_i$ AND LiCl TREATMENT ON ATP-SUPPORTED AND SUCCINATE-SUPPORTED PYRIDINE NUCLEOTIDE TRANSHYDROGENASE REACTIONS

The conditions employed were as described in MATERIALS AND METHODS except that inhibitors were added as indicated. $P_i$ was omitted when indicated. LiCl-treated membrane fragments were prepared as described previously[7].

| *Inhibitors added* | *Concentration* ($\mu M$) | % *Inhibition* | |
|---|---|---|---|
| | | *ATP-supported* | *Succinate-supported* |
| Oligomycin | 13 | 63 | 10 |
| Oligomycin | 63 | 100 | 23 |
| Tributyltin chloride | 0.88 | 63 | 11 |
| Tributyltin chloride | 1.75 | 85 | 19 |
| CCCP | 2.5 | 99 | 34 |
| Pentachlorophenol | 50 | 67 | 46 |
| Pentachlorophenol | 100 | 92 | 58 |
| Oleate | 355 | 61 | 45 |
| Oleate | 710 | 93 | 85 |
| $P_i$ omitted | | 51 | 6 |
| 1.0 M LiCl treated, then washed | | 95 | 60 |

*Transhydrogenase reaction supported by succinate oxidation*

It has been shown that in mitochondria energy required for enhanced reduction of $NADP^+$ by NADH can be supplied not only by ATP but also by the oxidation of succinate or ascorbate (mediated by tetramethyl-*p*-phenylenediamine (TMPD)) without the participation of ATP (ref. 3). This finding has been generally taken as an indication of the fact that a non-phosphorylated intermediate of oxidative phosphorylation is the direct source of energy for the transhydrogenase reaction[3]. As may be seen from Table V, the addition of succinate to the rotenone-treated membrane fragments resulted in a rapid reduction of $NADP^+$ by NADH; fumarate and L-malate could not drive the reaction, ruling out the possibility of direct reduction of $NADP^+$ by the products of succinate oxidation. The bacterial reaction could not be

TABLE V

REQUIREMENTS FOR SUCCINATE-SUPPORTED REDUCTION OF $NADP^+$ BY NADH

The conditions employed were as described in MATERIALS AND METHODS except that 1.84 mg protein of the membrane fragments were used and additions and omissions were made as indicated.

| *Reaction medium* | *NADPH formed (mμmoles/min per mg protein)* |
|---|---|
| Complete | 16.2 |
| *minus* succinate | 2.4 |
| *minus* succinate *plus* L-malate (12.5 mM) | 1.9 |
| *minus* succinate *plus* fumarate (12.5 mM) | 1.9 |
| *minus* succinate *plus* ascorbate (37.5 mM) *plus* TMPD (0.1 mM) | 2.4 |
| *minus* $NADP^+$ | 0.0 |
| *minus* NADH | 0.6 |
| *plus* KCN (1.25 mM) | 0.7 |
| *plus* KCN *plus* ATP (1.25 mM) | 12.5 |
| *minus* $P_i$ | 15.2 |
| *minus* $P_i$ *plus* arsenate (1.25 mM) | 16.8 |

supported by the oxidation of ascorbate *plus* TMPD, in accordance with the finding that this oxidation by the bacterial preparation was not coupled to phosphorylation[7]. That the reduction observed was of transhydrogenase nature could be concluded from requirement of both NADH and $NADP^+$. Inhibition of succinate oxidase by cyanide resulted in almost complete abolishment of the transhydrogenase activity, indicating that the mere presence of succinate was not sufficient for the reaction. The activity of the cyanide-inhibited system could be restored by a supply of energy in the form of ATP. The most remarkable finding salient from Table V is that the transhydrogenase activity supported by succinate oxidation, in sharp contrast to the ATP-linked reaction, was stimulated neither by phosphate nor arsenate.

*Differential effects of several reagents on two types of energy-linked transhydrogenase reactions*

Besides $P_i$ and arsenate which stimulated only the ATP-linked transhydrogenase activity, several uncouplers and energy-transfer inhibitors exerted differential effects on the ATP- and succinate-linked reactions. Thus, oligomycin, tributyltin chloride and CCCP strongly inhibited the ATP-linked activity as mentioned above,

but the reaction supported by succinate oxidation was affected only slightly by these reagents as may be seen from Table IV. Treatment of the membrane fragments with high concentrations of LiCl, a condition causing apparent uncoupling of phosphorylation from oxidation[7], also inhibited the ATP-linked reaction in a relatively specific manner. Oleate, which has been shown to be a potent uncoupler in this system[7], and pentachlorophenol inhibited both reactions, though the ATP-linked reaction was always somewhat more sensitive than the succinate-supported process.

*Participation of the same transhydrogenase in energy-linked and energy-independent reactions*

Mammalian mitochondria contain an energy-independent pyridine nucleotide transhydrogenase catalyzing the reduction of $NAD^+$ by NADPH (NAD(P) transhydrogenase, EC 1.6.1.1)[12]. Since the mitochondrial energy-linked transhydrogenase (NAD(P) transhydrogenase) reaction has been shown to be inhibited by anti-NAD(P) transhydrogenase serum[16] and triiodothyronine[17], an inhibitor of the energy-independent reaction, it has been concluded that the same transhydrogenase enzyme is involved in both the energy-linked and energy-independent reactions acting in different directions. As shown in Table VI, 3,3′,5-triiodo-L-thyronine was found to inhibit the ATP-linked reduction of $NADP^+$ by NADH also in the bacterial preparation. Furthermore, other thyronine derivatives such as L-thyroxine and 3,5-diiodo-DL-thyronine also inhibited the two types of reaction to similar extents. However, neither the ATP-linked reaction nor the energy-independent process were effectively inhibited by thyronine itself, tyrosine, and iodinated tyrosine derivatives. These results suggested the participation of the same transhydrogenase enzyme in the two types of bacterial reactions.

TABLE VI

EFFECT OF THYROXINE DERIVATIVES ON ATP-SUPPORTED REDUCTION OF $NADP^+$ BY NADH AND NON-ENERGY-LINKED REDUCTION OF $NAD^+$ BY NADPH

The conditions employed were as described in MATERIALS AND METHODS except that 2.56 mg protein of the membrane fragments were used and thyroxine derivatives were added as indicated.

| *Addition* | *Concentration* ($\mu M$) | % *Inhibition* | |
|---|---|---|---|
| | | *ATP-supported* | *Non-energy-linked* |
| L-Thyroxine | 200 | 88 | 78 |
| 3,3′,5-Triiodo-L-thyronine | 37.5 | 11 | - |
| 3,3′,5-Triiodo-L-thyronine | 100 | 64 | 47 |
| 3,3′,5-Triiodo-L-thyronine | 200 | 97 | 76 |
| 3,5-Diiodo-DL-thyronine | 200 | 40 | 74 |
| 3,5-Diiodo-DL-thyronine | 400 | 75 | 84 |
| DL-Thyronine | 400 | 6 | 6 |
| 3,5-Diiodo-L-tyrosine | 200 | 7 | 7 |
| 3-Iodo-L-tyrosine | 200 | 5 | 3 |
| L-Tyrosine | 200 | 1 | +10 |

## DISCUSSION

The data reported in this paper demonstrate clearly that energy-linked pyridine nucleotide transhydrogenase reactions occur in the phosphorylating membrane

fragments of *M. denitrificans*. A closer investigation of these reactions, coupled with our previous studies on electron transfer and oxidative phosphorylation in the same preparation[7], shows that the energy-transfer system in this bacterium is closely similar to, though not identical with, that postulated for mitochondrial energy transfer[3].

In the bacterial system, $P_i$ and arsenate stimulate the ATP-linked, but not the succinate-supported, transhydrogenase reaction. These anions, therefore, appear to stimulate the reaction by increasing the rate of formation from ATP of a non-phosphorylated high-energy intermediate. In the mitochondrial transhydrogenase reaction, no such stimulation has yet been observed. However, since the ATPase activity of submitochondrial particles is several-fold higher than that of the bacterial preparation, it is possible that phosphate is rapidly formed from ATP in submitochondrial particles and obscures the effect of added phosphate. Using intact pigeon-heart mitochondria, CHANCE[18] has actually reported that the ATP-linked reduction of pyridine nucleotides and oxidation of cytochrome *c* in the presence of succinate and sulfide were accelerated by 26 % and 56 %, respectively, by the addition of 3.6 mM phosphate.

The reason why phosphate and arsenate stimulate the conversion of ATP to the non-phosphorylated intermediate is not yet clear. The phenomenon may be related to the stimulation of ATP–ADP exchange by phosphate observed in mitochondrial preparations[19,20]. Since a lag time of 1–1.5 min is needed for the action of phosphate in the ATP-supported transhydrogenase reaction, it is also likely that the addition of phosphate may in some way increase the level of high-energy intermediates.

An ATP-stimulated reduction of $NADP^+$ by NADH has also been found in a particulate fraction of *Escherichia coli*[4]. The presence of particulate transhydrogenases in bacteria may raise a question concerning their relationship to the well-known soluble transhydrogenases of bacterial origin[12]. Dr. M. UEDA of Tottori University, in collaboration with us, has found the occurrence of both particulate and soluble transhydrogenases in *M. denitrificans* and is now attempting to characterize the two enzymes.

Recently, energy-linked reduction of pyridine nucleotides has been shown to occur in cell-free extracts of chemoautotrophs[21,22]. A similar energy-linked transhydrogenase reaction has also been reported in a photosynthetic bacterium, *Rhodospirillum rubrum*[23], after the completion of the present investigation.

Finally, it may be worthwhile to emphasize again the similarity between the energy-transfer systems of mammalian mitochondria and *M. denitrificans* membrane fragments. This fact, besides being of interest from the viewpoint of comparative biochemistry, enables us to use the bacterial membrane fragments as a material for studies of the mechanism of oxidative phosphorylation and related energy-linked processes. The bacterial preparation actually has notable advantages as a material in view of its low ATPase activity, tight binding of coupling factors to the system[7], and availability of cells grown under a wide variety of conditions. It has been shown that cells of *M. denitrificans* grown anaerobically in the presence of nitrate could yield a preparation capable of energy-linked pyridine nucleotide transhydrogenase reaction*.

* A. ASANO, unpublished observation.

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