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ACTION OF THE ADENOSINE TRIPHOSPHATE ANALOG, ADENYLYL IMICODIPHOSPHATE IN MITOCHONDRIA

by

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ABSTRACT

Adenylyl imidodiphosphate (AMP-PNP), an analog of adenosine triphosphate (ATP), is a potent competitive inhibitor of mitochondrial ATPase activity. It inhibits both the soluble oligomycin insensitive ATPase ($K_i = 9.2 \times 10^{-7}$ M) and the bound oligomycin sensitive ATPase ($K_i = 1.3 \times 10^{-6}$ M). ATPase activity of inside-out submitochondrial preparations are more sensitive to AMP-PNP in the presence of an uncoupler ($K_i = 2.0 \times 10^{-7}$ M). Mitochondrial ATP dependent reactions (reversed electron transfer and potassium uptake) do not proceed if ATP is replaced with AMP-PNP; however, the analog does affect these systems. Oxidative phosphorylation of whole mitochondria and submitochondrial preparations were unaffected by AMP-PNP.

INTRODUCTION

The interaction of adenosine triphosphate (ATP) with rat liver mitochondrial ATPase was studied using adenylyl imidodiphosphate (AMP-PNP), an analog of ATP. Yount et al. (1) synthesized AMP-PNP and showed that it was chemically and structurally similar to ATP, the sole difference being the replacement of the third oxygen bridge of the triphosphate chain with an imido bridge. The analog binds Mg^{++} as does ATP and thus is a suitable replacement for a reaction involving Mg-ATP (1). Another study by Yount et al. (2) showed that AMP-PNP is a strong competitive inhibitor of myosin ATPase. The analog interacts at the ATP binding site of the myosin ATPase; however, it is not hydrolyzed nor does its binding elicit contraction in glycerinated muscle fibers. These findings prompted us to investigate the interaction of AMP-PNP with mitochondrial ATPase, during ATP hydrolysis and ADP phosphorylation, as an indicator of the influence of ATP binding to ATPase during different active states of mitochondria.

METHODS

Preparation of mitochondria, submitochondrial preparations, and F_1-ATPase

Rat liver mitochondria were prepared by the method of Stancliff et al. (3) and submitochondrial preparations were prepared by sonication of the mitochondria according to Kielly and Bronk (4). Soluble F_1-ATPase was prepared by the method of Catterall and Pederson (5).

Abbreviations: AMP-PNP, adenylyl imidodiphosphate; FCCP, carbonylcyanide p-trifluoromethoxy phenylhydrazone.

All systems were studied at 30°C and at pH 8.0. Duke and Morales (6) have noted that AMP-PNP is more similar to ATP at pH 7.5-8.0 than at pH 6.5-7.0. ATP hydrolysis was measured on a Cary 14 spectrophotometer by following the oxidation of NADH at 340 nm in a coupled reaction. The concentrations of reaction components were 5 mM $MgSO_4$, 3.3 mM phosphoenol pyruvate, 0.2 mM NADH, 5 units/ml of lactate dehydrogenase, 8 units/ml of pyruvate kinase, 250 mM sucrose and 50 mM tris-acetate buffer. Membrane-bound ATPase assays were performed in the presence of 2 mM KCN. 1 μM concentration of the uncoupler FCCP was present where indicated.

Reversed electron transfer and mitochondrial phosphorylation

Reversed electron transfer was measured as described by Dannielson and Ernster (7) except that 1 mM ATP was used. Phosphorylation in whole mitochondria was measured polarographically using a Clark oxygen electrode (Chance and Williams (8)). The concentrations of reaction components were, 10 mM KCl, 3 mM $MgCl_2$, 3 mM $NaPO_4$, 5 mM sodium succinate and 5 mM tris buffer; where indicated, ADP was added to make a final concentration of 144 μM.

ATP-dependent potassium uptake

Mitochondrial swelling was measured by observing changes in percent light transmission due to potassium uptake (Gooch and Packer (9)). The concentrations of assay components were 2 mM ATP, 2 μg/ml rotenone, 0.01 μg/ml valinomycin, 28.6 mM potassium acetate, 100 mM sucrose, and 5 mM tris buffer.

Phosphorylation in submitochondrial preparations

To determine the phosphorylation capacity (P/O ratio) of submitochondrial preparations, NADH oxidation was quantitated by measuring the decrease in absorbancy at 340 nm. The ATP formed during NADH oxidation was trapped as glucose-6-phosphate using hexokinase and glucose. The concentrations of reactants were 7 units/ml hexokinase, 17 mM glucose, 2.5 mM $NaPO_4$, 3.3 mM $MgSO_4$, 0.25 mM NADH, 250 mM sucrose, and 50 mM tris buffer. Upon completion of NADH oxidation, EDTA (8 mM) and KCN (2 mM) were added to inhibit adenylate kinase and transhydrogenase, respectively. The ATP that was trapped as glucose-6-phosphate was quantitated by measuring the formation of NADPH (at 340 nm) upon addition of NADP (0.5 mM) and glucose-6-phosphate dehydrogenase (2 units/ml). The ratio of NADPH formed to NADH oxidized was used to indicate the phosphorylation capacity of the preparations. A blank was subtracted to account for adenylate kinase activity during the NADH oxidation. Protein was determined by the method of Lowry et al. (10) using bovine serum albumen as a standard.

MATERIALS

ATP, NADH, pyruvate kinase, lactate dehydrogenase, phosphoenol pyruvate, NAD, rotenone, hexokinase, NADPH, glucose-6-phosphate dehydrogenase, and ADP were purchased from Sigma Chemical Co. Valinomycin was obtained from Calbiochem. FCCP was a gift from DuPont. All other compounds were of reagent grade.

RESULTS

As shown in Figs. 1, 2 and 3 AMP-PNP inhibits mitochondrial ATPase as it inhibits ATP hydrolysis. In all of our studies hydrolysis of AMP-PNP forming ADP was never detected. The analog was found to be a potent competitive inhibitor of the ATP hydrolyzing systems. Figure 1 indicates the effect of AMP-PNP on the hydrolytic activity of the soluble oligomycin-insensitive (F_1) ATPase. The Michaelis constant of 9.7×10^{-4}M is similar to that reported by Hammes and Hilborn (11) for solubilized ATPase from beef heart mitochondria. Our study showed that AMP-PNP was a strong competitive inhibitor of this enzyme with a K_i of 9.0×10^{-7}M.

The effect of AMP-PNP on the bound oligomycin-sensitive ATPase was studied with inside-out submitochondrial preparations made by sonication of whole mitochondria (Figs. 2, 3). (The use of such preparations eliminates the possibility of the analog's affecting translocation of ATP rather than ATP hydrolysis.) ATP hydrolysis by the submitochondrial preparations was insensitive to atractyloside. The ATPase reaction was studied with and without the uncoupler FCCP. In both cases, the analog acted as a strong competitive inhibitor of the ATP hydrolytic reaction. These results (K_i without FCCP = 1.3×10^{-6} M; K_i with FCCP = 2.0×10^{-7} M) indicate that relative affinity of ATPase for the analog differed in the coupled and uncoupled states. The Michaelis constants for the bound ATPase were also similar to those reported for bound ATPase from beef heart mitochondria.

Since AMP-PNP binds to an ATP binding site and was not hydrolyzed, it seemed to be a suitable probe for testing whether the ATP-dependent

reactions were due to ATP hydrolysis or to binding of ATP. AMP-PNP was substituted for ATP to study the ATP-driven reversed electron transfer reaction from succinate to the reduction of NAD^+. In this system NADH did not form. If there is only one ATP binding site, then this finding would discount the possibility that binding of adenine nucleotide at the ATPase activity site represents the driving force, i.e., ATP hydrolysis would be necessary. An interesting observation was noted when ATP was added to this reaction system. In the absence of AMP-PNP, NADH formed almost instantaneously and the reaction went to completion as the ATP became depleted. ATP hydrolysis was determined separately under the same conditions. In the presence of AMP-PNP, the ATPase activity was markedly decreased; however, the effect on the reversed electron transfer was not comparably affected (Fig. 4). In the latter system the reversed electron transfer reaction showed an initial lag, which was dependent on the concentration of analog, followed by NADH formation at a rate only slightly less than the initial rate elicited by the control. The reversed electron transfer reaction also was observed to continue for a longer time as compared to the control, probably because the lower ATPase activity inhibited the depletion of the ATP supply, yet increased the efficiency of ATP-driven NAD^+ reduction.

The effect of the analog was also investigated on an ATP-dependent reaction of whole mitochondria. Potassium uptake driven by ATP was followed by measuring changes in light transmission caused by mitochondrial swelling (Fig. 5). This reaction was dependent on ATP and did not proceed if ATP was replaced by AMP-PNP. However, if the analog was added prior to ATP, then the system was markedly inhibited. The inhibition could be either at the site of ATP hydrolysis or at the

site of translocation of the ATP.

Whole mitochondria were also studied to see whether AMP-PNP interferes with oxidative phosphorylation. As seen in Fig. 6, even at 0.45 mM AMP-PNP, there was no significant change in the phosphorylating capacity of whole mitochondria. If the analog is translocated (12), then the catalytic site of the ATPase has a much greater affinity for ADP than AMP-PNP under phosphorylating conditions.

The possibility still remains that AMP-PNP did not affect oxidative phosphorylation of whole mitochondria because it did not cross the inner mitochondrial membrane during succinate oxidation. To determine the influence of the possible presence of a membrane barrier or of translocation, we studied the effect of AMP-PNP on phosphorylation in "inside-out" submitochondrial preparations (Table I), in which the ATPase is exposed to the external medium. Once again, phosphorylation of ADP was unaffected by the analog. If AMP-PNP can bind at the active site of mitochondrial ATPase, then the lack of detectable inhibition may be due to the enzyme having a higher affinity for ADP. Catterall and Pederson reported a K_M(ADP) of 3.8×10^{-6} M during oxidative phosphorylation of rat liver submitochondrial preparations, a value much lower than the K_M(ATP) for the hydrolytic reaction. This difference makes physiological sense as the primary role of the enzyme is to synthesize ATP.

DISCUSSION

It is generally believed that the ATPase complex in mitochondria functions reversibly in the synthesis and hydrolysis of ATP. The studies reported here show that although the analog, AMP-PNP, interacts

with the ATPase to competitively inhibit the hydrolytic reaction, it does not affect the rate or efficiency of the phosphorylation of ADP to ATP in whole mitochondria or submitochondrial preparations.

In order to competitively inhibit ATP hydrolysis, the analog must bind to an ATP binding site. If there is only one adenine nucleotide binding site and if it is the ATPase catalytic site, then from our studies on the effect of the analog on ATP hydrolysis it would be expected that AMP-PNP would interfere with the phosphorylation of ADP. Because no inhibition was observed, it could be that ADP out-competes AMP-PNP for the ATPase active site under energized conditions, or else the presence of AMP-PNP does not effect the binding of ADP and release of ATP from the enzyme. This prediction implies that AMP-PNP is bound during ATP synthesis which may not be the case. The ATPase energy linked catalytic site may be made more accessible to AMP-PNP when mitochondria are uncoupled. A comparison of Figs. 2 and 3 shows that FCCP renders the ATPase more sensitive to inhibition by AMP-PNP. The possibility still remains, however, that there is more than one ATP binding site on the mitochondrial ATPase and that the analog interacts at a non-energy linked ATP binding site when mitochondria are energized. Thus under phosphorylation conditions AMP-PNP would not interfere with ADP phosphorylation.

The ATP-driven reversed electron transfer reaction in submitochondrial preparations requires coupling between ATP hydrolysis and NAD reduction. The greater production of NADH noted in the presence of AMP-PNP indicates that in terms of coupling ATP hydrolysis to NAD^+ reduction this system is more efficient. The paradox of an inhibitor which promotes a more efficient system can be resolved if it is assumed that the mitochondrial membrane contains two different ATPase enzymes,

or one ATPase in two different states -- one state being energy-linked and the other being non-energy linked. The latter situation could occur if there is a mixed population in the submitochondrial preparations, coupled and uncoupled systems. Only the coupled or energy linked system can drive the reversed electron transfer. Since uncoupling with FCCP rendered the ATPase more sensitive to inhibition by the analog, it may be that the analog preferentially inhibits the non-energy linked system, leaving the energy linked system available to drive the reversed electron transfer.

Potassium uptake with valinomycin in the presence of ATP also requires a coupled mitochondrial system. In this case the analog had a strong inhibitory effect. Inhibition, though, may be at the site of adenine nucleotide translocation rather than at the ATPase catalytic site. Although Klingenberg et al. (12) have shown that AMP-PNP interacts with the adenine nucleotide carrier of the inner mitochondrial membrane, it may be that the analog interferes with ATP translocation, thus inhibiting ATP-driven potassium uptake. In order to explain how the analog works in whole mitochondria more information will be needed concerning the interaction of AMP-PNP with the adenine nucleotide carrier and its subsequent effect on ADP and ATP translocation. From this discussion it is inevitable that further studies of the interaction of AMP-PNP with ATPase are warranted. Such a study may resolve some of the problems of the mechanism and reversibility of the mitochondrial ATP synthetase complex.

Preliminary results of such work have recently been reported (14, 15). Garrett and Penefsky (15), Penefsky (16) noted that AMP-PNP competitively inhibits beef heart mitochondrial ATPase without effecting oxidative phosphorylation.

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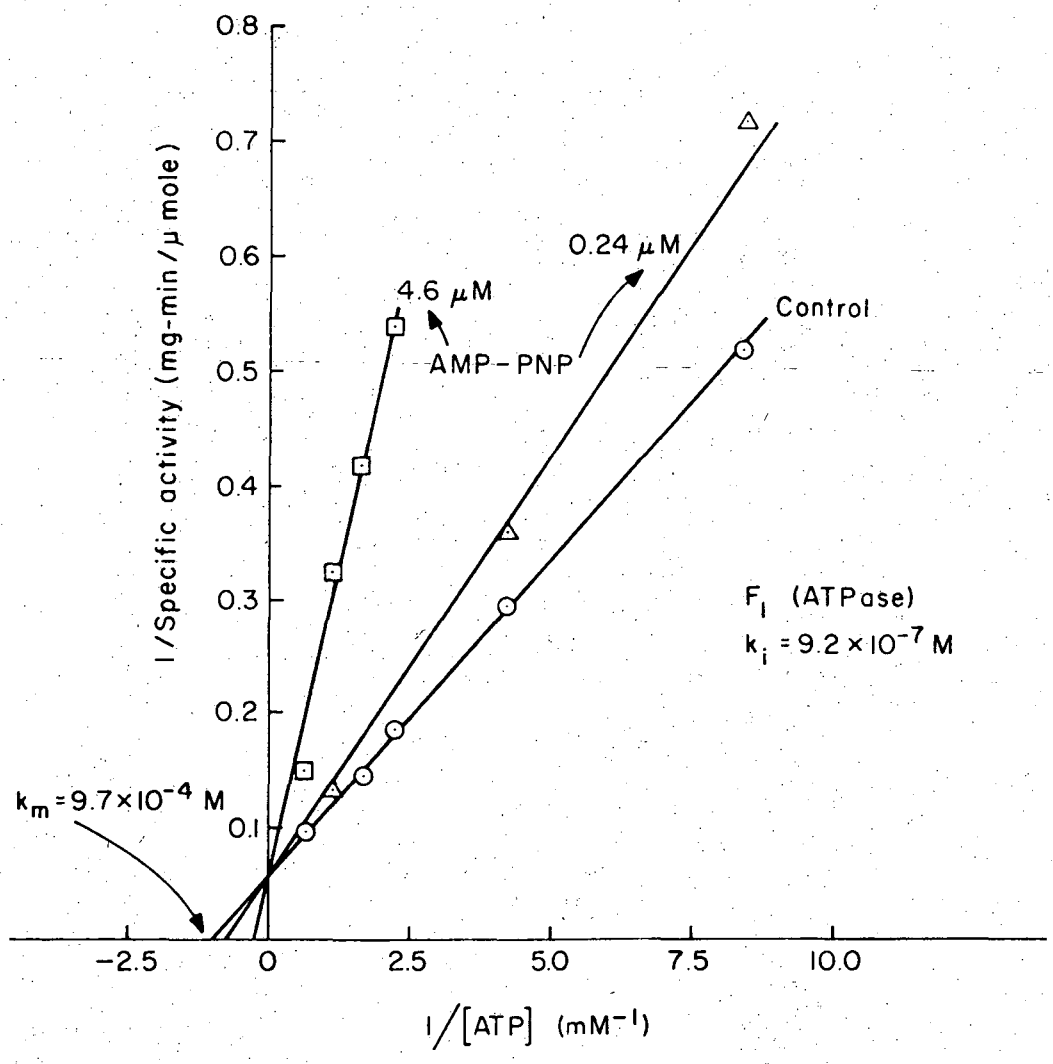

Figure 1

Lineweaver-Burk plot of F_1 (soluble ATPase) with and without AMP-PNP.

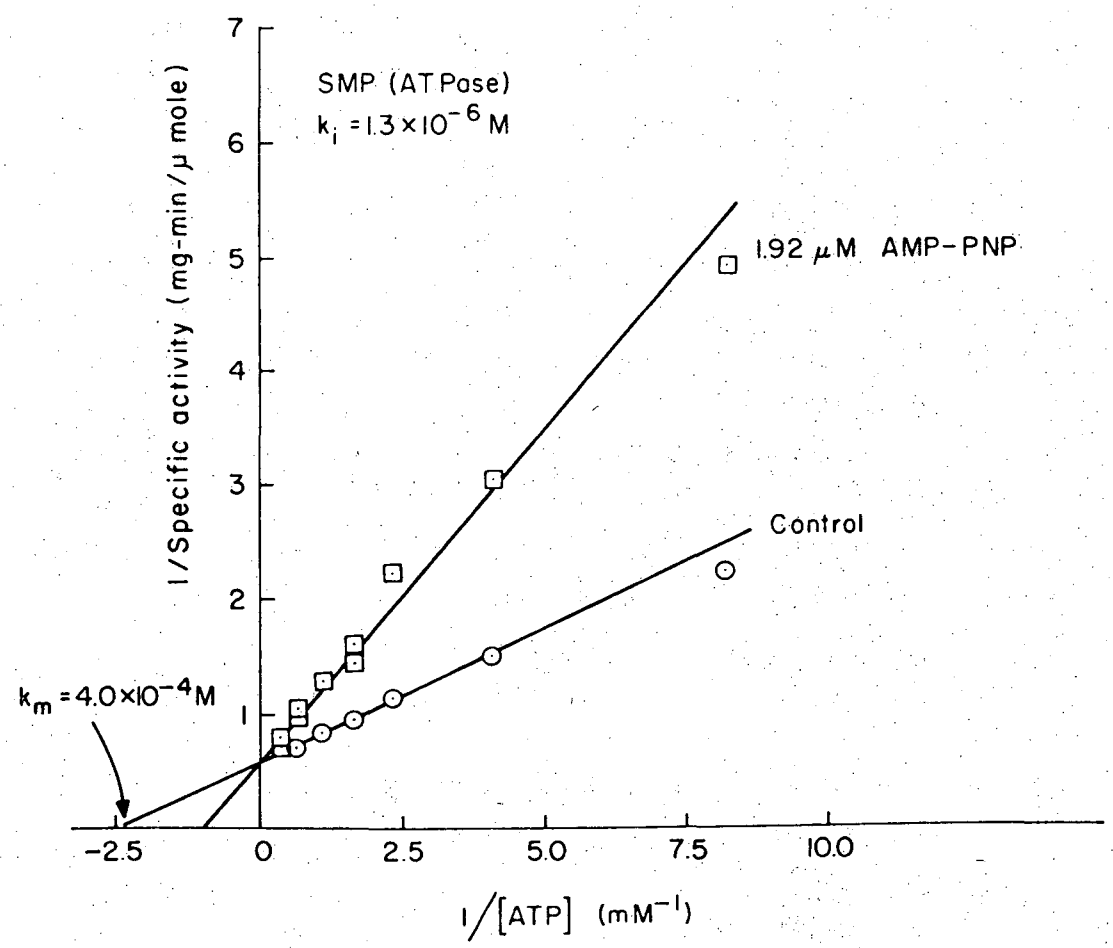

Figure 2

Lineweaver-Burk plot of SMP ATPase with and without AMP-PNP.

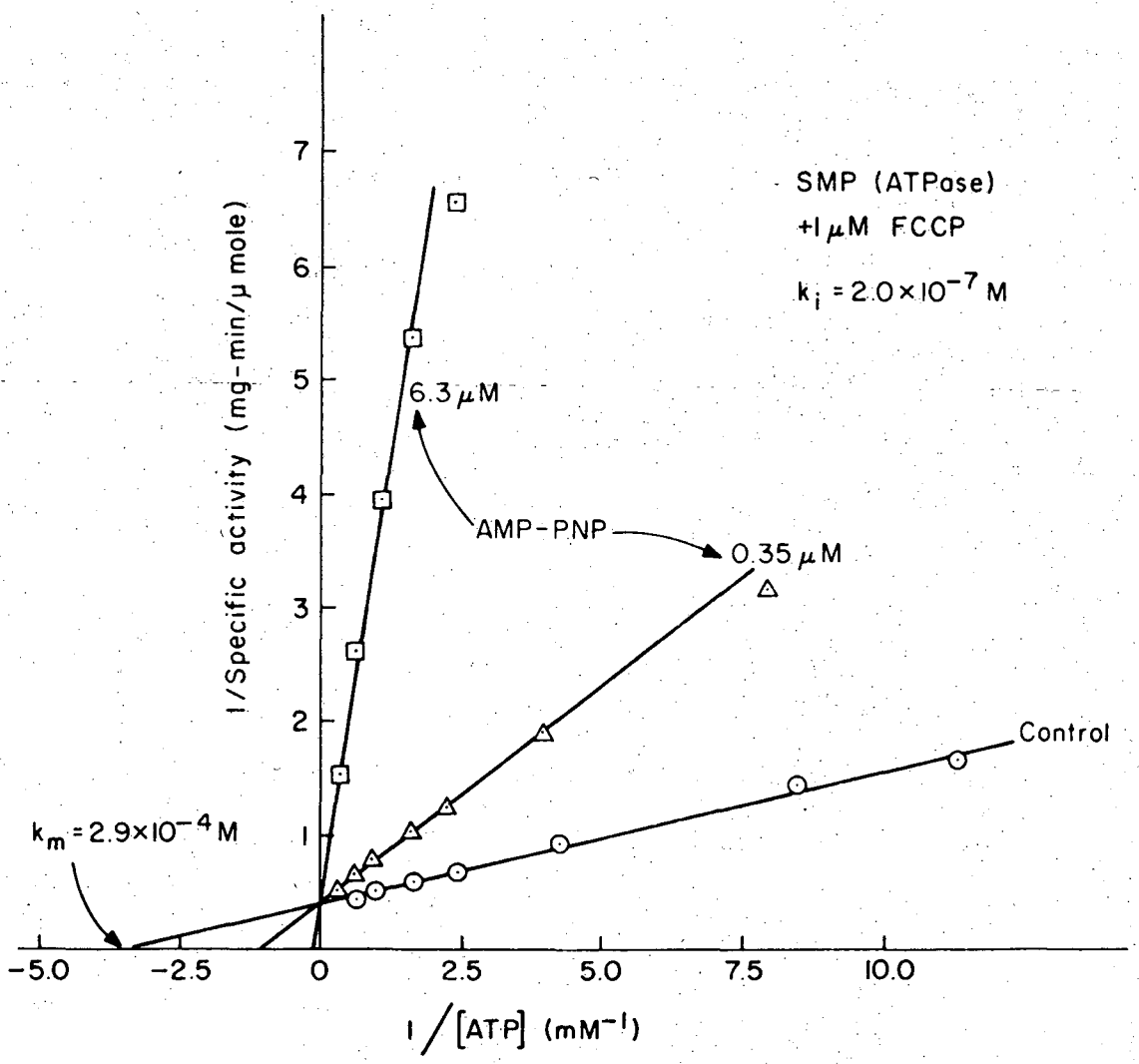

Figure 3

Lineweaver-Burk plot of SMP ATPase with and without AMP-PNP and the uncoupler FCCP.

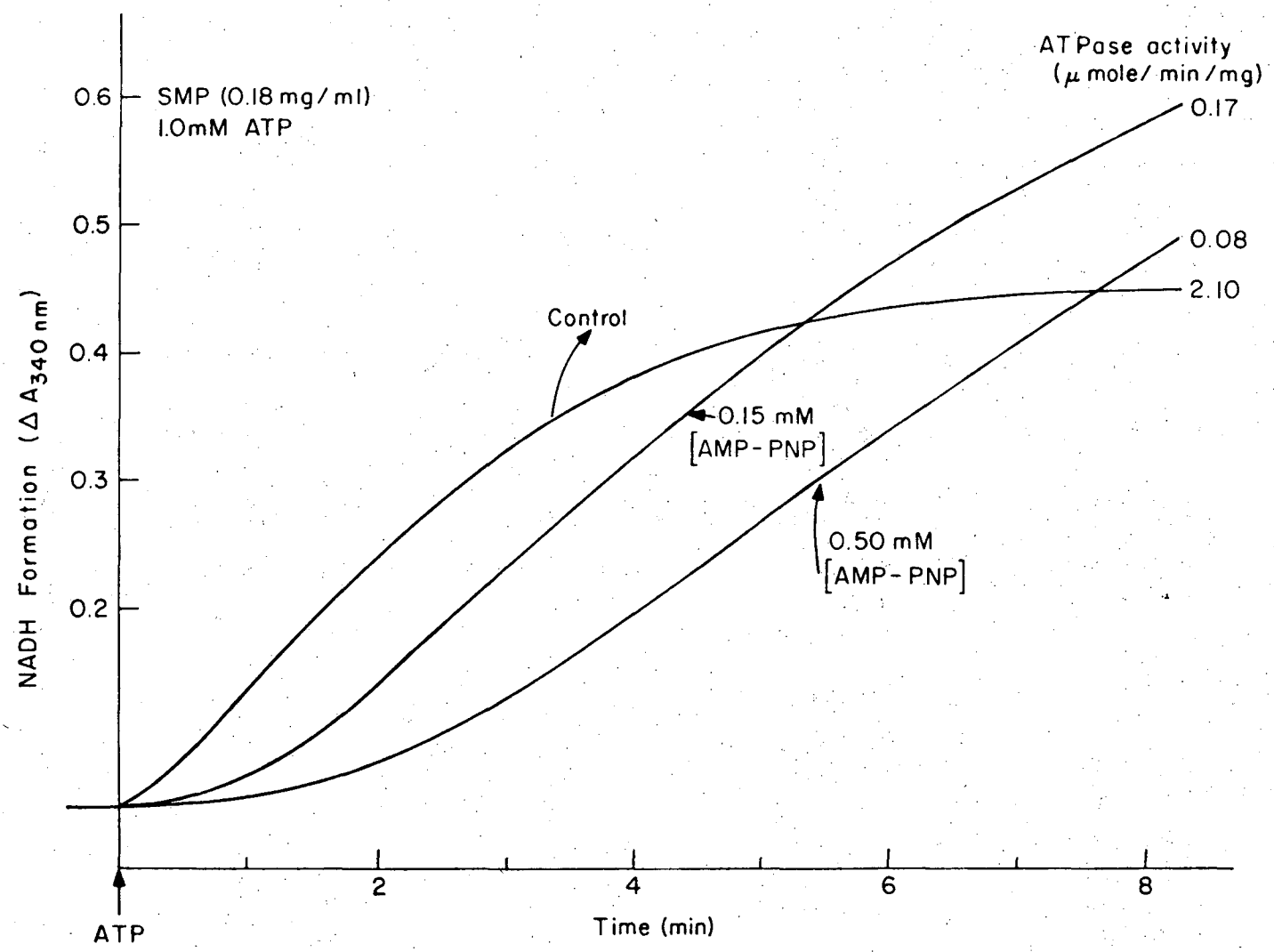

Figure 4

Succinate reduction of NAD^+ driven by ATP hydrolysis with and without AMP-PNP.

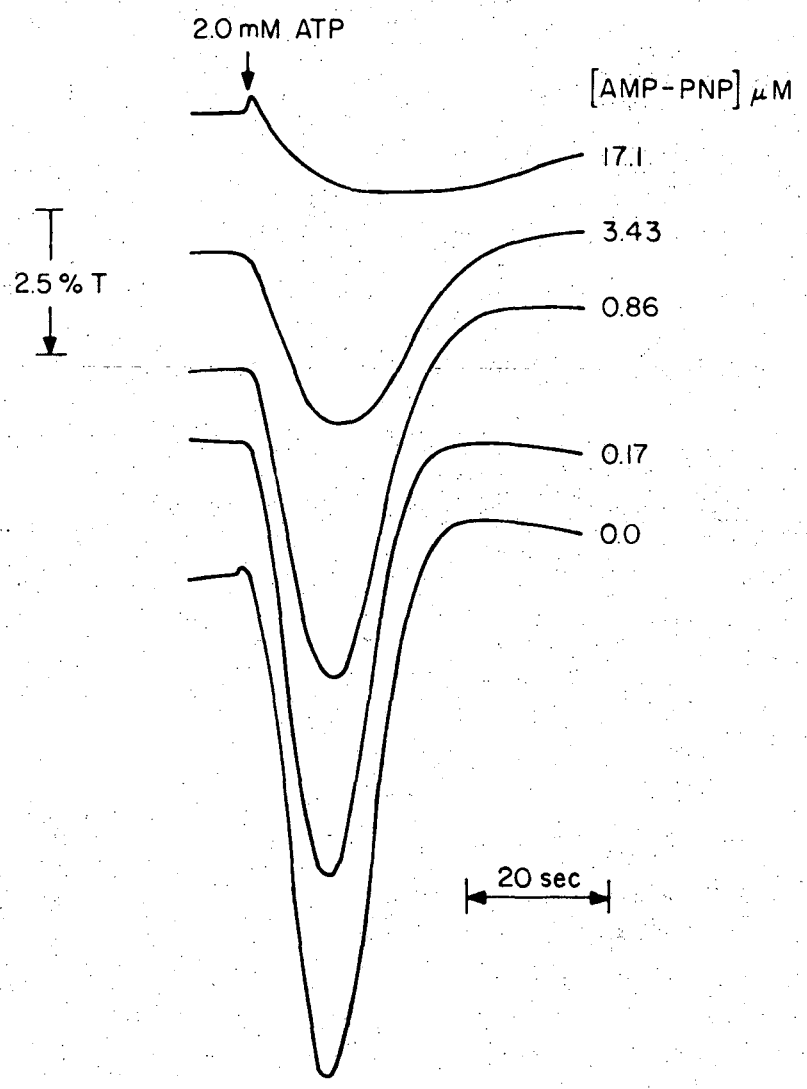

Figure 5

ATP dependent potassium uptake by whole mitochondria with and without AMP-PNP.

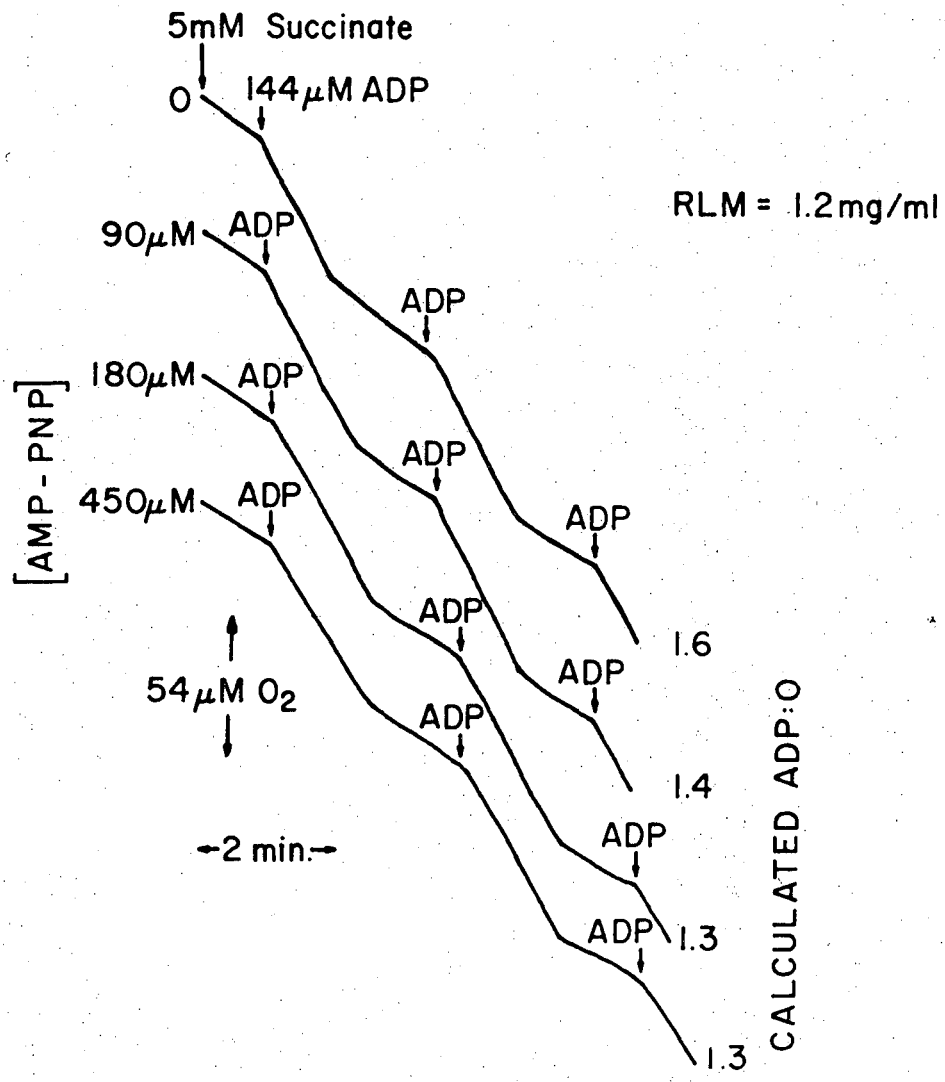

Figure 6

Effect of AMP-PNP on phosphorylation in whole mitochondria.

TABLE I
EFFECT OF AMP-PNP ON PHOSPHORYLATION OF SMP's

ADP (μM)	AMP-PNP (μM)	FCCP (1 μM)	P/O
1,400	0	-	0.44
1,400	600	-	0.44
140	0	-	0.24
140	260	-	0.25
140	0	+	0.04

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