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ATP SYNTHESIS DURING EXOGENOUS NADH OXIDATION

A REAPPRAISAL

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This paper reports a reinvestigation on the pathway for mitochondrial oxidation of exogenous NADH and on the related ATP synthesis, first reported 30 years ago (Lehninger, A.L. (1951) J. Biol. Chem. 190, 345–359). NADH oxidation, both in intact and in water-treated mitochondria, is 90% inhibited by mersalyl, an inhibitor of the outer membrane NADH-cytochrome b_5 reductase, and 10% inhibited by rotenone. The mersalyl-sensitive, but not the rotenone-sensitive, portion of NADH oxidation is stimulated by exogenous cytochrome *c*. Part of ATP synthesis is independent of exogenous NADH and cytochrome *c*, and is inhibited by rotenone and antimycin A, and is therefore due to oxidation of endogenous substrates. Another part of ATP synthesis is dependent on exogenous NADH and cytochrome *c*, is insensitive to rotenone and antimycin A, and is due to operation of cytochrome oxidase. It is concluded that (i) oxidation of exogenous NADH in the presence of cytochrome *c* proceeds mostly through NADH-cytochrome b_5 reductase and cytochrome b_5 on the outer membrane and then through cytochrome oxidase via the cytochrome *c* shuttle, and (ii) ATP synthesis during oxidation of exogenous NADH is partly due to oxidation of endogenous substrates and partly to operation of cytochrome oxidase receiving electrons from the outer membrane via cytochrome *c*.

Introduction

In 1951 Lehninger [1] showed that the oxidation of exogenous NADH by rat liver mitochondria was coupled to the synthesis of ATP. Exogenous NADH oxidation and ATP synthesis were stimulated by water treatment of the mitochondria and were found to be dependent on exogenous cytochrome *c*, unlike oxidation of NAD-linked stubstrates [1]. The water treatment was assumed to abolish a permeability barrier impeding the oxidation of exogenous NADH [1]. Oxidation of exogenous NADH was suggested to proceed through two pathways: one through the phosphorylating respiratory chain and another, not coupled to phosphorylation, occurring on the mitochondrial surface and requiring exogenous cytochrome *c* [2–4]. Only the 'internal', phosphorylating pathway was blocked by amytal [3,4], antimycin A [5–7] and rotenone [8].

In 1961, Mitchell [9] proposed that energy conservation involves the formation of a proton electrochemical gradient, $\Delta\tilde{\mu}_H$, across the inner mitochondrial membrane and showed that discharge of $\Delta\tilde{\mu}_H$ leads to abolition of ATP synthesis (reviewed in Ref. 10). In 1967, Sottocasa et al. [11] demonstrated that the outer mitochondrial membrane exhibits a rote-

Abbreviations: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid; Mops, 4-morpholinopropanesulfonic acid; TMPD, tetramethyl-*p*-phenylenediamine; FCCP, carbonyl cyanide *p*-trifluoromethyoxyphenylhydrazone.

none-insensitive NADH-cytochrome *c* reductase activity, and a few years later it was shown that the outer mitochondrial membrane is not permeable to cytochrome *c* [12,13]. Recently, Bernardi and Azzone [14] showed that endogenous cytochrome *c* can function as an electron shuttle between the outer and inner membranes of intact mitochondria (cf. Refs. 15–17), and that the oxidation of exogenous NADH proceeds through the rotenone- and antimycin A-insensitive respiratory chain of the outer membrane leading to cytochrome b_5 reduction. From cytochrome b_5 electrons are then channeled via intermembrane cytochrome *c* to cytochrome oxidase [14].

By comparing the latter observations with those of the early 1950's a number of questions arise: Is it correct that the water treatment facilitates the passage of NADH through the inner membrane? Can a membrane permeable to NADH still maintain the low H^+ permeability required for a $\Delta\tilde{\mu}_H$-driven ATP synthesis? Why is cytochrome *c* required for the oxidation of exogenous NADH and not for the oxidation of NAD-linked substrates? In the present paper it is shown that the synthesis of ATP in intact mitochondria oxidizing exogenous NADH is due partly to the oxidation of endogenous substrate and partly to the transfer of electrons via NADH-cytochrome b_5 reductase on the outer membrane and cytochrome oxidase on the inner membrane, with cytochrome *c* as electron shuttle. The hypotonic treatment removes a permeability barrier for exogenous cytochrome *c* rather than for exogenous NADH, and this barrier is at the level of the outer membrane. Finally, the use of artificial electron carriers, such as TMPD [18], is illustrated. A short report of this work has already been published [19].

Materials and Methods

Rat liver mitochondria were prepared by standard centrifugation procedures in 0.25 M sucrose, 10 mM Tris-HCl, pH 7.4, and 0.1 mM EGTA [20]. The last washing was carried out in an EGTA-free medium and mitochondrial protein was assayed with the biuret method using bovine serum albumin as a standard.

The water treatment was as follows: 1 ml of concentrated mitochondrial suspension was diluted to a final volume of 20 ml with distilled water, at 0°C. After 5 min, 5 ml of 1 M sucrose were added and the suspension centrifuged at 30 000 × *g* for 10 min. The pellet was resuspended in 0.25 M sucrose, 10 mM Tris-HCl, pH 7.4, 1 mg/ml bovine serum albumin.

Experiments on ATP synthesis were carried out at 30°C in a thermoequilibrated vessel equipped with a magnetic stirrer. Mitochondria (1 mg protein/ml) were preincubated for 1 min under the specified conditions, and the reaction was started by the addition of 0.5 mM ADP. At intervals, 1-ml samples were withdrawn and quenched with 0.2 ml of 5 M $HClO_4$. The acid extracts were centrifuged at 30 000 × *g* for 3 min and the clear supernatants were neutralized with 0.9 ml of 0.55 M triethanolamine buffer, pH 7.4, +2.22 M KOH. After precipitation of $KClO_4$, the clear solutions were assayed for ATP content using the enzymatic assay of Greengard [21]. The amount of ATP found was corrected for oligomycin-insensitive ATP synthesis due to adenylate kinase activity.

Experiments of ATP hydrolysis were performed with an Aminco DW 2a dual-wavelength spectrophotometer, equipped with magnetic stirring and thermostatic control. ATP hydrolysis was followed as the decrease in absorbance at 340 minus 374 nm, due to NADH oxidation, in the presence of phospho*enol*pyruvate, pyruvate kinase, ATP, lactate dehydrogenase and NADH. Mitochondria (1 mg/ml) were incubated under the specified conditions and the reaction started with 0.5 mM phospho*enol*pyruvate. The method allows an accurate determination of the rate of ATP hydrolysis at constant ATP levels, due to the continuous regeneration of ATP at the expense of phospho*enol*pyruvate.

TMPD and cytochrome *c* reduction rates were measured by means of dual-wavelength spectrophotometry with the Aminco DW 2a spectrophotometer. For the measurements of TMPD formation, mitochondria (1 mg/ml) were preincubated for 3 min under the specified conditions in order to allow complete oxidation of TMPD to Wurster blue. Reduction was then started by the addition of 0.2 mM NADH, and followed as the decrease in absorbance at 610 minus 680 nm (chart speed 2.5 cm/s). Exogenous cytochrome *c* reduction was measured as the increase of absorbance at 546 minus 536 nm following addition of 0.1 mM NADH (chart speed 1.25 cm/s). These wavelengths for cytochrome *c* were selected in order to avoid interference with cytochrome b_5, as discussed previously [14].

Oxygen consumption was measured with a Clark oxygen electrode (Yellow Springs Instruments, OH) in a water-jacketed thermostatically controlled vessel equipped with a magnetic stirrer.

Pyruvate kinase, lactate dehydrogenase and horse heart cytochrome *c* were purchased from Boehringer (Mannheim). All chemicals were of analytical grade.

Results

The pathway of oxidation of exogenous NADH

In a recent paper, evidence has been presented that mersalyl is a strong inhibitor of the NADH-cytochrome b_5 reductase operating on the outer mitochondrial membrane [14]. Mersalyl therefore lends itself as a useful tool to ascertain whether the oxidation of exogenous NADH proceeds through the respiratory chain of the inner or outer mitochondrial membrane.

Fig. 1 shows the effect of mersalyl on the oxidation of exogenous NADH in intact or in water-treated mitochondria inhibited with rotenone and antimycin A in the presence of 20 μM cytochrome *c*. Mitochondria were preincubated in the presence of increasing concentrations of mersalyl and then supplemented with exogenous NADH. It is seen that the oxidation of exogenous NADH is about 50% inhibited below 10 nmol mersalyl/mg protein and completely at 15 nmol mersalyl/mg protein. Fig. 1 also shows that the water treatment resulted in a marked increase in the rate of NADH oxidation, in agreement with that observed by Lehninger [1]. However, the oxidation of exogenous NADH by water-treated mitochondria was inhibited by mersalyl at the same concentrations as by intact mitochondria.

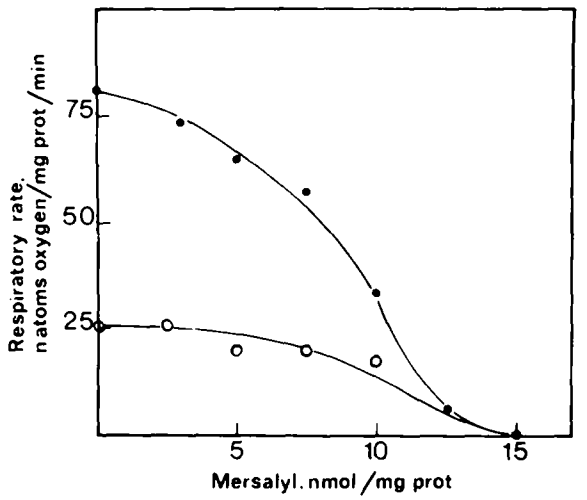

Fig. 1. Effect of mersalyl on rotenone-insensitive NADH oxidation. The incubation medium contained 0.1 M KCl, 10 mM Tris-Mops, pH 7.4, 5 mM $MgCl_2$, 2 mM P_i, 1 mg/ml bovine serum albumin, 2 μM rotenone, 0.5 μg antimycin A/ml, 20 μM cytochrome *c* and mersalyl as indicated on the abscissa, 2 mg/ml mitochondria; final volume 2 ml, 25°C. After 8 min of preincubation, respiration was started by the addition of 0.2 mM NADH. (•——•) water-treated (○——○) untreated mitochondria.

In experiments complementary to that of Fig. 1, mitochondria were supplemented with fully inhibitory concentration of mersalyl and tested for the rotenone-sensitive oxidation of exogenous NADH. The rate of oxygen uptake was only 2.5 and 10.5 ng-atom/mg protein per min in intact and water-treated mitochondria, respectively. This rate was not stimulated by exogenous cytochrome *c*; thus, the rotenone-sensitive pathway for exogenous NADH oxidation accounts only for 10% of the total, and is not stimulated by exogenous cytochrome *c*. On the other hand, the rate of the mersalyl-sensitive NADH oxidation via cytochrome b_5 is increased by added cytochrome *c* (see below) and accounts for 90% of the total. These observations strongly suggest that the oxidation of exogenous NADH proceeds mostly through the outer membrane electron-transfer pathway, via NADH-cytochrome b_5 reductase and cytochrome b_5.

The synthesis of ATP during oxidation of exogenous NADH

Fig. 2 shows the synthesis of ATP in intact mitochondria oxidizing either endogenous substrates or exogenous NADH in the absence or presence of exogenous cytochrome *c* and rotenone. Consider first the oxidation of endogenous substrates (Fig. 2A). After an initial rapid phase, a linear rate of ATP synthesis of about 10 nmol ATP/mg protein per min was established. This rate was slightly enhanced by exogenous cytochrome *c* (not shown) and slightly lowered by the addition of exogenous NADH [1].

As expected, ATP synthesis was inhibited by rotenone. Addition of NADH together with cytochrome *c* resulted in a considerable stimulation of the overall rate of ATP synthesis (Fig. 2B). In the presence of cytochrome *c* + NADH, rotenone-treated mitochon-

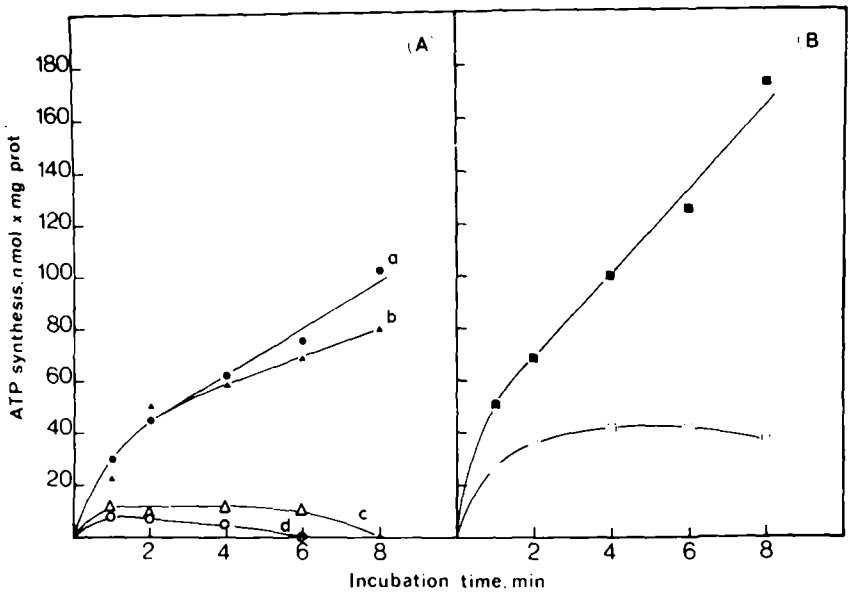

Fig. 2. ATP synthesis during oxidation of exogenous NADH and/or endogenous substrates. The incubation medium contained 0.1 M KCl, 10 mM Tris-Mops, pH 7.4, 5 mM $MgCl_2$, 2 mM P_i, 1 mg/ml bovine serum albumin and 0.1 mM EGTA. (A) (●———●, trace a) No further addition, (▲———▲, trace b) 0.5 mM NADH, (○———○, trace c) 2 μM rotenone, (△———△, trace d) 2 μM rotenone and 0.5 mM NADH. (B) (■———■) 0.5 mM NADH and 20 μM cytochrome *c*, (□———□) 0.5 mM NADH, 20 μM cytochrome *c* and 2 μM rotenone. 1 mg/ml mitochondria, final volume 6 ml, 30°C. After 1 min of preincubation ATP synthesis was started by the addition of 0.5 mM ADP (time 0). At the indicated times, 1-ml aliquots were withdrawn and ATP synthesis was determined as described in Materials and Methods. Each point is the average of two independent experiments. The amount of ATP found was corrected for the oligomycin-insensitive ATP formation, due to adenylate kinase activity.

dria synthesized ATP although the synthesis levelled off after 3–4 min (Fig. 2B). It may be noted that the rate of ATP synthesis of the sample supplemented with NADH and cytochrome *c* was slightly higher (especially in the time span 4–8 min) than the sum of the two samples: endogenous substrates (Fig. 2A) and NADH + cytochrome *c* + rotenone (Fig. 2B). The results of Fig. 2 suggest that in the absence of inhibitors and in the presence of exogenous NADH and cytochrome *c* three processes occur: (i) ATP synthesis due to oxidation of endogenous substrates, (ii) ATP synthesis due to a rotenone-insensitive NADH-cytochrome *c* reductase-cytochrome oxidase pathway, and (iii) ATP hydrolysis due to Mg^{2+}-stimulated, and perhaps other, ATPases.

In other experiments similar to those of Fig. 2, not shown here, ATP was trapped with hexokinase/glucose and the experiment was carried out for a longer incubation period. Under these conditions, the synthesis of ATP was linear for at least 10 min also in the presence of NADH + cytochrome *c* + rotenone, suggesting that the levelling off of ATP synthesis observed in Fig. 2B is essentially due to a balance between rates of ATP synthesis and hydrolysis.

Fig. 3 shows the dependence of the rate ATP hydrolysis on ATP concentration at constant ATP levels. ATP hydrolysis was only partially inhibited by oligomycin. This suggests that the oligomycin-insensitive, Mg^{2+}-stimulated ATPase activity is located either on the outer membrane or on some lysosomal [22, 23] and/or microsomal contaminants. The oligomycin-insensitive fraction had a K_m of about 40 μM ATP, and approached a V of about 20 nmol/mg protein per min. The oligomycin-sensitive fraction had a much higher K_m, about 0.2 mM ATP, and approached a V of about 22 nmol/mg protein per min. The existence of these ATPase activities may contribute to explain the levelling off of ATP synthesis in the presence of NADH + cytochrome *c* + rotenone (Fig. 2B).

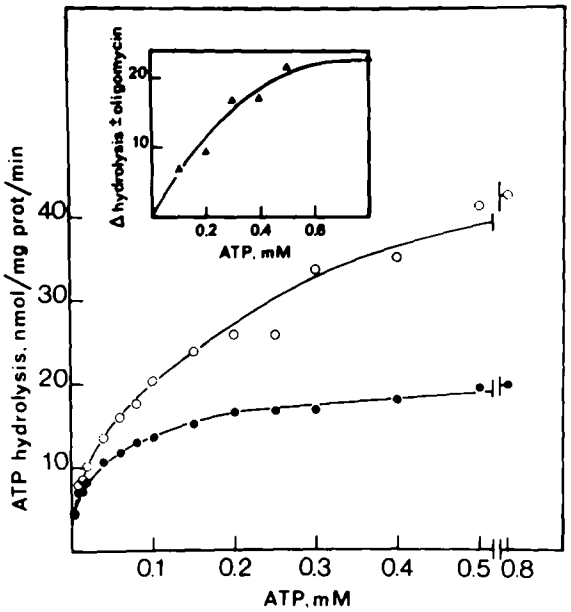

Fig. 3. Dependence of ATPase activities on ATP concentration. The incubation medium contained 0.1 M KCl, 10 mM Tris-Mops, pH 7.4, 5 mM $MgCl_2$, 0.1 mM EGTA, 2 μM rotenone, 0.1 μg antimycin A/ml, 0.125 mM NADH, and excess pyruvate kinase and lactate dehydrogenase and ATP as indicated. 1 mg/ml mitochondria, final volume 2 ml, 30°C. The reaction was started by the addition of 0.5 mM phospho*enol*-pyruvate in the absence (○———○) or presence (●———●) of 2 μg oligomycin. For further details see Materials and Methods.

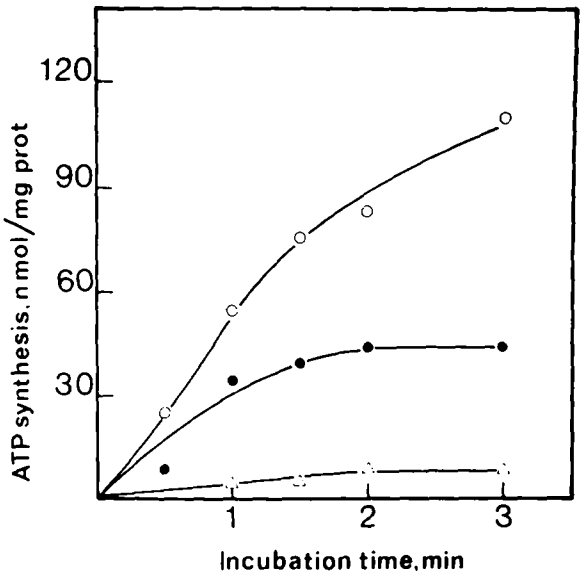

Fig. 4. ATP synthesis during exogenous NADH oxidation with different electron carriers. Experimental conditions as in Fig. 2, with 2 μM rotenone and 0.5 mM NADH in the incubation medium and (○———○) 100 μM TMPD, (●———●) 20 μM cytochrome *c*, (△———△) 1 mM $K_3Fe(CN)_6$. Each point is the average of two independent experiments. The amount of ATP was corrected for oligomycin-insensitive formation.

The synthesis of ATP during oxidation of exogenous NADH in the presence of rotenone with different electron carriers

Once established, the pathway for exogenous NADH oxidation and its extent of coupling via the natural electron carrier cytochrome *c* pose the problem as to why the rate of ATP synthesis is so low and tends to level off rapidly when oxidation of endogenous substrate is blocked by rotenone. In principle, the rate-limiting step may be at the level of either the reduction or oxidation of cytochrome *c*. The latter step is a likely candidate in view of the restricted permeation of cytochrome *c* through the outer mitochondrial membrane [13].

Fig. 4 shows a comparison of the rates of ATP synthesis with TMPD, cytochrome *c* and $K_3Fe(CN)_6$ as electron shuttles between exogenous NADH and cytochrome oxidase. While ferricyanide was very inefficient, TMPD was more efficient than cytochrome *c*. As we will show below, the rate of ATP synthesis cannot be increased by increasing the concentration of cytochrome *c*.

Fig. 5A and B shows the Linewaever-Burk plots for the rate of cytochrome *c* and Wurster blue reduction by exogenous NADH. In the case of cytochrome *c*, *V* was 570 nmol/mg protein per min and the apparent K_m 19 μM; in the case of Wurster blue, *V* was 703 nmol/mg protein per min and the apparent K_m 12 μM. Since during NADH oxidation both TMPD and cytochrome *c* are completely reduced (not shown), the rate-limiting step in electron transfer is in both cases at the level of cytochrome oxidase. Furthermore, the experiments rule out the possibility that the higher efficiency of TMPD in ATP synthesis, with respect to cytochrome *c* (see Fig. 4), is due to a higher rate of reduction of the former.

Figs. 6 and 7 show results of experiments where rates of ATP synthesis and of oxygen uptake during oxidation of exogenous NADH were measured at increasing concentration of TMPD (Fig. 6) or of cyto-

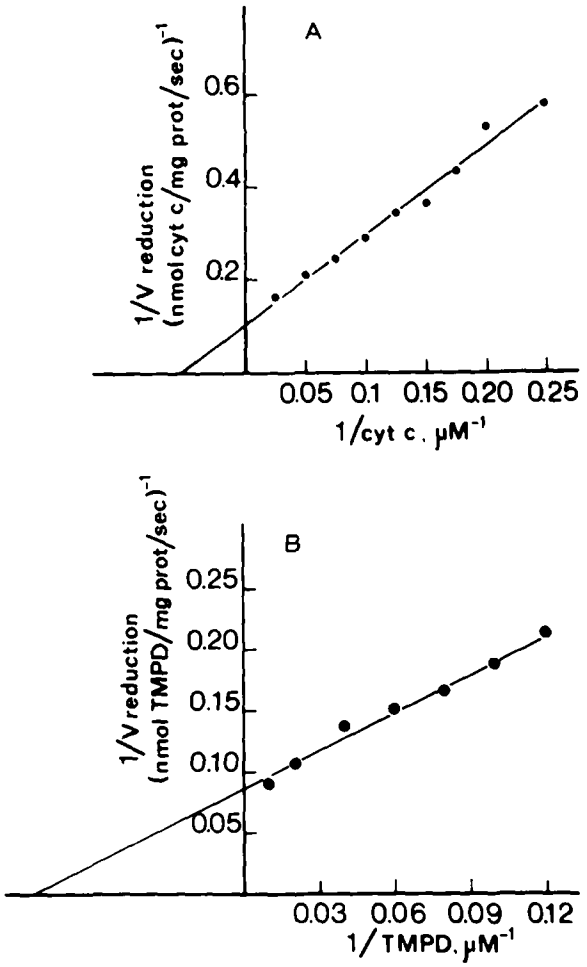

Fig. 5. Lineweaver-Burk plots for cytochrome *c* and TMPD reduction by exogenous NADH. The incubation medium contained 0.1 M KCl, 10 mM Tris-Mops, pH 7.4, 5 mM $MgCl_2$, 0.1 mM EGTA, 2 μM rotenone and 0.1 μg antimycin A/ml. 1 mg/ml mitochondria, final volume 2 ml, 30°C. (A) Cytochrome *c* as indicated, and cytochrome *c* reduction was started by the addition of 0.1 mM NADH. (B) TMPD as indicated: after complete oxidation to Wurster blue, reduction was started by the addition of 0.2 mM NADH. For further details see Materials and Methods. cyt, cytochrome.

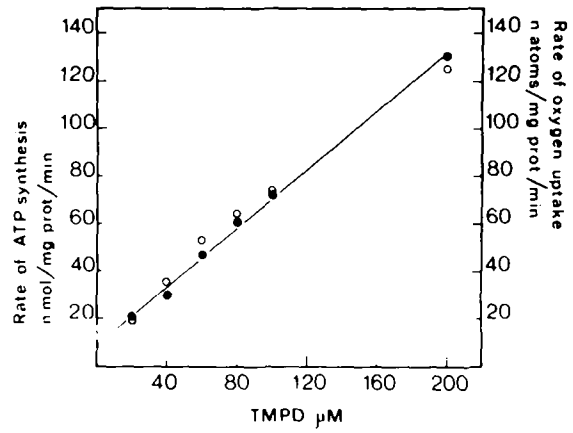

Fig. 6. Effect of the TMPD concentration on the rate of respiration and phosphorylation. The incubation medium contained 0.1 M KCl, 10 mM Tris-Mops, pH 7.4, 2 mM P_i, 1 mg/ml bovine serum albumin, 1 mM EDTA, 2 μM rotenone, 0.5 μg antimycin A/ml and TMPD as indicated. 1 mg/ml mitochondria, final volume 2 ml, 30°C. After 3 min of preincubation, 0.5 mM NADH and 0.5 mM ADP were added. ATP synthesis (●——●) and oxygen uptake (○——○) were determined on parallel samples. Each point is the average of two independent experiments. For further details Materials and Methods.

chrome *c* (Fig. 7). It is seen that the rate of NADH oxidation and the rate of ATP synthesis increased linearly with the concentration of TMPD, with a constant P : O ratio of about 1, whereas both the rate of NADH oxidation and the rate of ATP synthesis exhibited saturation kinetics at low concentrations of cytochrome *c*. Moreover, the rate of ATP

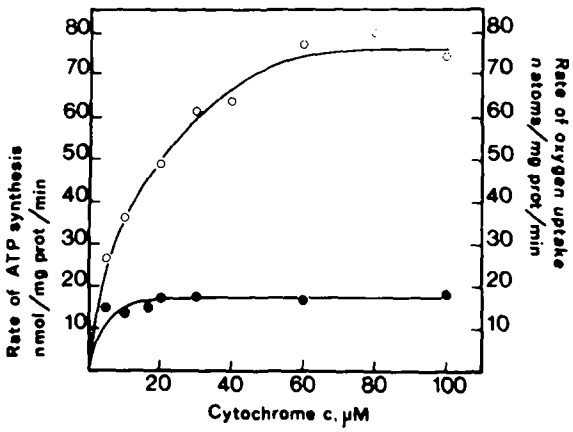

Fig. 7. Effect of the cytochrome *c* concentration on the rate of respiration and phosphorylation. Experimental conditions as in Fig. 6, with 5 mM $MgCl_2$ and 0.1 mM EGTA instead of EDTA, 0.5 mM NADH and cytochrome *c* as indicated. After 1 min of preincubation ATP synthesis was started by the addition of 0.5 mM ADP. ATP synthesis (●——●) and oxygen uptake (○——○) were determined on parallel samples, and the data were corrected for oligomycin-insensitive ATP synthesis, Each point is the average of two independent experiments. For further details see Materials and Methods.

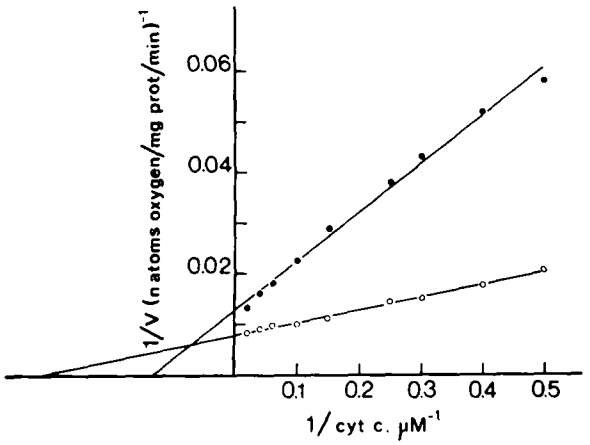

Fig. 8. Effect of the water treatment on exogenous NADH oxidation with cytochrome *c* as intermembrane electron shuttle. The incubation medium was the same as that of Fig. 6, plus 2 μM FCCP and 0.5 mM NADH. Cytochrome (cyt) *c* as indicated, 1 mg/ml mitochondria, final volume 2 ml, 30°C. (●——●) intact and (○——○) water-treated mitochondria.

synthesis reached its maximum at very low concentrations of cytochrome *c*: the P : O ratio was much lower than 1 and tended to decline further with the increase of cytochrome *c*. Figs. 6 and 7 agree with the concept that exogenous cytochrome *c* does not interact freely with cytochrome oxidase, due to the permeability barrier of the outer membrane.

This is further supported by the experiment of Fig. 8 which shows the effect of the water treatment on NADH oxidation in the presence of increasing cytochrome *c* concentration. The water treatment resulted in an increase in the apparent *V* from 77 to 130 nmol/mg protein per min and in a decrease in the apparent K_m from 7.5 to 3.2 μM. This finding is more striking if it is considered that the water treatment reduces dramatically the *V* of respiration from NADH to oxygen with TMPD as electron shuttle (not shown).

Discussion

The pathway for exogenous NADH oxidation

The demonstration that oxidation of exogenous NADH is coupled to ATP synthesis, provided by Lehninger in 1951 [1], has been of crucial importance in the history of oxidative phosphorylation. The higher P : O ratio found by Lehninger was 1.89, which left little doubt that the synthesis of ATP was entirely due to the operation of the electron-transport chain, above the level of NAD reduction, as previously suggested by Belitser and Tsibakova [24] and by Lipmann [25]. Exogenous NADH oxidation was dependent on exogenous cytochrome *c*, and rates of NADH oxidation and of ATP formation were increased by water treatment, thought to remove a permeability barrier for exogenous NADH, and perhaps for exogenous cytochrome *c* [1,26]. On the other hand, the dependence on exogenous cytochrome *c* has not been easy to interpret, since phosphorylation with NAD-linked substrates is not dependent on added cytochrome *c*.

The role of the inner mitochondrial membrane as an osmotic barrier between the matrix and the intermembrane space has gradually assumed a major importance. The concept is now accepted that energy conservation is coupled, directly or indirectly, to the formation of $\Delta\tilde{\mu}_H$ [9,10]. The inner mitochondrial membrane has a very low permeability for H^+, in order to restrict the energy drain due to diffusion of H^+ down the electrochemical gradient. When $\Delta\tilde{\mu}_H$ is decreased, ATP synthesis is also decreased. It is therefore difficult to visualize how a membrane can maintain a low permeability for H^+ and yet let a large molecule such as NADH move across the membrane. Furthermore, on the basis of Lehninger's experiments it is not possible to distinguish whether electrons coming from NADH travel along the inner or the outer mitochondrial membrane.

The present study demonstrates that 90% of NADH oxidation, either by intact or water-treated mitochondria, proceeds through the rotenone-insensitive NADH-cytochrome *c* reductase of the outer membrane, while NADH oxidation along NADH dehydrogenase of the inner membrane accounts for only 10% of the total. This finding explains the dependence of exogenous NADH oxidation on cytochrome *c*, which acts as an electron shuttle between cytochrome b_5 and cytochrome oxidase [14].

The permeability barrier which is overcome by the water treatment is at the level of the outer membrane, which in the intact mitochondrion limits the interaction of exogenous cytochrome *c* with cytochrome oxidase (Fig. 8). This concept can also be deduced by comparing the effect of TMPD and of

exogenous cytochrome *c* on oxygen uptake during oxidation of exogenous NADH. Oxygen uptake increased linearly with the TMPD concentration, which freely permeates the outer membrane, while saturation was obtained at rather low concentrations of cytochrome *c*.

Energy coupling during exogenous NADH oxidation

The present study reports two observations relevant to the interpretation of ATP synthesis during oxidation of exogenous NADH. First, part of ATP synthesis is due to oxidation of endogenous substrates, since it is independent of the addition of exogenous NADH or cytochrome *c* and is abolished by rotenone. Second, another part of ATP synthesis is rotenone insensitive and then independent of the oxidation of endogenous substrates, but is dependent on exogenous cytochrome *c*. The former part corresponds to electron transfer along the inner membrane, while the latter corresponds to electron transfer from NADH to cytochrome b_5 on the outer membrane and from cytochrome *c* to oxygen via cytochrome oxidase on the inner membrane [14]. In the absence of inhibitors the two pathways operate simultaneously, and the overall rate of ATP synthesis corresponds almost quantitatively to the sum of the two pathways measured independently.

Due to the matrix localization of the active site of NADH dehydrogenase, exogenous NADH is oxidized directly by inside-out inner membrane vesicles. The question then arises as to whether oxidation of exogenous NADH and ATP synthesis, observed in this and previous reports, could be due to a population of inside-out or 'scrambled' membranes. These membranes could in principle account for the mersalyl-insensitive oxidation of exogenous NADH which, however, is not coupled to synthesis of ATP in the absence of cytochrome *c* (Fig. 2A). Furthermore, ATP synthesis is completely inhibited by atractyloside (Bernardi, P. and Azzone, G.F., unpublished observations); these inside-out or scrambled membranes, then, if present, are nonphosphorylating.

The rate of ATP synthesis during operation of Site III alone, with cytochrome *c* as electron shuttle, tends to level off after a few minutes. We attribute this to the fact that the rate of charge separation at the level of cytochrome oxidase, which is limited by the concentration of free cytochrome *c* in the intermembrane space, may rapidly become unable to compensate for the various energy-draining reactions, in particular ATPase activities and H^+ leakages. What remains unclear at present is why the P : O ratio decreases with the increase in cytochrome *c* concentration (cf. Fig. 7). This finding cannot be explained by autooxidation of cytochrome *c*, since exogenous NADH and succinate oxidation are inhibited by the same titer of cyanide (Bernardi, P. and Azzone, G.F., unpublished observations).

The present study illustrates the use of the artificial electron shuttle, TMPD [18], for the study of energy conservation during exogenous NADH oxidation. A kinetic analysis of this system demonstrates that the rate of electron transfer through NADH-cytochrome b_5 reductase is considerably higher than the rate of electron transfer through Site III in the intact mitochondria. The NADH-TMPD system becomes then a useful tool for studying electron transfer and energy conservation at Site IIII.

The earlier findings of Lehninger [1,2] and of Ernster et al. [3,4] can then be rationalized in the following manner. When no inhibitor was added, such as amytal or rotenone, ATP synthesis was partly due to the oxidation of endogenous substrates along the inner mitochondrial membrane and partly to the oxidation of NADH through the outer membrane up to cytochrome b_5 and then to cytochrome oxidase via cytochrome *c*. The observed P : O ratio was a mixture of two processes, one reflecting electron transfer along the three coupling sites of the inner membrane and the other reflecting electron transfer only through Site III. The observed P : O ratios, ranging between 0.4 and 1.89 [1], presumably reflected the varying contributions of the two processes, together with the varying degree of coupling of different preparations of intact and water-treated mitochondria. Thus, Lehninger's mitochondria were indeed 'pretty bust-up' [27] to let cytochrome *c* through the outer membrane but not enough so to let NADH through the inner membrane, as expected for an H^+-driven ATP synthesis.

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