A SPECIFIC ADP REQUIREMENT IN THE COURSE OF Ca^{++} AND PHOSPHATE ACCUMULATION IN MITOCHONDRIA*.

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Summary. A stringent requirement of Ca^{++} and phosphate accumulation for ADP is demonstrated. The presence of this nucleotide seems necessary for Ca^{++} and/or phosphate uptake at the membrane level but is not specifically required for the intramitochondrial precipitation step of calcium phosphate (dense granules).

It is known that the respiration linked accumulation of Ca^{++} ions by isolated mitochondria in the presence of inorganic phosphate requires ATP or ADP to be present in the incubation medium. This requirement obtains even in the presence of oligomycin (1-3), which eliminates the possibility that these nucleotides may operate solely by generating energy rich intermediates via the oxydophosphorylating system. In rat liver mitochondria, adenylic nucleotides are taken up simultaneously with Ca^{++} and inorganic phosphate (4) and incorporated to a certain extent into the calcium phosphate deposits (dense granules) of the mitochondrial matrix (5). Moreover cristalline hydroxylapatite (which contains the same proportions of Ca^{++} and inorganic phosphate as the "dense granules") appears to bind ATP and ADP in vitro (6). These facts may be interpreted as signifying that these nucleotides are required to initiate or stabilize coprecipitation of calcium phosphate (7).

The present work aims to establish the specific requirements for adenylic nucleotides of the sequential events which eventually lead to calcium and phosphate accumulation. With the use of suitable ADP-traps it is shown that the overall accumulation of calcium and phosphate ions by hog heart sarcosomes, rat liver and kidney mitochondria strictly requires the presence of ADP. In hog heart sarcosomes there is no compulsory coupling between the uptake and precipitation of calcium phosphate and the uptake of adenylic nucleotides, which does not seem specific to any degree. Stabilization of the

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intramitochondrial deposits may be obtained with inorganic pyrophosphate (8) which has no effect on the accumulation as such. It is concluded that ADP is specifically required in the active uptake step of calcium and/or phosphate ions through the mitochondrial membrane.

Materials and Methods : Sarcosomes were prepared from hog hearts (immediately removed from freshly slaughtered 6 months old animals and chilled to 0°) by a slight modification of the method of Crane et al. (9) : the 30 % (w/v) homogenate in 270 mM sucrose, 60 mM KCl, 10 mM KH_2PO_4 was adjusted to pH 7.6, without further adjusting the pH of the 1,500 x g supernatant. The sarcosomes were washed twice with a medium containing 270 mM sucrose, 60 mM KCl, 10 mM Tris-HCl, pH 7.2, discarding light sarcosomes. The material was eventually suspended in 150 mM KCl, 10 mM Tris-HCl, pH 7.2 (20-30 mg protein/ml).

Rat liver and kidney mitochondria were prepared according to Hogeboom et al. (10). Fractionation was performed throughout in 270 mM sucrose, 60 mM KCl, 10 mM Tris-HCl, pH 7.2. The mitochondria were washed twice discarding the light fractions. Final suspension of the material in 150 mM in 150 mM KCl, 10 mM Tris-HCl, pH 7.2 (40-50 mg protein/ml).

Incubations were performed in the following medium : 60 mM sucrose, 60 mM Tris-HCl, 8 mM $MgCl_2$, 10 mM KH_2PO_4, 16 mM succinate, cytochrome c 1.2 mg, oligomycin 2 µg/mg protein, rotenone 3 µg. Final volume 4.2 ml, pH 7.0, 21°. Extenporaneous additions as indicated.

^{45}Ca (CEA, Saclay, France) accumulation was determined by separating the mitochondria from the incubation medium on a Schleicher and Schüll 0.6 µ membrane under negative pressure. The filters were washed once with 5 ml 100 mM sucrose, 100 mM Tris-HCl, pH 7.2, extracted with 1 ml HCOOH and a dried aliquot of the extract counted in a Nuclear Chicago gas flow detector.

Accumulation of adenylic nucleotides was measured by the same method using ($U^{14}C$) ATP (CEA Saclay, France). Distribution of the accumulated radioactive material among the individual nucleotides was performed after filtration by dipping a glass tube with the filter in a mixture of dry ice and acetone, grinding the frozen filter, extracting with 200 mM $HClO_4$, containing a mixture of unlabelled 0.7 mM ATP; ADP and AMP as carriers, precipitating perchlorate ions by KOH and separating the nucleotides by electrophoresis according to Wadkins and Lehninger (11). The UV absorbing spots corresponding to the various nucleotides were counted in a liquid scintillation counter.

AMP deaminase (EC 3.5.4.6) was partially purified from rabbit skeletal muscle, according to K.L. Smiley Jr. et al. (12) ; after elution of the purified enzyme from the cellulose phosphate column, it was precipitated at 0° by $(NH_4)_2SO_4$ at a final saturation of 45 % (v/v) and suspended in a solution of 500 mM KCl, 1 mM mercaptoethanol, pH 6.7. Enzymatic activity was tested by the method of Kalckar (13) : 1 µl of AMP deaminase solution converts 30 nmoles of AMP to IMP per minute at 20°, pH 6.5.

Proteins were determined according to Jacobs et al. (14), ovalbumine being used as a standard.

RESULTS.

1) Specific requirement for ADP of Ca^{++} accumulation linked to respiration in the presence of inorganic phosphate.

Experiments are performed in the presence of ATP, of oligomycin which inhibits ATP.ADP interconversion by the phosphorylating system, and of two traps : trap 1 for ADP, which is phosphorylated to ATP by phosphocreatine (PC) + creatine kinase (EC 2.7.3.2) and by phosphoenol pyruvate (PEP) + pyruvate kinase (2.7.1.40) ; trap 2 for AMP, which is deaminated to inosine 5'

phosphate : rabbit skeletal muscle AMP deaminase (EC 3.5.4.6). The latter system prevents phosphorylation of any AMP, which diffuses from the mitochondria, via the adenylate kinase (2.7.4.3) catalyzed reaction.

Figures 1A and 1B show the results obtained with rat liver and kidney mitochondria : almost no Ca^{++} accumulation occurs in the presence of the trapping devices ; it may be noted however that the use of trap 2 alone is sufficient to obtain this inhibition.

Figure 1C shows that with hog heart sarcosomes, AMP deaminase, at the concentration effective with liver and kidney mitochondria, slows down Ca^{++} uptake in the presence of ATP, but does not modify the amount finally accumulated ; both traps have to be used to obtain complete inhibition. No other nucleoside diphosphate tested, including AOPCP (Miles Inc.), the diphosphonic analog of ADP, stimulates Ca^{++} uptake, with the exception of CDP and GDP (Table I) ; the effect of the latter compounds is suppressed in the presence

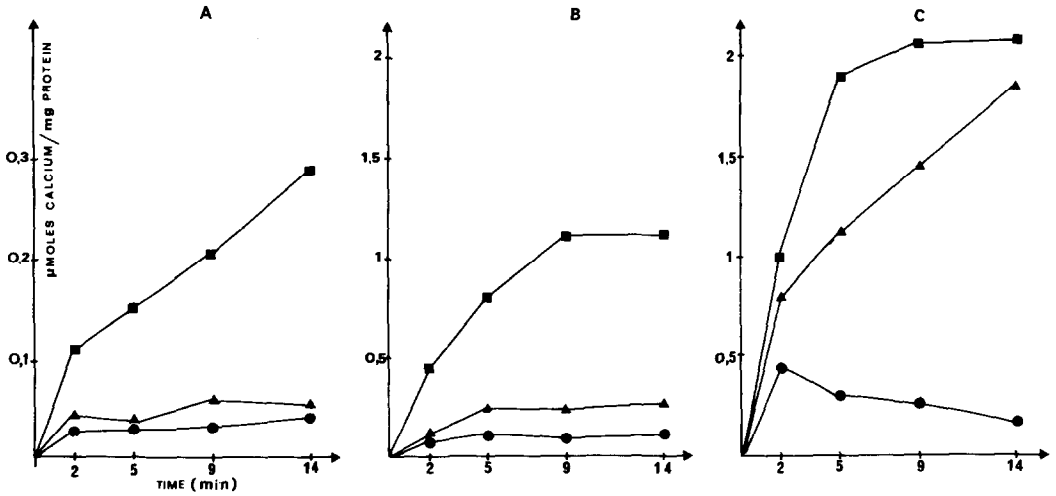

Fig.1 : Accumulation kinetics of Ca^{++} in mitochondria from various tissues, in the presence or absence of ADP and AMP traps.

A : Rat liver mitochondria 7.56 mg (protein content) ; addition of 5.3 mM $CaCl_2$ + ^{45}Ca 52,000 cpm after 2 min. preincubation ; final volume 4.3 ml, pH 7.0, 21° ; ATP 2.5 mM, PC 9 mM, PEP 2.5 mM. ■—■ without trap 1 and 2 ; suspension medium of AMP deaminase 200 µl. ▲—▲ Trap 2 (AMP deaminase preparation 200 µl). ●—● Trap 2 + trap 1 (creatine kinase 100 µg and pyruvate kinase 100 µg).

B : Rat kidney mitochondria 5.25 mg (protein content) ; same conditions as under (A) ; $CaCl_2$ 2.4 mM, $^{45}Ca^{++}$ 36,000 cpm.

C : Hog heart sarcosomes 4.7 mg (protein content) ; $CaCl_2$ 2.1 mM, $^{45}Ca^{++}$ 46,000 cpm ; preincubation time 7 min. ■—■ no PEP nor PC as sarcosomes contain creatine kinase (15) ; ▲—▲ Trap 2, no PC nor PEP ; ●—● Trap 1 (PEP, PC, Pyruvate kinase, creatine kinase) + Trap 2 ; otherwise as under (A).

Table I : Effect of various nucleoside diphosphates on the respiration linked Ca^{++} accumulation by sarcosomes in the presence of inorganic phosphate and oligomycin.

	Calcium uptake (μmoles/mg protein/14 min.incubation)			
	Experiment 1*		Experiment 2**	
	AMP deaminase		Phosphocreatine	
	-	+	-	+
None	0.2	-	0.62	-
ADP 2.5 mM	1.93	-	-	-
AMP 2 mM	-	-	0.62	2
AOPCP 3 mM	0.183	-	-	-
IDP 3 mM	0.130	-	-	-
UDP 2.5 mM	0.187	-	-	-
GDP 2.5 mM	0.450	0.09	-	-
CDP 2.5 mM	1.81	0.515	-	-

*Proteins 4.05 mg : final volume 3.95 ml, pH 7.0 ; $CaCl_2$ 2.5 mM ; $^{45}Ca^{++}$ 39,500 cpm ; glucose 4 mM ; hexokinase (EC 2.7.1.1) 100 μg. Conditions otherwise as under Fig.1C. This trap, combined with adenylate kinase keeps ATP and ADP concentrations at a minimal level, by converting them to AMP (and eventually IMP, when AMP deaminase is present).

**Proteins 5.0 mg ; final volume 5.45 ; pH 7.4 , $CaCl_2$ 2.4 mM ; $^{45}Ca^{++}$ 33,700 cpm. Conditions otherwise as under Fig.1C.

of AMP deaminase ; conversely the addition of AMP has no effect, but in the presence of this nucleotide plus PC, Ca^{++} uptake is enhanced.

2) Adenylic nucleotide (AN) uptake in the course of Ca^{++} and phosphate accumulation by the three mitochondrial preparations.

Figures 2A and 2B show that the results obtained with liver and kidney mitochondria, essentially agree with the data of E.Carafoli et al. (4). AN uptake occurs in considerable amounts and parallels Ca^{++} accumulation. If the latter event is inhibited, AN accumulation is stopped. Figure 2C shows that with hog heart sarcosomes, the results are different ; in the absence of any trap, very small amounts of nucleotides are taken up, whereas Ca^{++} accumulation is at its maximum ; in the presence of both traps, AN accumulation seems to be correlated with Ca^{++} uptake and stops when the latter phenomenon is inhibited ; when, however, trap 2 is used alone, thus slowing down Ca^{++} uptake, without modifying the amount eventually accumulated, AN are taken up massively. When analyzed (fig.3) AN are shown to consist mainly of ATP. Figure 4 shows that in all types of mitochondria, less than 30 % of the total AN taken up are rapidly exchangeable with extramitochondrial AN.

3) Respiration-linked Ca^{++} and phosphate accumulation in the presence of inorganic pyrophosphate (PPi).

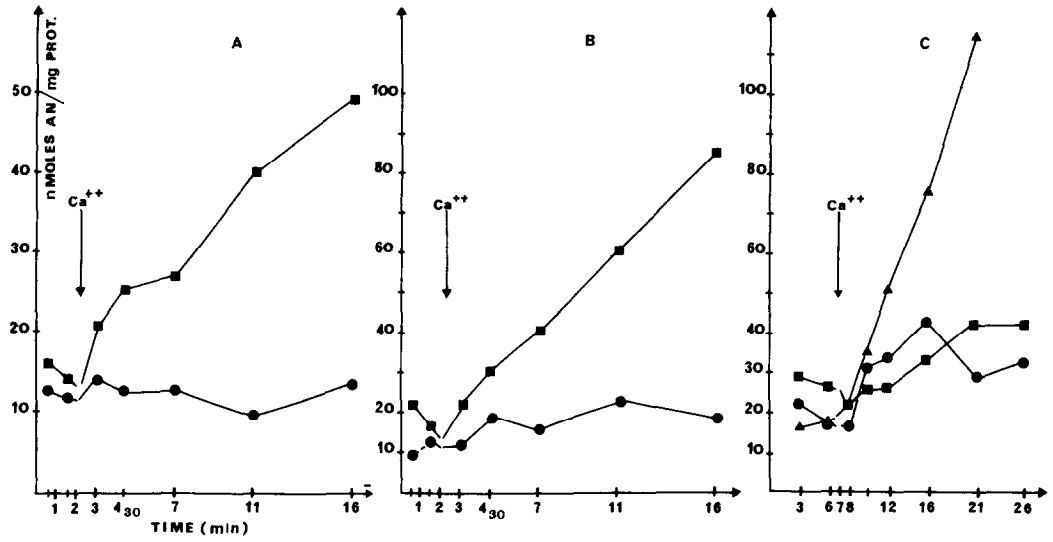

Fig. 2 : Accumulation kinetics of AN in mitochondria from various tissues, in the course of respiration linked Ca^{++} accumulation, in the presence or absence of ADP and AMP traps.

ATP 2.5 mM + (^{14}C) ATP 427,000 cpm in A, B and C.

A : Rat liver mitochondria 7.56 mg ; $CaCl_2$ 4 mM. Other conditions as under Fig. 1A.

B : Rat kidney mitochondria 5.25 mg, $CaCl_2$ 3.1 mM. Other conditions as under Fig. 1B.

C : Hog heart sarcosomes 4.16 mg ; $CaCl_2$ 2.1 mM. Other conditions as under Fig. 1C.

■—■ without trap ; ▲—▲ Trap 2 added ; ●—● both traps added.

In the absence of any AN addition, Ca^{++} uptake by hog heart sarcosomes exhibits biphasic kinetics (fig.5A) : a rapid, initial uptake is immediately followed by an accelerated release of accumulated Ca^{++} ions. In the presence of PPi the total amount of Ca^{++} taken up is slightly increased, but the main effect of this compound is to inhibit completely the subsequent Ca^{++} release. When ADP is added to the medium, Ca^{++} uptake is immediately resumed.

Figure 5B shows the effect of PPi in the presence of ATP and both trapping devices : basic uptake and accumulation occurs to a lesser extent than without any added AN or trap, but its release also is less rapid. PPi exhibits essentially the same action on this phenomenon, as mentioned above. Addition of ADP to the medium again provokes an immediate stimulation of Ca^{++} uptake.

DISCUSSION. The use of both ADP and AMP traps described above, results in demonstrating a stringently specific requirements for ADP in the course of

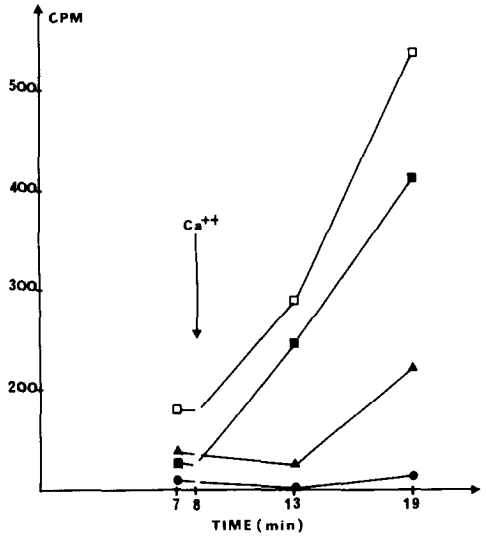

Figure 3 : Nature of AN accumulated in hog heart sarcosomes in the course of Ca^{++} accumulation in the presence of trap 2.

Sarcosomal proteins : 4.34 mg ; ATP 2.5 mM + (^{14}C) ATP 328,000 cpm, $CaCl_2$ 2.3 mM ; adenylate deaminase solution : 200 µl. Final volume 4.0 ml. For nucleotide extraction and separation see methods. Conditions otherwise as under Fig. 1C.

□—□ Total radioactivity accumulated ; ■—■ (^{14}C) ATP ; ▲—▲ (^{14}C) ADP ; ●—● (^{14}C) AMP.

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respiration linked Ca^{++} accumulation in the presence of phosphate and oligomycin. No other nucleotide may be substituted for ADP, not even the ADP analogue AOPCP, despite the fact that it acts as a substrate for the specific AN translocase (16) ; this interpretation is confirmed by the results observed with CDP and GDP, which support Ca^{++} accumulation only in the absence of AMP deaminase (17); it is well established that CDP is a substrate for adenylate kinase ; hence an ultimate phosphorylation of AMP from the mitochondrial material via CTP is entirely possible (the effect of GDP is less clear and is actually under investigation); similarly AMP supports accumulation only in the presence of phosphorylating systems which continuously regenerate ATP (hence ADP). This absolute requirement of Ca^{++} accumulation for ADP contrasts with earlier data of Carafoli et al. (4), and our own results with sarcosomes, concerning AN accumulation which, though somewhat preferential for ADP, is neither exclusive, nor even correlated with Ca^{++} uptake, at least in sarcosomes (Fig.2C), see also (18).

The possibility of separating the ADP requiring Ca^{++} accumulation from the less specific AN accumulation indicates that these steps may reflect two

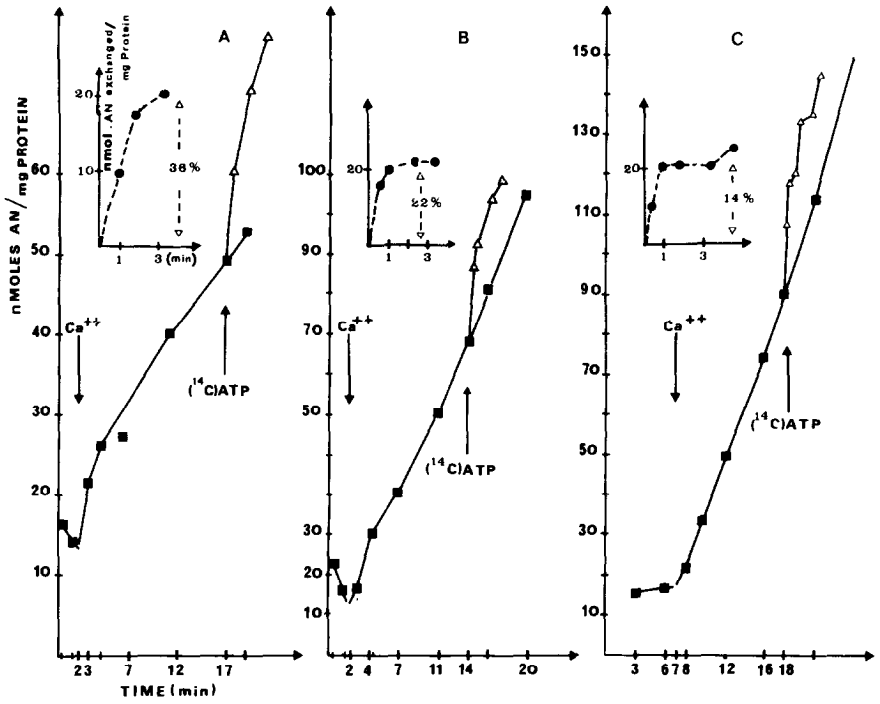

Fig. 4 : Exchange kinetics of AN accumulated by mitochondria of various tissues during respiration linked Ca^{++} accumulation.

A : Rat liver mitochondria. Conditions as under Fig. 1A without trap. ■—■ ATP 2 5 mM + (^{14}C) ATP added at 0 min. ; Δ—Δ ATP 2.5 mM at 0 min. and the mixture : (^{14}C) ATP 417,000 cpm, 1 μmole + PEP 2 μmoles + PEP kinase 20 μg + $MgCl_2$ 1 μmole in H_2O 0.09 ml added at indicated time.

B : Rat kidney mitochondria. Conditions as under Fig. 1B without trap. Experiment performed as 4A.

C : Hog heart sarcosomes. Conditions as under Fig. 1C with trap 2. Experiment performed as 4A.
Note : When (^{14}C) ATP addition was delayed (Δ—Δ), the onset of its accumulation was plotted starting from the point reached by the control (■—■) accumulation curve. Under these conditions the difference between both curves (●—●) accounts for net AN exchange.

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dissimilar but sequential events in the mitochondria : active transport of Ca^{++} and phosphate ions through the inner membrane and their precipitation within the mitochondrial matrix. The use of inorganic pyrophosphate confirms this view, as this compound appears to stabilize the accumulated calcium phosphate deposit or at least to enhance its intramitochondrial retention without increasing its total amount ; a similar phenomenon has already been described by Fleisch et al. (8) both in vivo and in vitro on bone tissue. In the presence of pyrophosphate, Ca^{++} uptake immediately resumes on addition of ADP (fig.5), a further indication in favour of its specific action on a step

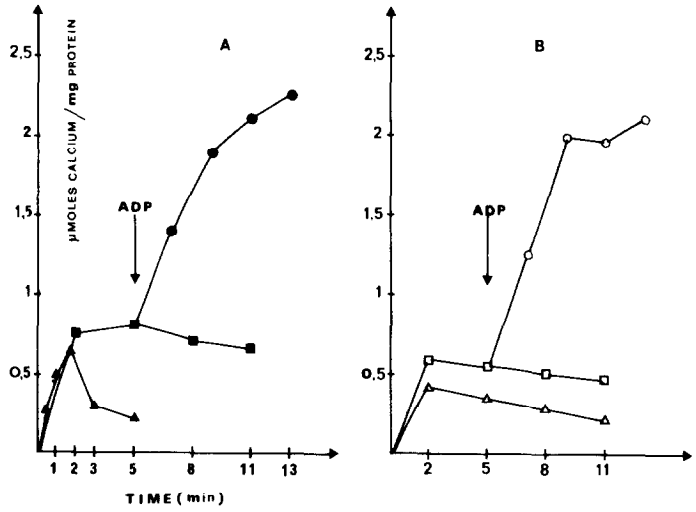

Fig. 5 : Accumulation kinetics of Ca^{++} in hog heart sarcosomes in the presence or absence of inorganic pyrophosphate and ATP (ADP and AMP traps present).

A : No AN added ; proteins 4.3 mg. Glucose 4 mM, hexokinase 100 µg (see Table I), adenylate deaminase solution : 200 µl ; $CaCl_2$ 2.1 mM ; $^{45}Ca^{++}$ 34,000 cpm. Conditions otherwise as under Fig. 1C.
▲—▲ no PPi ; samples withdrawn at 30 sec., 1 min., 2 min., 3 min., 5 min., following addition of Ca^{++} ; ■—■ PPi 0.5 mM ; ●—● PPi 0.5 mM, ADP 5 mM added 5 min. after $CaCl_2$.

B : ATP 2.5 mM, proteins 4.34 mg. Conditions as under Fig. 1C. $CaCl_2$ 2.1 mM, $^{45}Ca^{++}$ 39,000 cpm added after 7 min. preincubation :
△—△ no PPi ; □—□ PPi 0.5 mM ; ○—○ PPi 0.5 mM, ADP 5 mM added 5 min. after $CaCl_2$.

related to transport and not to precipitation of the ions accumulated. This interpretation of course does not contradict the assumption (7) according to which in the absence of pyrophosphate adenylic nucleotides are required, to initiate or stabilize calcium phosphate deposits : our own results (Fig.4) show that a large fraction of the AN accumulated are not readily exchangeable with extracellular AN, indicating that they may be tightly bound to the precipitates.

Hence it seems evident that the specific role of ADP on Ca^{++} and phosphate accumulation in the presence of oligomycin concerns the permeability and/or active transport phenomena of the mitochondrial membrane. It does not seem possible to decide, at the present time, if Ca^{++} or phosphate transport is primarily stimulated, since under our experimental conditions both ions are accumulated simultaneously,or if ADP intervenes in some specific coupling step linking respiratory energy to active Ca^{++} transport.

The requirement for ADP demonstrated in the present paper may however be correlated with an equally specific action of this nucleotide on mitochondrial integrity (19) and on the movements of cations other than Ca^{++} (20).

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