SOME PROPERTIES OF A NEW PHOSPHORYLATED DERIVATIVE OF NAD, AN INTERMEDIATE IN OXIDATIVE PHOSPHORYLATION.*

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In the accompanying paper we have presented evidence for a phosphorylated intermediate in oxidative phosphorylation (Griffiths & Chaplain, 1962) which accumulates when heart mitochondria are incubated with inorganic phosphate (P_i) and NAD in the presence of succinate. This compound has been purified by paper electrophoresis and shown to be a new derivative of NAD containing equimolar amounts of phosphate and NAD.

The purification of this new derivative has been complicated by its high lability and the fact that at least one other labile form of NAD is present in the incubation mixture. In particular, a highly labile form of NAD which absorbs at 315-318 mμ has been detected after separation by paper electrophoresis The relationship of this compound to the '315 mμ pigment' described by Chance & Hagihara (1961), and to the new phosphorylated derivative is under investigation.

The phosphorylated derivative of NAD is isolated from incubation mixtures containing $^{32}P_i$ and NAD by paper electrophoresis at pH 7.2 in Triethanolamine-HCl buffer. The compound is readily separated from NAD and P_i and can be further purified by repeating the paper electrophoresis step. Large losses of the compound were always observed, indicating that the compound is highly labile even at neutral pH.

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Purified preparations of this intermediate contain equimolar amounts of NAD and P_i as shown in Table 1.

Table 1

Composition of the purified intermediate

Component	μmoles/ml
Total phosphate - after ashing	- 15.9
NAD - by ADH assay*	- 4.1
NAD - by 260 mμ absorption	- 5.1
Phosphate - by Fiske-Subbarow method	- 5.3
Phosphate present initially - by Fiske-Subbarow*	- 0.9
NAD present initially - by ADH assay**	- 1.93

* NAD and phosphate levels increase when the compound is allowed to stand at 0°. The lower figure for total NAD is due to the partial conversion to a NAD derivative absorbing at 290-295mμ which does not react in the ADH assay.

** The high initial figure for NAD is probably due to hydrolysis at the alkaline pH of the alcohohol dehydrogenase (ADH) assay.

The amount of NAD and phosphate as measured by the ADH assay and the Fiske-Subbarow method respectively, increases on standing at 0°. During this period marked changes on the absorption spectrum of the compound occur at 260mμ and 310mμ. The absorption at 260 mμ was found to increase on standing at 0° concomitant with a release of P_i (as estimated by the Fiske-Subbarow method) and the formation of NAD (as assayed by alcohol dehydrogenase). The compound is reactive initially in a system containing ADP, Mg^{++}, Antimycin A and sub-mitochondrial particles, giving rise to ATP and $NADH_2$. This

property is gradually lost on standing at 0° concomitant with an increase in the P_i concentration as measured by the Fiske-Subbarow method.

On incubation of the intermediate in the presence of Antimycin A (5μg) with phosphorylating sub-mitochondrial particles (ETP_H of Linnane & Ziegler, 1958), which had been treated with Antimycin A so as to block electron transport, the following reactions occur;

(a) On incubation with low levels of ADP in the presence of a glucose-hexokinase trap $NADH_2$ was rapidly formed and P_i was released. Initially the amounts of P_i and $NADH_2$ formed was stoichiometric (Fig. 1). ATP formation was also observed. In the absence of ADP and hexokinase the initial rate of P_i release was almost doubled (Fig. 1) but the rate of formation of $NADH_2$ was unchanged.

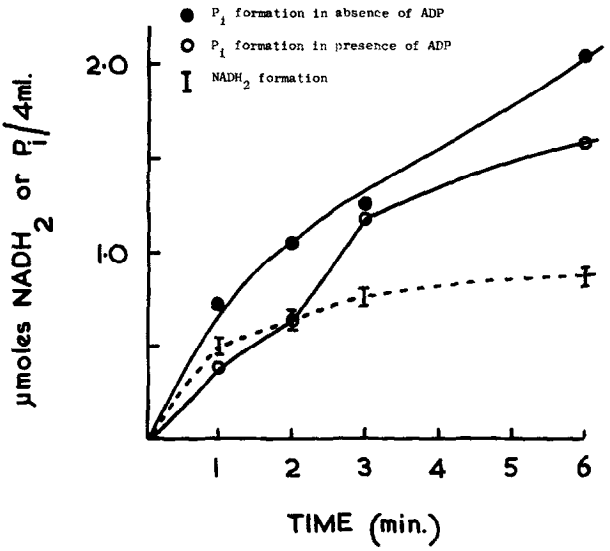

FIGURE 1.

The incubation mixture contained 3.18 μmoles of the phosphorylated intermediate; 200 μmoles Tris-sulphate buffer, pH 7.4; 4 μmoles $MgCl_2$; 20 μmoles glucose; 0.5 μmoles ADP; 250 μg crystalline hexokinase; Antimycin A, 5 μg; sub-mitochondrial particles which had been treated with Antimycin A, 0.92 mg protein; total volume 4.0 ml. Temperature 20 - 22°. The experimental procedure is described in the footnote to Table 2.

(b) On incubation with high levels of ADP the rate of $NADH_2$ formation was essentially the same as with low ADP concentrations whereas the rate of P_i release showed a marked decrease. In the presence of a glucose-hexokinase trap, rapid ATP formation occurred (Table 2). The formation of ATP was inhibited by Oligomycin but no effect on $NADH_2$ formation was observed.

TABLE 2

Formation of ATP from the phosphorylated intermediate

Experimental: The incubation mixture contained 1.59 µmoles of the phosphorylated intermediate; 100 µmoles Tris-sulphate buffer, pH 7.4; 2 µmoles $MgCl_2$; 10 µmoles glucose; 5 µmoles ADP; 100 µg crystalline hexokinase; 5 µg Antimycin A; sub-mitochondrial particles (ETP_H) which had been treated with Antimycin A - 0.46 mg protein; total volume 2.0 ml. Temperature 20 - 22°.

The reaction was carried out in a cuvette placed in an Optica CF4 recording spectrophotometer and initiated by addition of enzyme. $NADH_2$ formation was followed by the increase in optical density at 340 mµ and its identity was checked by reaction with acetaldehyde/alcoholdehydrogenase and pyruvate/lactic dehydrogenase. Aliquots were removed for assay of glucose 6-phosphate by the Zwischenferment assay and phosphate by the Fiske-Subbarow method and pipetted into perchloric acid and acid molybdate, respectively.

	$NADH_2$ formed µmoles/2 min.	ATP formed µmoles/2 min.
A. Complete system	0.312	0.695 (0.590)
B. Plus 5 µg Oligomycin	0.322	0.105 (0.0)*
C. Minus hexokinase	0.226	0.0

* The Oligomycin-insensitive formation of ATP is probably due to the myokinase activity of sub-mitochondrial particles. The figures in the brackets are corrected for this activity.

When Mg^{++} was omitted from the incubation mixture no detectable amounts of $NADH_2$ or ATP were observed. No ATP or $NADH_2$ was formed with samples of

the intermediate where complete breakdown to inorganic phosphate had already occurred. The reaction products of both the non-enzymic and enzymic reactions included a derivative of NAD which has maximal absorption in the region of 290-295 mμ. This compound may be related to the 'hydrated' form of $NADH_2$ described by Meinhart, Chaykin & Krebs (1956) which has been suggested to be a compound formed by opening of the nicotinamide ring (Burton, 1960).

The formation of this new phosphorylated derivative of NAD on incubation of mitochondria with succinate and the further formation of ATP and $NADH_2$ leads us to believe that this compound is a high energy intermediate in oxidative phosphorylation which also plays a key role in the reduction of mitochondrial NAD by succinate. The compound is as yet only partially characterised but analysis of the spectral changes which occur on hydrolysis coupled with the fact that one of the products of the reaction is similar to the compound described by Meinhart et. al. (1956) leads us to suggest that the formation of this compound involves opening of the nicotinamide ring.

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