ATRACTYLOSIDE-SENSITIVE TRANSLOCATION OF PHOSPHONIC ACID ANALOGUES OF ADENINE NUCLEOTIDES IN MITOCHONDRIA

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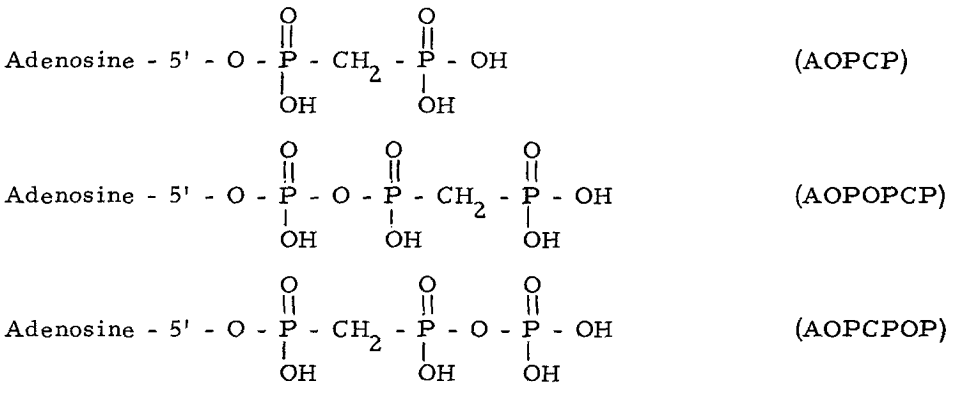
Radioactive nucleotides have been useful to elucidate many functions of adenine nucleotides in mitochondria. In particular, they made possible the study of adenine nucleotide translocation across mitochondrial membranes (Bruni et al., 1964 ; Pfaff et al., 1965 ; Brierley and Green, 1965 ; Brierley and O'Brien, 1965 ; Duée and Vignais, 1965 ; Klingenberg and Pfaff, 1966 ; Vignais and Duée, 1966). Mitochondria loaded with labelled adenine nucleotides are not metabolically different from normal mitochondria. Although this has been often taken as an advantage, it may constitute a limitation to investigate some aspects of mitochondrial reactions involving adenine nucleotides. This limitation may be overcome by the use of adequate adenine nucleotide analogues. In the present paper it will be shown that phosphonic acid analogues of adenine nucleotides, the synthesis of which has been described by Myers et al. (1963, 1965), are a valuable tool for the study of adenine nucleotides dependent reactions in mitochondria since they are rapidly exchanged with internal adenine nucleotides by an atractyloside-sensitive reaction but do not participate to phosphorylation or transphosphorylation reactions within mitochondria.

Abbreviations : AOPCP : Adenosine-5'-methylenediphosphonate
AOPOPCP : 5'-adenylyl methylenediphosphonate
AOPCPOP : Adenosine-5'-methylenediphosphono-P^2-phosphate

Materials and Methods : (^{32}P)-orthophosphate was obtained from the "Commissariat à l'Energie Atomique, Saclay", (^{14}C)-ADP from Schwarz Bioresearch, Inc.. The following analogues : AOPCP (ADP analogue), AOPOPCP and AOPCPOP (ATP analogues), were purchased from Miles and Co.



Rat liver mitochondria were isolated according to Hogeboom (1955). Digitonin particles were prepared as described by Devlin and Lehninger (1958). AMP and ADP were determined enzymatically by the method of Adam (1963) and ATP by the method of Lamprecht and Trautschold (1963).

Tests to measure the exchange between intramitochondrial and added adenine nucleotides were carried out at 0° in 110 mM KCl, 20 mM Tris-HCl, pH 7.3, 1 mM EDTA ; this medium will be referred to as KCl-Tris-EDTA. The incubation period was ended by centrifugation for 5 min. at 10 000 x g, the maximum centrifuge force being attained within 30 sec. ; under these conditions, the mean sedimentation time for mitochondria was calculated as being 45 sec..

Mitochondria loaded with (^{14}C)-adenine nucleotides were prepared as follows : rat liver mitochondria (15 mg/ml) were incubated at 0° in KCl-Tris-EDTA supplemented with 0.25 mM (^{14}C)-ADP ; after 20 min. of incubation, mitochondria were centrifuged for 10 min. at 10 000 x g. The mitochondrial pellet was washed three times by resuspension in 0.25 M sucrose and centrifugation. After the third washing, mitochondria were resuspended in 0.25 M sucrose.

Results : The reactivity of AOPCP as an analogue of ADP on the one hand and of AOPOPCP and AOPCPOP as analogues of ATP on the other has been tested towards different enzymes commonly used for assays of adenine nucleotides (Adam, 1963), (Lamprecht and Trautschold, 1963). The ADP or ATP analogues are not substrates for adenylate kinase, pyruvate kinase or hexokinase, but they do not inhibit the reaction of ADP or ATP with these enzymes. While AOPOPCP is not a substrate for phosphoglycerate kinase, AOPCPOP reacts only very slowly with this enzyme and AOPCP is inhibitory. Therefore none of the analogues can be estimated by enzymatic tests but it is possible however to measure AMP, ADP and ATP in the presence of either of these analogues. The lack of reactivity of phosphonic acid analogues reported here extends previous findings which showed that AOPOPCP was unable to replace ATP in hexokinase reaction (Flesher et al., 1960) and was inhibitory in actomyosin (Moos et al., 1960) and polynucleotide phosphorylase (Simon and Myers, 1961) systems.

The comparative effect of ADP and AOPCP on the content of mitochondria in adenine nucleotides is shown in Table I. The adenine nucleotides content (AMP + ADP + ATP) of mitochondria incubated with ADP and then washed with a saline medium does not vary ; this is in agreement with previous data (Pfaff et al., 1965), (Duée and Vignais, 1965). On the contrary, upon addition of AOPCP, the sum of AMP + ADP + ATP remaining inside mitochondria is substantially decreased suggesting that a fraction of internal adenine nucleotides has been released upon addition of AOPCP. Since AOPCP cannot be assayed enzymatically, but has the same extinction coefficient as ADP at 260 mμ, the amount of intramitochondrial adenine nucleotides present after incorporation of AOPCP (AOPCP + AMP + ADP + ATP) was determined spectrophotometrically at 260 mμ. Mitochondria which have been incubated with AOPCP and then washed with saline medium were extracted by perchloric acid. The absorbance at 260 mμ of the extracts after neutralization is given in Table I. The absorbance data indicate that the amount of compounds absorbing at 260 mμ is the same, independent of whether the mitochondria were incubated with ADP or with AOPCP. This result suggests that the release of internal adenine nucleotides upon addition of AOPCP is compen-

Table I : Effect of ADP and AOPCP on the content and the distribution of adenine nucleotides in mitochondria.

Time	Addition	Adenine nucleotides found (mμmoles)				Absorbance at 260 mμ
		AMP	ADP	ATP	Sum	
Zero	None	280	250	60	590	0.750
20 min.	None	260	270	60	590	0.760
20 min.	ADP	290	300	60	650	0.770
20 min.	AOPCP	220	140	40	400	0.760

Mitochondria (48 mg of protein) were incubated at 0° in KCl-Tris-EDTA (cf. Materials and Methods). Final volume : 5.5 ml. Additions were either 0.44 mM ADP or 0.36 mM AOPCP. Incubation was stopped by centrifugation ; the mitochondrial pellet was washed with KCl-Tris-EDTA and extracted with 2 ml of 2.5 N perchloric acid. Adenine nucleotides were determined enzymatically on the neutralized extract (cf. Materials and Methods) and absorbance was taken at 260 mμ after a six-fold dilution.

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sated by the uptake of an equal amount of AOPCP.

A direct demonstration of the release of (^{14}C)-adenine nucleotides from (^{14}C)-adenine nucleotide-loaded mitochondria upon addition of either ADP or AOPCP is given in Fig.1. The nucleotide release induced by AOPCP, although slower than the one induced by ADP, is much more inhibited by atractyloside. It was shown previously (Vignais et al., 1967) that atractyloside allows to distinguish between net leakage of internal adenine nucleotides and exchange of internal with external adenine nucleotides, the latter being inhibited by atractyloside whereas the former is atractyloside-insensitive. The inhibition by atractyloside of the release of internal (^{14}C)-adenine nucleotides induced by addition of AOPCP may be taken as an indication that internal (^{14}C)-adenine nucleotides are exchanged with external AOPCP. Similar results were obtained when mitochondria were incubated with AOPCPOP or AOPOPCP.

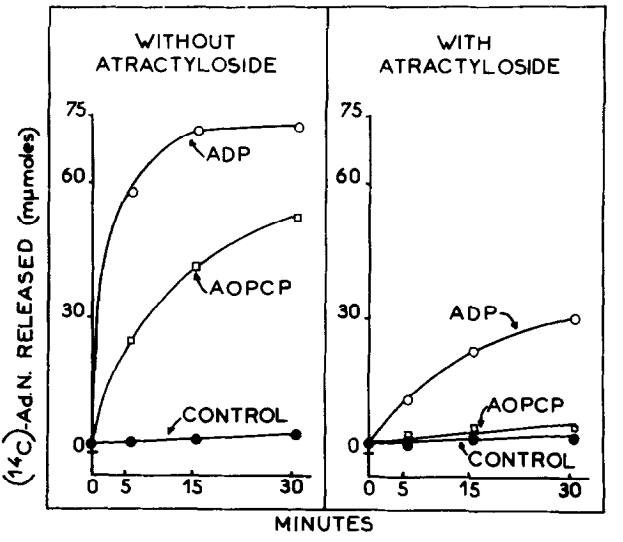

Fig.1 - Exchange of intramitochondrial (^{14}C)-adenine nucleotides with external ADP or AOPCP.
Mitochondria were previously loaded with (^{14}C)-adenine nucleotides, as described in Materials and Methods. 19 mg of mitochondria containing 110 mμmoles of (^{14}C)-adenine nucleotides were incubated at 0° in KCl-Tris-EDTA. Additions were either 0.23 mM ADP or 0.23 mM AOPCP ; atractyloside, when present, was 23 μM. Final volume 2.2 ml. Incubation was stopped by centrifugation and the amount of (^{14}C)-adenine nucleotides present in the supernatant fluid was measured by liquid scintillation counting.

Since AOPCP and AOPOPCP are translocated into mitochondria by the same atractyloside-sensitive process as ADP and ATP, it was interesting to test whether AOPCP could replace ADP as phosphate acceptor in oxidative phosphorylation and if AOPOPCP could be hydrolyzed in the same manner as ATP by uncoupled mitochondria. In order to diminish the interference of permeability factors, this study was carried out with submitochondrial particles obtained by action of digitonin on mitochondria. As shown in Table II, ATP is rapidly hydrolyzed by digitonin particles supplemented with Mg^{++}, whereas AOPOPCP remains unaltered. Furthermore, AOPOPCP does not inhibit ATP hydrolysis. AOPCPOP is also not hydrolyzed. However, owing to some AOPCP contaminant in the preparation of AOPCPOP, the

Table II : Reactivity of phosphonic acid analogues of ADP and ATP towards reactions of oxidative phosphorylation.

Oxidative phosphorylation				ATP-ase	
Additions	Acetoacetate formed (mμmoles)	$^{32}P_i$ esterified (mμmoles)	$\frac{P}{Acetoac.}$	Substrate	P_i formed (μmoles)
None	70	0	0		
ADP	130	100	0.77	ATP	2.4
AOPCP	70	12	0.14	AOPOPCP	0.0
ADP + AOPCP	110	90	0.82	ATP + AOPOPCP	1.9

Conditions : Oxidative phosphorylation : the reaction medium contained : 82 mM KCl, 15 mM Tris-HCl, 8.3 mM phosphate labelled with ^{32}P, 18 mM β-hydroxybutyrate, 4 mg/ml BSA and digitonin particles (2.7 mg of protein). ADP and AOPCP were 1.5 mM. Final volume : 2.6 ml ; temperature 28°. Incubation was stopped after 5 min. by addition of 0.2 ml of 30% trichloracetic acid (w/v). $^{32}P_i$ esterified was determined by the method of Nielsen and Lehninger (1955), acetoacetate according to Walker (1964).

ATP-ase : digitonin particles (1.2 mg of protein) were incubated for 5 min. at 28° in the following medium : 100 mM KCl, 10 mM Tris-HCl, pH 7.3, 5 mM $MgCl_2$. Additions were either 5.2 mM ATP or 5.2 mM AOPOPCP or both. Final volume was 1.9 ml. Incubation was stopped by the addition of 0.2 ml of 30% trichloracetic acid. Inorganic phosphate was determined as described by Fiske and Subbarow (1925).

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significance of this result cannot be assessed. On the other hand, AOPCP is a poor phosphate acceptor : when β-hydroxybutyrate is oxidized by digitonin particles, AOPCP is phosphorylated much slower than ADP (8 times). Separation of ADP, ATP, AOPCP, and AOPCPOP by paper chromatography (Duée, 1967) revealed that the small amount of (^{32}P)-phosphate esterified in the presence of AOPCP was actually present in AOPCPO^{32}P. Furthermore, data in Table II show that AOPCP does not apparently interfere with the phosphorylation of ADP. Another experiment not presented here and bearing on the respiratory control of rat liver mitochondria showed that ADP is still capable to stimulate the respiration of mitochondria previously loaded with AOPCP. The

same experiment extended to yeast mitochondria prepared according to Mattoon and Balcavage (1967), gave identical results.

In conclusion, AOPCP and AOPOPCP which are the phosphonic acid analogues of ADP and ATP respectively, are rapidly exchanged with intramitochondrial adenine nucleotides through an atractyloside-sensitive translocation. Although easily translocated into mitochondria, AOPCP and AOPOPCP are metabolized only very slowly, if at all. Based on the above observations, it is possible to prepare mitochondria loaded with phosphonic acid analogues in which the amount of total adenine nucleotides is kept constant while the amount of enzymatically reactive adenine nucleotides is strikingly decreased. Such a system is now being used in our laboratory to study the compartmentation of adenine nucleotides in mitochondria.

Experiments recently reported in the literature (Klingenberg and Pfaff, 1966), (Kemp and Groot, 1967), have led to the conclusion that the specificity of the overall process of oxidative phosphorylation for ADP is caused by the specificity of the translocation for the adenine nucleotides at the level of the inner membrane of mitochondria. The experiments reported here indicate that this conclusion does not apply to the phosphonic acid analogues of adenine nucleotides.

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REFERENCES

Adam, H. (1963), in Bergmeyer, H.U., Methods of Enzymatic Analysis, Academic Press, New York, p.573.
Brierley, G. and Green, D.E. (1965), Proc. Natl. Acad. Sci. U.S., 53, 73.
Brierley, G. and O'Brien, R.L. (1965), J. Biol. Chem., 240, 4532.
Bruni, A., Luciani, S. and Contessa, A.R. (1964), Nature, 201, 1219.
Delvin, T.M. and Lehninger, A.L. (1958), J. Biol. Chem., 233, 1586.
Duée, E.D. and Vignais, P.V. (1965), Biochim. Biophys. Acta, 107, 184.
Duée, E.D. (1967), Bull. Soc. Chim. Biol. (submitted).
Fiske, S.H. and Subbarow, Y. (1925), J. Biol. Chem., 66, 375.
Flesher, J.W., Oester, T.Y. and Myers, T.C. (1960), Nature, 185, 772.
Hogeboom, G. (1955) in Colowick, S.P. and Kaplan , N.O., Methods in Enzymology, Academic Press, New York,, Vol.I, p.16.

Kemp, Jr., A. and Groot, G.S.P. (1967) Biochim. Biophys. Acta, 143, 628.
Klingenberg, M. and Pfaff, E. (1966), in Tager, J.M., Papa, S., Quagliariello, E. and Slater, E.C. (Editors), Regulation of Metabolic Processes in Mitochondria, Elsevier Publ. Co., Amsterdam, B.B.A. Library, Vol.7, p. 180.
Lamprecht, W. and Trautschold, I. (1963), in Bergmeyer, H.U., Methods of Enzymatic Analysis, Academic Press, New York, p. 543.
Mattoon, J.R. and Balcavage, W.X. (1967), in Colowick, S.P. and Kaplan, N.O., Methods in Enzymology, Academic Press, New York, Vol. X, p. 135.
Moos, C., Alpert, N.R. and Myers, T.C. (1960), Arch. Biochem. Biophys., 88, 183.
Myers, T.C., Nakamura, K. and Flesher, J.W. (1963), J. Am. Chem. Soc., 85, 3292.
Myers, T.C., Nakamura, K. and Danielzahed, A.B. (1965), J. Org. Chem., 30, 1517.
Nielsen, S.O. and Lehninger, A.L. (1955), J. Biol. Chem., 215, 555.
Pfaff, E., Klingenberg, M. and Heldt, H.W. (1965), Biochim. Biophys. Acta, 104, 312.
Simon, L.N. and Myers, T.C. (1961), Biochim. Biophys. Acta, 51, 178.
Vignais, P.V. and Duée, E.D. (1966), Bull. Soc. Chim. Biol., 48, 1169.
Vignais, P.V., Duée, E.D. and Huet, J. (1967), manuscript in preparation.
Walker, P.G. (1964), Biochem. J., 58, 699.