The Activation of Non-Phosphorylating Electron Transport by Adenine Nucleotides in Jerusalem-Artichoke (*Helianthus tuberosus*) Mitochondria

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In isolated plant mitochondria the oxidation of both succinate and exogenous NADH responded in the expected manner to the addition of ADP or uncoupling agents, and the uncoupled rate of respiration was often in excess of the rate obtained in the presence of ADP. However, the oxidation of NAD⁺-linked substrates responded in a much more complex manner to the addition of ADP or uncoupling agents such as carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone to mitochondria oxidizing pyruvate plus malate failed to result in a reliable stimulation; this uncoupled rate could be stimulated by adding AMP or ADP in the presence of oligomycin or bongkreikic acid. Spectrophotometric measurements showed that the addition of AMP or ADP resulted in the simultaneous oxidation of endogenous nicotinamide nucleotide and the reduction of cytochrome *b*. ADP was only effective in bringing about these changes in redox state in the presence of Mg²⁺ whereas AMP did not require Mg²⁺. It was concluded that AMP activated the flow of electrons from endogenous nicotinamide nucleotide to cytochrome *b*, possibly at the level of the internal NADH dehydrogenase.

Plant mitochondria frequently respond in a complex and often unreliable manner to the addition of ADP or weak acid uncoupling agents. There have been many reports that the addition of adenine nucleotides to uncoupled mitochondria results in the stimulation of electron transport. Wiskich & Bonner (1963) found that both ADP and ATP would stimulate succinic oxidase in the presence of dinitrophenol. They suggested that the stimulation of succinic oxidase under these conditions was due to the removal by ATP of oxaloacetate, a known inhibitor of succinate dehydrogenase; ADP would only become active after conversion to ATP by residual oxidative phosphorylation or by the action of adenylate kinase (Bonner, 1973). Drury *et al.* (1968) reported that the stimulation of electron transport by adenine nucleotides in the presence of dinitrophenol was not sensitive to oligomycin and concluded that it was not necessary to convert ADP into ATP in order to relieve the inhibition of the succinate dehydrogenase. Recent observations (Oestreicher *et al.*, 1973) have shown that the succinic dehydrogenase from plant mitochondria can be activated both by ATP and by ADP, thus explaining why it was unnecessary to convert ADP into ATP in order to bring about the stimulation.

Succinate is not the only substrate whose oxidation failed to respond predictably to the addition of uncoupling agents. The rate of oxidation of NAD⁺-linked substrates has often been found to be slow in the presence of uncoupling agents (Ikuma & Bonner,

1967). Laties (1973) showed that the uncoupled rate of oxidation of citrate, pyruvate-plus-malate or succinate-plus-pyruvate could be stimulated by ADP; he found the stimulation by ADP was insensitive to oligomycin and concluded that ADP was directly involved in bringing about 'uncoupler effectiveness'. Laties (1973) could find no evidence that either ATP or AMP had a similar effect in activating the uncoupled rate of oxidation, thus his conclusions appear to be in conflict with those of Bonner (1973) who stated 'the mitochondria must have had a small amount of ATP in order for the uncoupler to be effective'.

The papers discussed so far suggest that ADP stimulates oxidation in the presence of uncoupling agents. Observations have also been made which suggest that ADP could stimulate electron flow in the absence of uncoupling agents, other than by acting as a phosphate acceptor in oxidative phosphorylation. Raison *et al.* (1973*b*) have described the phenomenon of 'conditioning' in plant mitochondria. The main characteristic of this phenomenon was that, under certain conditions, the rate of oxidation in the presence of ADP, obtained in response to the first limited addition of ADP, was markedly lower than that obtained in response to subsequent additions of ADP. Raison *et al.* (1973*a*) showed that if mitochondria were incubated with substrate and ADP in the absence of phosphate, the rapid rate of oxidation initiated by the addition of phosphate was consider-

ably higher than if the mitochondria were incubated with substrate and phosphate and the oxidation initiated by the addition of ADP. Thus they concluded that incubation of mitochondria with ADP under non-phosphorylating conditions resulted in 'conditioning'. Incubation in presence of ATP did not have a similar effect.

Laties (1973) concluded that the ability of ADP to stimulate the uncoupled rate of oxidation was not dependent on its role in bringing about partial conditioning and thus he considered that ADP had two separate roles in stimulating non-phosphorylating electron flow in plant mitochondria. The purpose of the experiments described in the present paper was to examine the influence of adenine nucleotides on the non-phosphorylating rate of electron transport in Jerusalem-artichoke mitochondria and to attempt to establish where in the metabolic sequence they had their effect.

Materials and Methods

Chemicals

NADH, ATP, ADP, AMP and glutamate-oxaloacetate transaminase (EC 2.6.1.1) were obtained from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany; malate, pyruvate, glutamate, oxoglutarate, citrate, malonate, thiamin pyrophosphate, GMP, IMP and oligomycin were from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K.; sucrose, succinate, EDTA, $MgCl_2$ and potassium arsenite from BDH Chemicals, Poole, Dorset, U.K.; Mops [3-(*N*-morpholino)propanesulphonic acid], Tes [*N*-tris(hydroxymethyl)methyl-2-aminoethanesulphonic acid] and KH_2PO_4 from Hopkin and Williams Ltd., Chadwell Heath, Essex, U.K. Carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone and bongkreikic acid were generous gifts from Dr. P. G. Heytler and Dr. H. C. Passam respectively.

Isolation of mitochondria

Jerusalem-artichoke mitochondria were isolated from the tubers by using the method described by Palmer & Kirk (1974). Rat liver mitochondria were isolated from a 200 g young adult male Sprague-Dawley rat by using the method described by Weinbach (1961).

Activation of succinate dehydrogenase

Mitochondria were incubated at 26°C for 4 min with 20 μ l of 50 mM-ATP, pH 7.2, 20 μ l of 50 mM- P_i (both amounts are per mg of mitochondrial protein) and $MgCl_2$ to a final concentration of 5 mM. After this activation procedure the mitochondria were kept

in an ice bath and the succinate oxidase remained active through the course of each experiment. This method was that of Cowley (R. C. Cowley, personal communication).

Oxygen consumption

Oxygen uptake was measured in a small Rank oxygen electrode (Rank Bros., Bottisham, Cambridge, U.K.) with an internal diameter of 9 mm, in conjunction with a Servoscribe RE 511.20 potentiometric recorder. Reactions were carried out at 25°C. The standard reaction mixture consisted of 0.3 M-sucrose, 5 mM-potassium phosphate, 2.5 mM- $MgCl_2$ and 5 mM-Tes at pH 7.2. Mitochondrial suspension was added to give a final volume of 1 ml in the electrode.

Measurement of the levels of reduction of cytochrome *b* and nicotinamide nucleotide

Measurement of the levels of reduction of cytochrome *b* and nicotinamide nucleotide was carried out in an Aminco DW-2 dual-wavelength spectrophotometer, with a light-path of 1 cm at 432 and 340 nm, with 410 and 374 nm respectively as reference wavelengths. The mitochondria were suspended in the same reaction medium as that used in the oxygen electrode so that the final volume in the cuvette was 1 or 3 ml. Other additions were as indicated in the legends to the Figures.

Protein determination

Mitochondrial protein was determined by the method of Lowry *et al.* (1951) after first solubilizing the protein with deoxycholate; bovine serum albumin was used as standard.

Results

Investigation of succinate oxidase in uncoupled Jerusalem-artichoke mitochondria confirmed the findings of previous authors (Wiskich & Bonner, 1963; Oestreicher *et al.*, 1973) that it was activated by both ATP and ADP but not by AMP. This stimulation appeared to be different from that occurring with NAD^+ -linked substrates reported in the present paper. All assays of succinate oxidase activity reported in the present study were carried out with activated mitochondria, as described in the Materials and Methods section; mitochondria used with other substrates were not activated. The oxygen-electrode traces in Fig. 1 show that when mitochondria were supplied with either added NADH or succinate as the electron donor, the rate of oxygen uptake in the presence of the uncoupler carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone was equal to or

Fig. 1. Influence of the uncoupler carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) on the rate of oxidation

Oxygen-electrode traces showing the effect of the uncoupler ($0.2/\mu\text{M}$) on the rate of oxidation of NADH (1 mM), succinate (10 mM) or pyruvate (15 mM) plus malate (1 mM). When pyruvate-plus-malate were used as the substrate, $170\mu\text{g}$ of thiamin pyrophosphate was included in the reaction medium. The mitochondria were suspended in a total reaction volume of 1.0 ml and the respiration was stimulated by adding 100 nmol of ADP or the uncoupler ($0.2/\mu\text{M}$). For the oxidation of NADH or pyruvate-plus-malate, 1 mg of mitochondrial protein was used per assay, and 0.4 mg was used for succinate oxidation. Values under the traces are the rates of oxygen consumption in nmol of O_2/min per mg of protein.

greater than that obtained in the presence of ADP. The response was of equal magnitude if the uncoupler was added before ADP or after the exhaustion of a pulse of ADP (i.e. state 4 as defined by Chance & Williams, 1956). In contrast the oxidation of pyruvate-plus-malate was not fully stimulated by the uncoupler; this was especially apparent if the uncoupler was added before ADP. The rate of oxidation of pyruvate-plus-malate in the presence of the uncoupler was characterized by an initial fairly rapid phase followed by a second, more prolonged, slower phase. The addition of ADP or AMP 1 min after the uncoupler, i.e. in the early stages of the slow phase, resulted in a marked stimulation of the uncoupled rate. The addition of ATP was without effect. Fig. 2 shows that AMP was apparently more efficient than ADP in bringing about the stimulation. Parallel studies showed that GMP or IMP did not have a similar effect. Studies using oligomycin (Fig. 3) and bongkrekic acid (Table 1) showed that whereas these compounds readily inhibited the rate of oxidation in the presence of ADP (i.e. state 3 as defined by Chance & Williams, 1956) they were completely without effect on the adenine nucleotide-enhanced rate in the presence of the uncoupler.

The effect of AMP on the uncoupled rate of oxidation of other substrates is given in Table 2. Inhibitors were added where necessary to prevent the further oxidation of the products of the principal reaction from complicating the result. Stimulation was only observed when substrates using the endogenous nicotinamide-nucleotide pool were supplied.

In some of our preparations we observed the phenomenon of 'conditioning' (already defined in the introduction) described by Raison *et al.* (1973b). When conditioning was apparent we noticed that the rate of oxygen uptake obtained in the presence of substrate and no added adenine nucleotide was very much lower than the subsequent rate in state 4. This is apparent in trace (a), Fig. 4. The slow pre-ADP rate could be stimulated to a rate similar to that in state 4, in the presence of oligomycin, either by ADP or by AMP (Fig. 4). When succinate or exogenous NADH were supplied as the substrate the pre-ADP rate (hereafter referred to as the 'substrate rate') was not significantly different from the rate in state 4, and neither AMP nor ADP had any effect when added after oligomycin.

Attempts were made to find the locus of the stimulation brought about by AMP or ADP in the

Fig. 2. Stimulation of the uncoupled rate of oxidation of pyruvate-plus-malate by different concentrations of adenine nucleotides

The rates of oxygen uptake were calculated from oxygen-electrode traces. Mitochondrial protein (1 mg) was suspended in 1.0 ml reaction volume; the substrate added was pyruvate (15 mM) plus malate (1 mM) and 170 μg of thiamin pyrophosphate. The uncoupler carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) (0.2 μM) was added 1 min after the substrate and appropriate amounts of ADP (●) or AMP (○) were added 1 min after the uncoupler. The rate in the presence of uncoupler and absence of adenine nucleotide was taken to be that immediately before adding the nucleotide.

Fig. 3. Influence of oligomycin on the ADP-stimulated oxidation of pyruvate-plus-malate in the presence or absence of the uncoupler carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP)

Jerusalem-artichoke mitochondria (1 mg of protein) were suspended in 1.0 ml reaction volume containing pyruvate (15 mM) plus malate (1 mM) and 170 μg of thiamin pyrophosphate. When measuring the control treatment (○) 125 nmol of ADP was added 2 min after the substrate and the appropriate amount of oligomycin added once a linear state-3 rate had been obtained. When measuring the effect in the presence of the uncoupler (0.2 μM) (●) the uncoupler was added 1 min after the substrate and the ADP (125 nmol) was added 1 min after the uncoupler. The 100% values found in this experiment were 56 nmol of O₂/min per mg of protein for the control and 35 nmol of O₂/min per mg of protein in the presence of the uncoupler.

Table 1. Effect of bongkreikic acid (BA) on the stimulation of mitochondrial protein by AMP

The rates of oxygen uptake of Jerusalem-artichoke mitochondria were measured polarographically in a 1 ml-volume electrode. Mitochondria were suspended in reaction medium containing 0.3 M-sucrose, 5 mM-Tes, 5 mM-phosphate and 2.5 mM-MgCl₂ at pH 6.8 in the electrode for 10 min with or without bongkreikic acid at 2.5 nmol/mg of protein. The substrates were added to start respiration. In Expt. 1, 100 nmol of AMP was added to bring about state-3 rates and the uncoupler carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) (0.1 μM) was added in state 4. In Expt. 2, AMP was added 1 min after the uncoupler, and the uncoupled rates reported are those immediately before additions of AMP. For NADH oxidation, 0.43 mg of mitochondrial protein was used per assay and 0.86 mg for malate oxidation. The 'substrate rate' is that produced when substrate was added to mitochondria before the nucleotide addition.

		O ₂ uptake (nmol/min per mg of protein)							
		Expt. 1				Expt. 2			
Substrate		Substrate rate	State 3	State 4	Uncoupled (FCCP)	Substrate rate	Uncoupled rate	Uncoupled + AMP	
1.2 mM-NADH	-BA	75	156	85	188	75	206	206	
	+BA	61	61	—	160	60	157	154	
25 mM-Malate (potassium salt) + 10 mM glutamate	-BA	52	83	43	94	55	85	97	
	+BA	37	43	—	81	36	40	62	
+20 μg glutamate-oxaloacetate trans-aminase									

Table 2. Influence of AMP on the uncoupled rate of oxidation of various substrates

Substrates at the specified final concentrations were added to Jerusalem-artichoke mitochondria suspended in 1.0ml of reaction mixture; where indicated, thiamin pyrophosphate (TPP) or inhibitors were added before the reaction was started. In each assay 1 mg of mitochondrial protein was used, except when measuring the oxidation of succinate when 0.4 mg was used. The uncoupler carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) (0.2/ μ M) was added 1 min after the substrate and AMP (75 μ M) was added 1 min after the uncoupler. When NAD⁺-linked substrate was used, the rate of oxygen uptake in the absence of uncoupler or in the presence of the uncoupler and AMP was approximately linear with time. The rate in the presence of the uncoupler alone often decreased with time and the rate quoted was that obtained immediately before the addition of AMP.

Substrate	Rate of oxygen uptake (nmol of O ₂ /min per mg of protein)		
	-FCCP	+FCCP	+FCCP+AMP
1 mM-NADH	49.2	174.4	174.4
10 mM-Succinate	60	186	186
15 mM-Pyruvate+1 mM-malate+170 μ g of TPP	22	26.2	50.3
10 mM-2-Oxoglutarate+170 μ g of TPP	7.7	11.6	57.9
10 mM-2-Oxoglutarate+170 μ g of TPP+0.5 mM-malonnate	6.8	6.8	27
10 mM-Citrate	15	26	39
10 mM-Citrate+1 mM-arsenite	—	26	39
100 mM-Malate+170 μ g of TPP	12	29	57
100 mM-Malate+170 μ g of TPP+1.5 mM-arsenite	12	18	34.7

Fig. 4. Influence of adenine nucleotides on the rate of oxidation in presence of oligomycin and absence of carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone

Jerusalem-artichoke mitochondria (Mito.) (1 mg of protein) were added to 1.0 ml of reaction mixture containing pyruvate (15 mM) plus malate (1 mM) and 170 μ g of thiamin pyrophosphate. Oligomycin was added when necessary at the rate of 1.2 μ g per mg of protein. The respiration was then stimulated by adding either ADP or AMP as indicated. The values under the oxygen-electrode traces are the rates of oxygen consumption in nmol/min per mg of protein. (a) Control; (b) and (c), +oligomycin.

electron-transport chain. Measurements of the level of reduction of cytochrome *b* (Fig. 5) revealed the unexpected observation that the addition of pyruvate-plus-malate to Jerusalem-artichoke mitochondria

failed to reduce cytochrome *b* to any great extent (trace b), whereas in a similar experiment using rat liver mitochondria (trace a) the same substrate resulted in a marked reduction of cytochrome *b*. The

Fig. 5. Reduction of cytochrome *b* by pyruvate-plus-malate in either rat liver (a) or Jerusalem-artichoke (b) mitochondria

Trace (a), rat liver mitochondria (100 μ l containing 3 mg of protein) were added to the cuvette containing 2.9 ml of reaction medium (0.1 M-KCl, 0.025 M-sucrose, 0.01 M-MgCl₂, 0.001 M-EDTA, 510 μ g of thiamin pyrophosphate, 0.005 M-Tes and 0.01 M-KH₂PO₄ at pH 6.8). The additions during the experiment were pyruvate (15 mM) plus malate (1 mM) and ADP (0.5 μ mol). Reduction of cytochrome *b* was measured in an Aminco DW-2 dual-wavelength spectrophotometer at 432 nm (with 410 nm as the reference wavelength) a band-pass of 1 nm and light-path of 1 cm. Trace (b), Jerusalem-artichoke mitochondria (100 μ l containing 2.5 mg of protein) were added to 2.9 ml of standard reaction mixture, as defined in the Materials and Methods section, plus 510 μ g of thiamin pyrophosphate. The additions and conditions of measurement were as defined for trace (a). Increase in absorbance indicates reduction of cytochrome *b*.

addition of ADP to the two types of mitochondria had markedly different effects on cytochrome *b*, whereas the rate of oxygen uptake in both types increased. It resulted in oxidation of cytochrome *b* in rat liver mitochondria, presumably because respiratory control was released, allowing electrons to flow out from cytochrome *b*. In Jerusalem-artichoke mitochondria the response was exactly opposite, suggesting that ADP allowed electrons to flow more readily into cytochrome *b*. Once the ADP had become converted into ATP by oxidative phosphorylation, respiratory control was imposed and cytochrome *b* became further reduced as expected. In these mitochondria, cytochrome *b* could not be kept reduced long after the exhaustion of ADP; the absorbance slowly decreased until oxygen was all consumed and the cytochrome became fully reduced (Fig. 5). In rat liver mitochondria the level of reduction of cytochrome *b* was constant in state 4 until it became further reduced when oxygen was exhausted. Fig. 6 shows the changes in the level of reduction of cytochrome *b* when either ADP or AMP was added in the absence or presence of oligomycin. The addition

of both ADP and AMP resulted in the rapid reduction of cytochrome *b* in the presence of oligomycin, indicating that the enhanced flow of electrons into cytochrome *b* was independent of oxidative phosphorylation. In the absence of oligomycin the response to AMP was more complex than the response to ADP. AMP caused a more rapid and more extensive reduction of cytochrome *b* than did ADP. The slow oxidation to the state-3 level which followed this initial rapid phase of reduction could be due to the conversion of AMP into ADP via endogenous adenylate kinase and ATP. The ADP thus produced could then act as a phosphate acceptor in oxidative phosphorylation. If the experiment was repeated in the presence of oligomycin, but in the absence of Mg²⁺ (Fig. 7), only AMP was capable of promoting the reduction of cytochrome *b*, thus it seemed as if AMP was primarily responsible for promoting electron flow into cytochrome *b*, and ADP was only active if converted into AMP via the enzyme adenylate kinase.

To establish the mechanism by which AMP induced the reduction of cytochrome *b* and to

Fig. 6. Effect of oligomycin and adenine nucleotides on the reduction of cytochrome *b*

Jerusalem-artichoke mitochondria (Mito.) (100 μ l containing 2 mg of protein) were added to the cuvette containing 2.9 ml of standard reaction medium plus 510 μ g of thiamin pyrophosphate. The amount of oligomycin, when present (traces *a* and *c*), was 2.5 μ g per cuvette. Additions made during the experiments were pyruvate (15 mM) plus malate (1 mM) and 0.5 μ mol of ADP (traces *a* and *b*) or AMP (traces *c* and *d*). Other conditions of assay were as described in Fig. 5.

investigate whether this phenomenon was related to the stimulation of electron transport by AMP in the presence of carbonyl cyanide *p*-trifluoromethoxy-

phenylhydrazone, simultaneous measurements of the level of reduction of endogenous nicotinamide nucleotide and cytochrome *b* were made. The traces of both components shown in Fig. 8 were obtained during consecutive runs using malate in the presence of glutamate, and glutamate-oxaloacetate transaminase to remove oxaloacetate. This substrate system ensured the efficient operation of the malate dehydrogenase (EC 1.1.1.37) which is believed not to be allosterically regulated by adenine nucleotides. The experiment was also carried out in the presence of carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone and oligomycin to prevent any interaction between the phosphorylating system and the redox components. The traces (Fig. 8) show that the addition of AMP resulted in the immediate reduction of cytochrome *b* and oxidation of the nicotinamide nucleotide.

Discussion

Results presented in the present paper suggest that the ability of adenine nucleotides to stimulate the oxidation of succinate or NAD⁺-linked substrates in the presence of uncoupling agents is the result of different mechanisms. The oxidation of succinate could be stimulated by ATP and ADP or partially stimulated by using glutamate and glutamate-oxaloacetate transaminase. Thus it would appear that the stimulation resulted from the removal of an inhibition caused by oxaloacetate. The oxidation of NAD⁺-linked substrates was stimulated by ADP or AMP whereas ATP was ineffective. All of our results point to AMP and not ADP being primarily responsible for activating the uncoupled rate of pyruvate oxidation. First, AMP was effective at lower concentrations than ADP; second, the rate of reduction of cytochrome *b* was more rapid when induced by adding AMP than by ADP. Finally the most compelling evidence was that ADP was less effective in stimulating respiration and reducing cytochrome *b* if Mg²⁺ was omitted from the medium. The lack of Mg²⁺ would prevent the activity of adenylate kinase from converting ADP into AMP. These observations suggest that ADP had to be converted into AMP before it was able to activate the uncoupled flow of electrons from NAD⁺-linked substrates. We cannot explain why our observations differ from those of Laties (1973), who reported that AMP would not stimulate in the presence of uncoupler. We repeated the experiments by using potato (*Solanum tuberosum*) mitochondria, as used by Laties, but nevertheless found AMP active. However, it seems significant that Laties (1973) found that the activation by ADP was second-order with respect to the concentration of ADP; thus the adenylate kinase enzyme system may also be active in bringing about the stimulation under the conditions he used.

Fig. 7. Effect of Mg^{2+} and adenine nucleotides on the reduction of cytochrome *b*

Jerusalem-artichoke mitochondria (Mito.) (100 μ l containing 2 mg of protein) were added to the cuvette containing 2.9 ml of magnesium-free reaction medium containing 510 μ g of thiamin pyrophosphate and 2.5 μ g of oligomycin. The concentration of Mg^{2+} added was 2.5 mM. Additions made during the experiment were pyruvate (15 mM) plus malate (1 mM) and ADP or AMP (0.5 μ mol). Other conditions of assay were as described in Fig. 5.

Fig. 8. Effect of AMP on the reduction of cytochrome *b* and endogenous nicotinamide nucleotide of Jerusalem-artichoke mitochondria oxidizing malate

In a 1 ml cuvette with 1 cm light-path, mitochondria (Mito.) (2.2 mg of protein) were suspended in standard reaction mixture plus 10 mM glutamate, 20 μ g of glutamate-oxaloacetate transaminase, 3 μ g of oligomycin and 0.1 μ M-carbonyl cyanide *p*-trifluoromethoxyphenylhydrazine. The reduction of cytochrome *b* was followed at 432 nm with 410 nm as the reference wavelength. The reduction of endogenous nicotinamide nucleotide was followed at 340 nm with 3.0 nm slit width and 374 nm as the reference wavelength in an Aminco DW-2 dual-wavelength spectrophotometer (Chance & Williams, 1956).

Our results showed that carbonyl cyanide *p*-trifluoromethoxyphenylhydrazine uncouple oxidative phosphorylation from electron transport efficiently when either succinate or exogenous NADH was supplied as the electron donor. Since electrons from both substrates only traverse two terminal sites

of oxidative phosphorylation (Coleman & Palmer, 1972) it seems that the uncoupler was capable of uncoupling both these sites of oxidative phosphorylation. If AMP is therefore to bring about the stimulation of oxidation of NAD^+ -linked substrates by increasing uncoupler effectiveness, as suggested

by Laties (1973), its effect must be restricted to the first site of phosphorylation, associated with the NADH dehydrogenase. Such a possibility seems unlikely because it is thought that the non-phosphorylated high-energy state associated with each coupling site is ultimately in equilibrium with a common non-phosphorylated high-energy state and therefore efficient uncoupling at one site results in uncoupling at all other sites. It therefore seems more likely to us that AMP is necessary, not to bring about uncoupling, but to have the uncoupled state reflected in an enhanced flux of electrons along the respiratory chain. The observation that addition of AMP resulted in the simultaneous oxidation of the nicotinamide nucleotide pool and reduction of cytochrome *b* is consistent with this theory. It also rules out the alternative theory that the stimulation was the result of the allosteric activation of the NAD⁺-linked dehydrogenase, thereby causing the stimulation by producing more endogenous NADH. The simultaneous oxidation of the nicotinamide nucleotide and reduction of cytochrome *b* by AMP indicate the existence of some form of accessibility barrier (Lee & Slater, 1972; Lambowitz & Bonner, 1974) between these components which is lowered by the addition of AMP. Studies using piericidin A [which inhibits electron transfer between NADH and cytochrome *b* (Jeng *et al.*, 1968)] and antimycin A [which inhibits between cytochrome *b* and *c* (Chance & Williams, 1956)] show that the cytochrome *b* reduced on the addition of AMP was reduced via a piericidin A-sensitive pathway and oxidized via an antimycin A-sensitive pathway, providing evidence that it was a normal member of the electron-transport chain. We thus conclude that the activation of the uncoupled rate of oxidation of NAD⁺-linked substrates by AMP was the result of activation of electron flow through the piericidin A-sensitive NADH dehydrogenase.

We have attempted to demonstrate AMP activation directly by showing that the oxidation of exogenous NADH by submitochondrial particles, the polarity of whose membranes is reversed, was stimulated by AMP. This experiment failed because AMP appears to act on the outside of the inner membrane. It is thought that AMP cannot enter intact mitochondria using the normal adenine-nucleotide translocator (Klingenberg, 1970), thus any stimulation of electron flow caused by AMP, and not ADP, resulting from the action of adenylate kinase situated in the intermembrane space, has to result from AMP interacting on the outside of the inner membrane. This situation is confirmed by the data in Table 1,

which show that the state-3 rate of oxidation, resulting from the formation of ADP from AMP, was inhibited by bongkreic acid whereas the stimulation of the uncoupled rate of malate oxidation was unaffected by bongkreic acid. Since bongkreic acid has been shown to inhibit the adenine-nucleotide translocator in Jerusalem-artichoke mitochondria (Passam *et al.*, 1973) it seems as if AMP does not have to enter the mitochondria to bring about the enhancement of electron flow. This observation could provide the basis of an interesting physiological control mechanism whereby the AMP concentration in the cytosol could regulate the rate of oxidation of NADH inside the inner mitochondrial membrane.

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