ORIGIN OF PHOSPHORYLATION COUPLED TO THE OXIDATION OF EXTRAMITOCHONDRIAL NADH

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1. Introduction

Demonstration of ATP synthesis coupled to the oxidation of extramitochondrial NADH by Lehninger in the early 1950s constitutes important evidence for the occurrence of phosphorylations in the respiratory chain [2–4]. A current textbook of biochemistry summarizes this evidence as follows [5]:

> 'Pure NADH was incubated aerobically with water-treated mitochondria, phosphate, and ADP in the absence of tricarboxylic acid cycle intermediates or any other added organic metabolite. (The hypotonic water treatment was necessary to make the mitochondria permeable to NADH.) The NADH was rapidly oxidized to NAD^+ at the expense of molecular oxygen, simultaneously, up to three molecules of ATP were formed from ADP and phosphate. Such experiments indicate that at three points in the chain of electron carriers leading from NADH to oxygen, oxidation–reduction energy is transformed into phosphate-bond energy.'

Since it is now generally believed that a proton-impermeable inner mitochondrial membrane is a prerequisite for the coupling of respiration to phosphorylation, and since it is unlikely that a proton-impermeable membrane is permeable to NADH, the question arises as to the origin of the phosphorylations observed in the above experiments. The purpose of the present study was to answer this question.

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This work was presented at the 14th FEBS Meeting [1]

2. Results and discussion

Addition of NADH to rat liver mitochondria resulted in no measureable increase in the rate of endogenous respiration and accompanying phosphorylation (table 1); the P/O ratio resulting from the oxidation of endogenous substrate varied from 0.8–2.7 and was not significantly altered by added NADH. Addition of cytochrome *c*-stimulated the oxidation of added NADH. This respiration, which was ~1/2 as rapid as that obtained with succinate, and about equal to that obtained with β-hydroxybutyrate, was accompanied by a phosphate uptake with a P/O ratio <1. The cytochrome *c*-stimulated respiration and the accompanying phosphorylation were virtually insensitive to rotenone and antimycin. This respiration most probably proceeds via the NADH-cytochrome *c* reductase associated with the outer membrane [8] and cytochrome oxidase, and the phosphorylation thus originates exclusively from coupling site 3 of the respiratory chain. Removal of the outer membrane by digitonin treatment resulted in a preparation of 'mitoplasts' [9] that no longer exhibited a rotenone-insensitive NADH oxidation in the presence of cytochrome *c*.

Brief exposure of the mitochondria to hypotonicity according to Lehninger [2] resulted in an increase of the cytochrome *c*-dependent stimulation of NADH oxidation (fig.1). The rate of oxidation of NADH in the presence of cytochrome *c* was unaffected by the hypotonic treatment. The results indicate that the effect of hypotonic treatment probably was to enhance the permeability of the outer membrane to cytochrome *c* rather than enhancing the permeability of the inner membrane to NADH.

When NADH was replaced by NAD^+ + ethanol (EtOH) + alcohol dehydrogenase (ADH), the ensuing

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Table 1
Respiration and phosphorylation of rat liver mitochondria with NADH as substrate

Additions	Without cytochrome *c*		With cytochrome *c*	
	Respiration	P/O ratio	Respiration	P/O ratio
None	4.3	1.47		
Succinate	62.7	1.69		
β-Hydroxybutyrate	28.0	2.69		
NADH	4.3	0.83	31.4	0.53
NADH + rotenone	1.7		25.6	0.42
NADH + antimycin	2.1		22.8	0.23
NAD^+ + EtOH + ADH	9.3	2.72	25.9	1.41
NAD^+ + EtOH + ADH + rotenone	1.5		15.9	0.64
NAD^+ + EtOH + ADH + antimycin	1.5		19.8	0.30
NADH + EtOH + ADH	5.2			

Rat liver mitochondria were prepared as in [6]. The assay system consisted of 50 mM Tris acetate (pH 7.4), 100 mM KCl, 3.7 mM $MgCl_2$, 2.7 mM $^{32}P_i$, 1 mM ADP, 5 mM glucose, 14 units yeast hexokinase, and 4.2 mg mitochondrial protein in a final volume of 2.7 ml. Additions when indicated were: 5 mM succinate or β-hydroxybutyrate, 2 mM NADH, 2 mM NAD^+, 42 mM ethanol (EtOH), 45 units liver alcohol dehydrogenase (ADH), 3.3 μM rotenone, 2 μg antimycin, 50 μM cytochrome *c*. Oxygen uptake was measured at 30°C polarographically with a Clark oxygen electrode. Phosphate uptake was determined by the isobutanol–benzene extraction method [7]. Respiration is expressed in nmol $O_2 \cdot min^{-1} \cdot$ mg mitochondrial $protein^{-1}$.

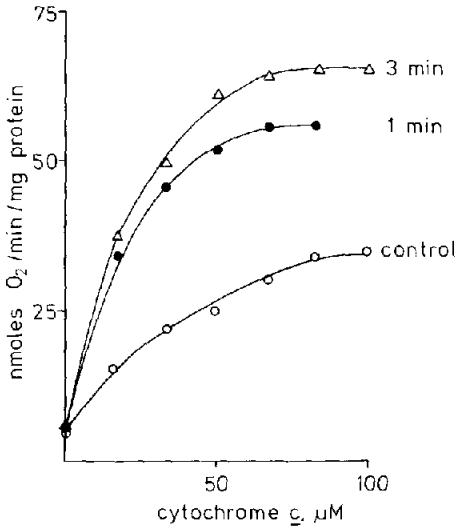

Fig.1. Effect of hypotonic treatment of rat liver mitochondria on the cytochrome *c*-dependent oxidation of NADH. A tightly-packed mitochondrial pellet (~40 mg protein) was suspended in 0.5 ml ice-cold water and kept in the cold for 1 or 3 min, after which 0.17 ml 1 M sucrose was added. Control mitochondria were suspended in 0.67 ml 0.25 M sucrose. Respiration was measured with 2 mM NADH as substrate as described in table 1. Cytochrome *c* was added as indicated.

respiration was more rapid than in case of externally added NADH and was accompanied by a phosphorylation with a P/O ratio >2 (table 1). This respiration was sensitive to rotenone and antimycin. Addition of cytochrome *c* to this system caused an increase in respiration which was virtually insensitive to rotenone and antimycin and was accompanied by an overall decrease in P/O ratio. These data suggest that NAD^+ + EtOH + ADH give rise to an oxygen uptake that proceeds via the internal, rotenone- and antimycin-sensitive NADH-cytochrome *c* reductase, including coupling sites 1 and 2. This system requires no added cytochrome *c* and when cytochrome *c* is added both the external and the internal pathways operate in parallel. These conditions are similar to those used in experiments in which the selective effects of amytal [10] and rotenone [11] on the phosphorylating pathway of NADH oxidation were first described.

When NAD^+ in the presence of EtOH + ADH was replaced by NADH, the respiration was almost as low as in the presence of the regenerating system (table 1, last line). This finding suggested that the increased respiration observed in the presence of EtOH + ADH may not be due to a continuous generation of NADH (in which case NADH + EtOH + ADH

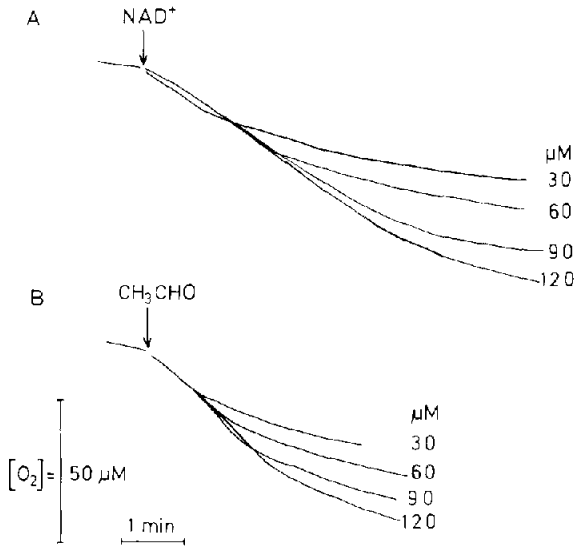

Fig.2. Respiration of rat liver mitochondria with NAD^+ + EtOH + ADH or acetaldehyde as substrate. NAD^+ or CH_3CHO was added in concentrations as indicated. Other conditions were as described in table 1.

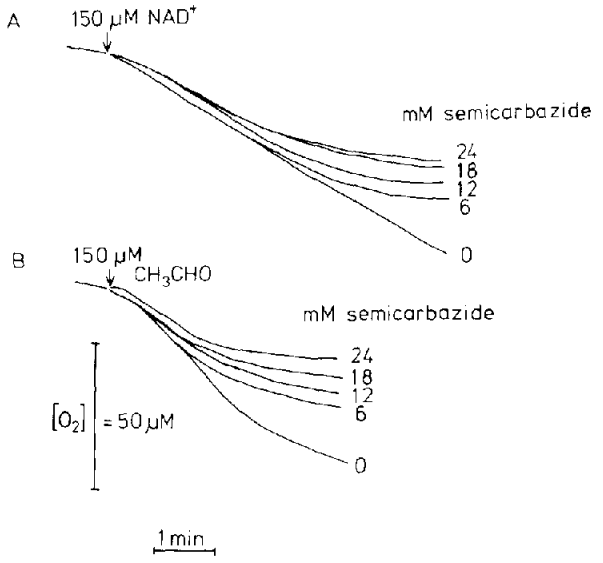

Fig.3. Effect of semicarbazide on the respiration of rat liver mitochondria with NAD^+ + EtOH + ADH or acetaldehyde as substrate. Semicarbazide was added to the reaction medium in the concentrations indicated. Other conditions were as in table 1.

should give the same respiration as NAD^+ + EtOH + ADH) but rather to the initial formation of acetaldehyde, which subsequently is oxidized by way of intramitochondrial NAD^+.

This possibility has earlier been considered by Maley [12] but was discarded on the account that:

(i) Semicarbazide, which would trap acetaldehyde, did not inhibit respiration; and

(ii) Acetaldehyde, which is volatile, was assumed to be efficiently removed from the reaction medium under the conditions of the manometric assay of respiration.

We have re-examined this possibility and found that under our conditions, acetaldehyde, formed initially, most probably was responsible for the respiration (and phosphorylation) observed in the presence of NAD^+ + EtOH + ADH. The evidence supporting this conclusion was as follows:

(i) When the amount of NAD^+ (and thereby the amount of initially-formed acetaldehyde) was decreased, this resulted in a decrease in both the initial rate and the total amount of stimulated oxygen uptake (above that observed with NADH alone), similar to that observed when an equivalent amount of acetaldehyde was used as a substrate instead of NAD^+ + EtOH + ADH. In both cases the amount of oxygen uptake was roughly stoichiometric with the amount of acetaldehyde (generated or added). Fig.2 summarizes these results.

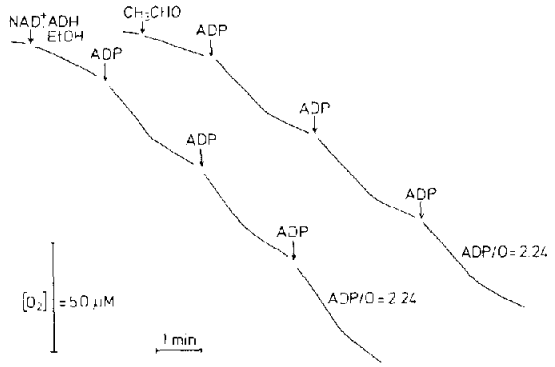

Fig.4. Effect of ADP on respiration of rat liver mitochondria with NAD^+ + EtOH + ADH or acetaldehyde as substrate. The concentration of CH_3CHO was 2 mM. Other conditions were as in table 1, except that glucose, hexokinase and $MgCl_2$ were omitted and 200 μM ADP was added when indicated.

(ii) Semicarbazide inhibited only weakly the NAD^+ + EtOH + ADH induced respiration, and the same was true for the respiration induced by acetaldehyde (fig.3). Apparently, acetaldehyde, generated or added, is rapidly taken up by the mitochondria and thus escapes trapping by semicarbazide. Similar results were obtained with hydrazine (not shown).

(iii) NAD^+ + EtOH + ADH and acetaldehyde gave respiratory control ratios of similar extents (fig.4). The ADP/O ratios exceeded 2. The oxidation of acetaldehyde was sensitive to rotenone and antimycin (not shown).

(iv) An NADH-regenerating system not giving rise to a substrate for mitochondrial oxidation, consisting of an NAD^+-linked glucose-6-phosphate dehydrogenase from *Leuconostoc mesenteroides* (which converts glucose-6-phosphate to 6-phosphogluconate), gave no increase in respiration similar to that observed with NAD^+ + EtOH + ADH.

4. Conclusions

1. Addition of NADH to rat liver mitochondria results in a respiration, the rate of which is not significantly different from that due to endogenous substrates. Added cytochrome *c* stimulates the oxidation of added NADH, with a P/O ratio ≤ 1. This respiration is rotenone- and antimycin-insensitive and probably proceeds via the NADH-cytochrome *c* reductase associated with the outer membrane and cytochrome *c* oxidase. The accompanying phosphorylation thus most probably originates from the reoxidation of reduced cytochrome *c* by way of cytochrome *c* oxidase, i.e., coupling site 3 of the respiratory chain.

2. Brief exposure of mitochondria to hypotonicity enhances the cytochrome *c*-dependent oxidation of added NADH but not that occurring in the absence of added cytochrome *c*. This effect obviously is due to an increased permeability of the outer membrane to cytochrome *c* rather than that of the inner membrane to NADH.

3. Addition of NAD^+ + EtOH + ADH results in a respiration that is rotenone- and antimycin-sensitive and is accompanied by a phosphorylation with a marked ADP-control and a P/O ratio approaching 3. The evidence presented indicates that the respiration and phosphorylation obtained under these conditions originate from the oxidation of acetaldehyde by way of intramitochondrial NAD^+ and do not involve the oxidation of exogenous NADH.

4. It is concluded that the early results quoted in the Introduction [2–4] provide evidence for one site of phosphorylation in the respiratory chain, corresponding to what later became identified as coupling site 3.

Acknowledgements

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