NAD^+ INDUCED NUCLEOSIDE SPECIFICITY OF OXIDATIVE PHOSPHORYLATION

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P/O ratios obtained in submitochondrial systems with succinate or ascorbate plus TMPD as substrate and with IDP as phosphate acceptor were considerably decreased by the addition of NAD^+. With ADP as phosphate acceptor the P/O ratios remained unchanged under similar conditions. The NAD^+ effect was not due to the introduction of an energy-requiring NAD^+ reduction. The effect was abolished by rotenone when succinate was the substrate and by antimycin A when ascorbate plus TMPD were used as the source of electrons. The effect is suggested to be due to a direct interaction between the pyridine nucleotide and non-heme iron components of the respiratory chain.

Submitochondrial particles obtained by sonic treatment of heavy beef heart mitochondria can utilize IDP or GDP as phosphate acceptor provided succinate or ascorbate plus TMPD is the substrate (Löw et al 1963a, Vallin and Lundberg, 1969). The phosphorylative efficiency thus obtained is about 40 per cent of that where ADP is the acceptor with the same substrates. Addition of NADH instead of succinate does not increase the P/O ratio when a nucleoside diphosphate other than ADP is the acceptor. Nucleosidetriphosphates other than ATP can supply energy for the succinate linked NAD^+ reduction, but this low activity is to a major extent due to a nucleoside diphosphate kinase reaction involving the endogenous adenine nucleotide pool. The fact that a much higher nucleoside specificity could be demonstrated when NAD^+ or NADH was present then in other systems made us investigate the influence of pyridine nucleotides on oxidative phosphorylation reactions where succinate or TMPD plus ascorbate was the substrate, especially since

endogenous mitochondrial NAD^+ is lost during preparation of the particles.

MATERIALS

Thionicotinamide-adenine dinucleotide and nicotinamide-hypoxanthine dinucleotide were purchased fromPABST laboratories, Milwaukee, Wis., U.S.A. and other nucleotides from Sigma Chemical Co., St. Louis, Mo., U.S.A. Hexokinase was bought from Boehringer and Soehne Gmbh, Mannheim, Germany and N,N,N',N'tetramethyl-p-phenylene diamine (TMPD) from Eastman Organic Chemicals, Rochester, N.Y., U.S.A.

METHODS

Submitochondrial particles were prepared according to Löw and Vallin (1963a) with the minor modifications introduced by Vallin (1968). Protein content was determined according to Gornall et al (1949). Rates of respiration were measured polarigraphically at 30°C in a basic medium containing 50 mM Glycyl-glycine buffer (pH 7.5), 6 mM $MgCl_2$ 0.167 M sucrose, 3.33 mM phosphate buffer (pH 7.5) 2 mM nucleosidediphosphate as indicated, 20 mM glucose, 6.6 units of dialyzed hexokinase and 0.33 mg of particle protein per ml. 3.3 mM sodium succinate or 5 mM ascorbate plus 0.3 mM TMPD was added as substrate giving a final volume of 3.0 ml. Phosphate esterification was determined by the ^{32}P distribution method described by Lindberg and Ernster (1956).

RESULTS

NAD^+ added in increasing concentrations to oxidative phosphoryltion systems where succinate or ascorbate plus TMPD was the substrate and IDP the phosphate acceptor decreased the P/O ratios considerably, table 1. A similar effect was obtained when GDP was the phosphate acceptor in t these systems. When ADP was used as acceptor, the addition of NAD^+ had no effect however. Under conditions where the NAD^+ effect was obtained there was no measurable reduction of NAD^+ as checked fluorimetrically.

Since hexokinase reacts with ITP less efficient than with ATP

(Kaplan, 1957), the decreased P/O ratio could reflect a change of equilibrium due to a competition between an energy dependent reversed

TABLE I

Effect of NAD^+ on oxidative phosphorylation with succinate or ascorbate plus TMPD as the substrate

	NAD^+ concentration	Phosphorylative capacity (P/O ratio) with succinate	with ascorbate TMPD
	(M)	μmoles P_i esterified/min/mg protein	
IDP	-	0.110 (0.54)	0.112 (0.41)
	10^{-4}	0.090 (0.35)	0.027 (0.05)
	$5 \cdot 10^{-4}$	0.055 (0.25)	0.031 (0.06)
	10^{-3}	0.059 (0.28)	0.072 (0.13)
ADP	-	0.237 (1.19)	0.374 (0.67)
	$5 \cdot 10^{-4}$	0.234 (1.24)	0.346 (0.63)

Experimental conditions as described in Methods plus NAD^+ added in concentrations indicated.

TABLE II

Influence of hexokinase on the NAD^+ effect exerted on IDP phosphorylation

	NAD^+ concentration	Hexokinase concentration	Phosphorylative capacity (P/O ratio)
	(M)	units/ml	μmoles P_i esterified/min/mg protein
IDP	-	6.6	0.097 (0.47)
	10^{-4}	3.3	0.057 (0.27)
	10^{-3}	3.3	0.18 (0.07)
	10^{-4}	6.6	0.043 (0.19)
	10^{-3}	6.6	0.017 (0.07)
	10^{-4}	13.3	0.040 (0.17)
	10^{-3}	13.3	0.016 (0.07)

Experimental conditions as described in Methods with 3.3 mM succinate as substrate and 2mM IDP added. Hexokinase concentration varied and NAD^+ added as indicated.

TABLE III

Effect of rotenone on pyridine nucleotide induced decrease of IDP phosphorylation with succinate as substrate

Pyridine nucleotide concentration		Phosphorylative capacity (P/O ratio) - rotenone	+ rotenone
	(M)	(μmoles P_i esterified/min/mg protein)	
NAD^+	-	0.116 (0.53)	0.092 (0.47)
	$5 \cdot 10^{-5}$	0.076 (0.36)	0.093 (0.52)
	10^{-4}	0.058 (0.29)	0.072 (0.43)
	$5 \cdot 10^{-4}$	0.059 (0.28)	0.074 (0.46)
	10^{-3}	0.042 (0.21)	0.081 (0.48)
NADH	-		0.123 (0.66)
	$5 \cdot 10^{-5}$		0.140 (0.67)
	10^{-4}		0.110 (0.55)
	$5 \cdot 10^{-4}$		0.101 (0.50)
	10^{-3}		0.078 (0.43)

Conditions as described in Methods with NAD^+ or NADH added in concentrations indicated and 0.4 nanomoles of rotenone when added.

TABLE IV

Effect of rotenone and antimycin A on NAD^+ induced decrease of IDP phosphorylation with ascorbate TMPD as substrate

	Concentration of NAD^+ added	Phosphorylative capacity (P/O ratio) - inhibitor	+ inhibitor
	(M)	(μmoles of P_i esterified/min/mg protein)	
rotenone	-	0.244 (0.40)	0.235 (0.34)
	$5 \cdot 10^{-5}$	0.057 (0.10)	0.170 (0.27)
	10^{-4}	0.077 (0.12)	0.192 (0.31)
	$5 \cdot 10^{-4}$	0.074 (0.11)	0.114 (0.17)
	10^{-3}	0.019 (0.03)	0.137 (0.23)
antimycin A	-	0.597 (0.37)	-
	$5 \cdot 10^{-5}$	0.510 (0.22)	0.529 (0.37)
	10^{-4}	0.469 (0.13)	0.499 (0.33)
	$5 \cdot 10^{-4}$	0.491 (0.08)	0.484 (0.35)
	10^{-3}	0.529 (0.27)	0.495 (0.36)

Conditions as described in Methods. NAD^+ added in concentrations indicated and antimycin A as 0.3 μg/mg protein.

electron transfer and the high energy generating capacity. The existence of such a competition was however less likely since a variation in hexokinase concentration was without influence on the reduced phosphorylative capacity in presence of NAD^+, table 2.

With rotenone present, the NAD^+ induced P/O decrease was almost completely abolished. NADH added to a rotenone inhibited system gave a 35 per cent decrease in phosphorylative capacity as a maximum, table 3, compared to an average of about 65 per cent with NAD^+. Where ascorbate plus TMPD was the substrate, rotenone had only a slight effect on the NAD^+ induced effect, which on the other hand was abolished by an addition of antimycin A, table 4. There was no effect of NADH in this antimycin A inhibited system.

TABLE V

Effect of pyridine nucleotides with varied configration

Concentrations added	Phosphorylative capacity (P/O ratio) NAD^+	NHD^+	$TNAD^+$	$NADP^+$
(M)	μmoles P_i esterified/min/mg protein			
-	0.127 (0.64)	0.127 (0.64)	0.127 (0.64)	0.158 (0.58)
$5 \cdot 10^{-5}$	0.113 (0.54)	0.084 (0.41)	0.107 (0.58)	0.181 (0.69)
10^{-4}	0.081 (0.40)	0.080 (0.39)	0.105 (0.56)	0.141 (0.52)
$5 \cdot 10^{-4}$	0.064 (0.31)	0.062 (0.30)	0.09 (0.53)	0.111 (0.44)

Conditions as described in Methods with succinate as substrate and IDP as phosphate acceptor, NHD^+ denotes nicotinamide-hypoxanthine dinucleotide and $TNAD^+$ thionicotinamide-adenine dinucleotide.

The structural configuration of NAD^+ had some importance for the effect obtained. A change of the adenine part of the molecule to hypoxanthine did not influence the P/O reducing effect, table 5. With sulphur replacing oxygen in the carboxyl group of the nicotinamide part of the molecule no effect on the P/O ratios was achieved. These two NAD^+ homologues were reduced at the same rate during ATP dependent succinate linked NAD^+ reduction as shown previously (Löw and Vallin,

1963a). The effect of $NADP^+$ when added instead of NAD^+ was less marked or of about the same order as with NADH in the presence of rotenone. These particles are unable to reduce $NADP^+$ directly without NADH present to complete a transhydrogenase system (Danielson and Ernster, 1963). Nicotinamide or nicotinamide mononucleotide in oxidized or reduced form could not mimic the effect of NAD^+.

DISCUSSION

The marked decrease in capacity to phosphorylate IDP or GDP, induced by the addition of NAD^+ and some of its homologues to submitochondrial particles respiring with succinate or ascorbate plus TMPD as substrate can not be due to the introduction of a substantial energy requiring NAD^+ reduction. That rotenone abolished the NAD^+ effect when succinate was the substrate could suggest such a possibility. With ascorbate plus TMPD as substrate however, the effect exerted by NAD^+ was unchanged by the addition of rotenone whereas the presence of antimycin A extinguished the influence of NAD^+. This would sooner indicate a direct interaction between NAD^+ and, depending on the substrate used, a rotenone or antimycin A sensitive component. Earlier investigations concerning the effect of piericidin A have made us suggest a hypothesis where a non-heme iron component is believed to be involved in the generation of a primary high energy complex (Vallin and Löw, 1968). Such an interference between piericidin A or rotenone and non-heme iron has been verified by Light *et al* (1968) who also demonstrated the role of non-heme iron components in energy conserving reactions. Paramagnetic resonance experiments by Rieske (1964 a and b) and by Palmer *et al* (1968) have indicated a non-heme iron component in the cytochrome *b* - c_1 region of the respiratory chain closely related to the antimycin A sensitive site (see also Vallin and Löw, 1968). We would therefore attribute the effect of NAD^+ to an interference between the pyridine nucleotide and non-heme iron components in the respiratory

chain. The interference exerted upon the non-heme iron components seems to regulate the capacity by which nucleotides other than ADP are phosphorylated in the site which the non-heme iron component precedes. NAD^+ may interfere by formation of a complex with an excess of non-heme iron at the phosphorylation site concerned and thus inducing a respiratory control in a way similar to o-phenantroleine (Butow and Racker, 1965).

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