EVIDENCE FOR A PHOSPHORYLATED INTERMEDIATE IN OXIDATIVE PHOSPHORYLATION.*

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In the course of investigations of the general properties of phosphorylating sub-mitochondrial particles from heart mitochondria, some discrepancies in the assay of inorganic phosphate (P_i) in the presence of NAD were noted. (Griffiths, 1962). These observations led to the following investigations which have demonstrated the presence of a phosphorylated intermediate in oxidative phosphorylation which is a new derivative of NAD.

On incubation of heart mitochondria with $^{32}P_i$, NAD and succinate for short periods at 0° a highly labile ^{32}P-labelled derivative of NAD accumulates which was detected by paper chromatography and paper electrophoresis (Figure 1). The compound is well separated from both NAD and P_i by both methods. The radioactive, ultra-violet absorbing spot was cut out and the compound eluted. NAD was detected in the eluate by the alcohol dehydrogenase assay and P_i estimated by the Fiske and Subbarow method. The content of NAD and P_i as assayed by these methods increased with time, suggesting the breakdown of a labile phosphate derivative into NAD and P_i. When the solution was rechromatographed only a ultra-violet spot corresponding to NAD and a radioactive spot corresponding to P_i was observed, again suggesting the breakdown of a labile phosphorylated derivative of NAD. This phosphorylated derivative is not NADP. Figure 1 shows that the compound is not detected if the incubation medium does not contain succinate. A number of other substrates, including pyruvate,

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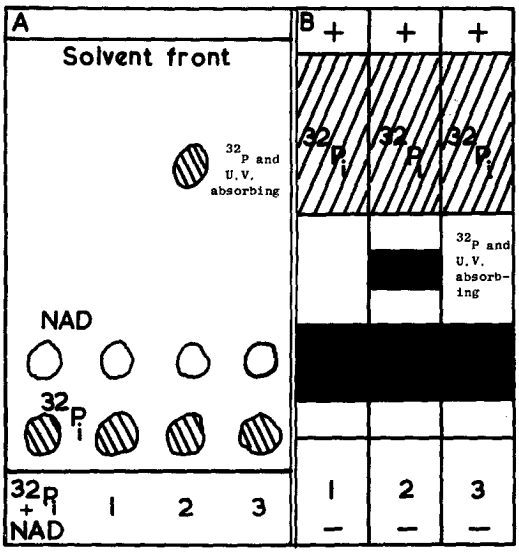

Figure 1. Paper chromatography and paper electrophoresis of deproteinised supernatants from Step 1 (see Table 1 for details of incubation mixture) 1 = succinate omitted; 2 = complete system; 3 = complete system + 5μg Antimycin A. A - Paper chromatography in isobutyric/ammonia/water (Krebs & Hems, 1953) B - Paper electrophoresis; Whatman 3mm or 17 paper, 0.05 M triethanolamine HCl buffer pH 7.2, 6-8 ma, 6-8hr. This method is preferable as the intermediate breaks down rapidly during paper chromatography.

α-oxoglutarate and glutamate have been tested but so far only succinate has been found to be effective. The compound is not detected if electron transport is blocked by Antimycin A. Thus this new derivative of NAD satisfies the requirement of a phosphorylated intermediate in oxidative phosphorylation, i.e. it is a phosphorylated derivative of a oxido-reduction component in the respiratory chain whose formation is dependent on electron transport.

The ability to form ATP from the phosphorylated intermediate was demonstrated as follows. After protein precipitation, an aliquot of the incubation mixture containing the phosphorylated derivative of NAD was transferred to a second incubation medium containing ADP, Mg^{++}, Antimycin A (5-10μg) and mitochondria or phosphorylating sub-mitochondrial particles (Linnane & Ziegler, 1958) which had been treated with Antimycin A (1 μg/mg protein). (For convenience we will refer to the first incubation, the formation of the intermediate, as Step 1 and the second incubation, the transphosphorylation

step as Step 2). Under conditions where the phosphorylated intermediate is formed in Step 1, ATP and $NADH_2$ were detected in Step 2. The relative amounts of ATP and $NADH_2$ were variable but the formation of both compounds was always observed (Table 1). Increased formation of ATP, as measured by glucose-6-phosphate formation, was observed in the presence of a glucose-hexokinase trap especially when sub-mitochondrial particles were used as these particles contain an active ATP-ase. The formation of ATP and $NADH_2$ has also been demonstrated with highly purified preparations of the intermediate.

TABLE 1

ATP and $NADH_2$ formation from the phosphorylated intermediate

Omissions or additions to Step 1** or Step 2** as indicated	Step 1 plus Step 2 ATP formed+ μmoles	Step 1 plus Step 2 $NADH_2$ formed+ μmoles
1 (a) Step 1 (- succinate)	0.00	0.00
(b) Step 1 (- phosphate)	0.00	0.00
(c) Step 1 (+ Antimycin A, 5 μg)	0.00	0.00
(d) Step 1 (none)	0.08-0.56*	0.06-0.3*
(e) Step 1 (+ p-CMB, 0.01 mM)	0.00	0.00
2 (a) Step 2 (none)	0.14	0.1
(b) Step 2 (+ 2 μg Oligomycin)	0.00	0.07

* Results of 10 experiments

** Incubation mixture for Step 1: 100 μmoles Tris-sulphate buffer, pH 7.4; 10 μmoles NAD; 5 μmoles potassium phosphate buffer, pH 7.3 containing $^{32}P_i$; 5 μmoles succinate; heart particles, 2.5-3.0 mg. protein. Total volume 1.7 ml. Incubated 3 min. at 0-1°.

** Incubation mixture for Step 2: 0.85 ml. of deproteinised supernatant from Step 1, 2 μmoles ADP; 1 μmole $MgCl_2$; 5 μmoles Tris-sulphate, pH 7.4; Antimycin A 5 μg; heart particles treated with Antimycin A, 1.25-1.5 mg. protein. Total volume 1.0 ml. Incubated 3-5 min. at 37°.

\+ $NADH_2$ was estimated by its absorption at 340 mμ and reaction with with acetaldehyde and alcohol dehydrogenase.

\+ ATP was assayed by specific enzyme methods using:
(a) Glyceraldehyde 3-phosphate dehydrogenase and 3-phosphoglyceric kinase;
(b) Hexokinase and glucose-6-phosphate dehydrogenase;
(c) Firefly luciferin-luciferase assay.

While the formation of the intermediate in Step 1 is inhibited by Antimycin A, a specific inhibitor of electron transport, the formation of ATP in Step 2 is inhibited by Oligomycin, a specific inhibitor of transphosphorylation reactions associated with oxidative phosphorylation, but not by Antimycin A. These results demonstrate the separation of the reaction mechanism at one of the sites of oxidative phosphorylation into a step involving electron transport and generating a high energy phosphate intermediate (Step 1), and a transphosphorylation step leading from this intermediate to ADP (Step 2). The phosphorylated intermediate has been purified and partially characterised. It is a hitherto undescribed phosphorylated derivative of NAD, containing equimolar amounts of NAD and phosphate, which is highly labile and which breaks down to form NAD and inorganic phosphate. The relationship of this compound to a high energy derivative of NAD reported by Pinchot (1960) and the "extra DPN" of Purvis (1960) is being investigated. Some chemical properties and enzymic reactions involving this new compound are described in the accompanying communication.

References

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