Biochemical Pharmacology, 1961, Vol. 7, pp. 165–168. Pergamon Press Ltd., Printed in Great Britain.

INHIBITION OF OXIDATIVE PHOSPHORYLATION BY AMINOSIDINE AND OTHER ANTIBIOTICS, IN RAT LIVER MITOCHONDRIA

U. BARDI, GIULIA BORETTI and A. DI MARCO

Laboratori Ricerche Farmitalia, Milano

(*Received* 6 *February* 1961)

Abstract—Several antibiotics, belonging to the class of basic polysaccharides, such as streptomycin, kanamycin, neomycin, and aminosidine, inhibit the phosphorylation coupled to ketoglutarate oxidation in rat liver mitochondria. The effect of aminosidine has been compared to that of its N-methaneosulphonate derivatives and of the products obtained by methanolysis. Results show that the uncoupling activity depends both on the presence of free amino groups and on the moelcular complexity of the antibiotic. The mechanism of the uncoupling effect of aminosidine is different from that of dinitrophenol.

THE present paper* deals with the study of the action of the antibiotics streptomycin, kanamycin, neomycin, aminosidine,[1] and trehalosamine[2] on the phosphorylation coupled to ketoglutarate oxidation in rat liver mitochondria. The antibiotics examined have in common the structure of basic oligosaccharides.

The relation between chemical structure and uncoupling effect on the oxidative phosphorylation of mitochondria has been studied particularly with regard to aminosidine. This antibiotic is a pentabasic oligosaccharide having the formula $C_{23}H_{45}N_5O_{14}$. It is split, by acid hydrolysis, into a glucoside ($C_{12}H_{25}N_3O_7$) and an aminodisaccharide (probably $C_{11}H_{22}N_2O_8$). Two substances, a crystalline glucoside ($C_{12}H_{25}N_3O_7 \cdot 3HCl$) and the methylglucoside of an aminodisaccharide [$C_{11}H_{21}N_2O_7(OCH_3)$, $2HCl$][3] have been obtained by methanolysis. Analogous products have been obtained from paromomycin[4] and hydroxymicin.[5] The treatment of the antibiotic with sodium bisulphate and formaldehyde gives rise to methanesulphonates, analogously to what occurs with kanamycin.[6]

MATERIAL AND METHODS

We used the following products: adenosinetriphosphate (sodium salt) (Boehringer), hexokinase Sigma type II, cytochrome *c* (Boehringer), ketoglutarate (Biosintex), β-hydroxybutyric acid (Fluka), streptomycin sulphate (Farmitalia), kanamycin sulphate (Merck), neomycin sulphate (Farmitalia.) Aminosidine sulphate, trehalosamine sulphate, as well as the products obtained by methanolysis of the latter and the N-methanesulphonates of aminosidine, have been prepared in our laboratory.

Rat liver mitochondria were prepared according to Schneider.[7] The oxidative phosphorylation activity was determined in suspensions of mitochondria in 3 ml of medium containing sucrose 250 μM, EDTA 2μM, K malonate 30 μM, K ketoglutarate 30 μM, KH_2PO_4 40 μM, cytochrome *c* 0·06 μM, ATP 6 μM, KF 120 μM, $MgSO_4$

* The following abbreviations are used: EDTA = ethylenediaminotetra-acetic acid; ADP = adenonine diphosphate; ATP = adenosine triphosphate; Pi = inorganic phosphorus; DNP = dinitrophenol.

50 μM, glucose 40 μM, hexokinase 0·9 mg. The mixture was incubated in Warburg flasks for 20 min at 30 °C. Oxygen uptake was measured by Warburg manometric technique. Inorganic phosphate was determined, according to Marsh,[8] in the supernatant of the suspension deproteinized by 0·5 ml 20% TCA, at zero time and at the end of incubation. Experiments in phosphate or phosphate acceptor deficient systems were performed using glutamate as substrate, according to Lardy and Wellman's method.[9] ATPase activity of mitochondria was determined according to Myers and Slater in Tris buffer at pH 7·4.[10] Hexokinase activity was determined by Colowick and Kalkar's method.[11] Ion-complexing activity of aminosidine was determined by Albert's method.[12]

RESULTS

Among the antibiotics tested, streptomycin, kanamycin, aminosidine and neomycin, at concentrations ranging from 10 to 50 μg/ml, inhibit the phosphorylation coupled to ketoglutarate oxidation in rat liver mitochondria and show about the same degree of activity (Table 1). Trehalosamine, which is a monobasic disaccharide, is inactive.

TABLE 1. INFLUENCE OF SOME ANTIBIOTICS ON P : O OF RAT LIVER MITOCHONDRIA

No additions		Aminosidine		Streptomycin		Neomycin		Kanamycin		Trehalosamine	
O_2 μatoms	P:O	O_2 μatoms	P:O	O_2 μatoms	P:O	O_2 μatoms	P:O	O_2 μatoms	P:O	O_2 μatoms	P:O
3·65	3·13	3·82	1·84	4·39	1·85						
3·98	2·66	3·65	1·69			3·65	1·52				
3·46	2·32	3·38	1·53					3·40	1·34		
5·10	2·56	5·31	1·63							5·73	2·31

Substrate: ketoglutarate. Concentrations of added antibiotics = 50 μg/ml.

TABLE 2. INFLUENCE OF N-METHANESULPHONATE DERIVATIVES OF AMINOSIDINE ON P : O OF RAT LIVER MITOCHONDRIA

	No additions	Aminosidine 50 μg/ml	MS_4 50 μg/ml	MS_5 50 μg/ml
P:O	3·08	1·62	3·12	2·86

Substrate: ketoglutarate.
MS_4 = aminosidine-di-N-methanesulphonate.
MS_5 = aminosidine-tetra-N-methanesulphonate.

The action of aminosidine has been compared to that of its methanesulphonate derivatives and to that of the products obtained by methanolysis and we have observed that none of the derivatives of aminosidine interferes with the processes of oxidative phosphorylation (Tables 2 and 3). Aminosidine inhibits oxidative phosphorylation also with β-hydroxybutyrate and glutamate as substrates. Its action is not due to an interference with the mechanism of transfer of the terminal phosphate of ATP on glucose; indeed it does not inhibit hexokinase at the concentrations used in the tests for oxidative phosphorylation, moreover, in this case it would cause an inhibition of O_2 consumption due to a depletion of ADP. Aminosidine neither stimulates the QO_2 of mitochondria in Pi or ADP deficiency (Table 4), nor influences QO_2 stimulation induced by dinitrophenol (DNP) under these conditions. Aminosidine, contrary to

TABLE 3. INFLUENCE OF AMINOSIDINE AND OF THE SUBSTANCES OBTAINED BY METHANOLYSIS OF AMINOSIDINE ON P:O OF RAT LIVER MITOCHONDRIA

	No additions	Aminosidine 50 μg/ml	α	β	α + β
P:O	2·35	1·21	2·37		
	2·67	1·40		2·20	
	2·78	1·58			2·54

Substrate: ketoglutarate.
α and β are, respectively, $C_{12}H_{25}N_3O_7 \cdot 3HCl$ and $C_{11}H_{21}N_2O_7(OCH_3) \cdot 2HCl$ obtained by methanolysis of aminosidine. α and β were added to give concentrations of 48 μg/ml and 82 μg/ml, respectively.

TABLE 4. INFLUENCE OF DNP AND OF AMINOSIDINE ON O_2 UPTAKE BY RAT LIVER MITOCHONDRIA IN DEFICIENCY OF ADP AND OF PI

Additions	ADP deficiency (μl O_2)	Pi deficiency (μl O_2)
None	116	172
DNP 10^{-4}M	248	314
Aminosidine 0·8–10^{-4}M	96	184
DNP 10^{-4}M + aminosidine 0·8–10^{-4}M	277	268

Substrate: glutamate; incubation time 60 min.

TABLE 5. INLFUENCE OF DNP AND OF AMINOSIDINE ON ATPASE ACTIVITY OF RAT LIVER IN MITOCHONDRIA

Additions	Fresh mitochondria % hydrolysed ATP	Aged mitochondria* % hydrolysed ATP
None	4·9	48
DNP, 10^{-4}M	58	51
Aminosidine, 50 μg/ml	8·5	44

Incubation for 10 min at 30 °C.
* Sucrose suspensions of mitochondria kept for 2 h at 37 °C.

TABLE 6. REVERSAL BY Mg^{2+} OF THE INHIBITING ACTION OF AMINOSIDINE ON P:O OF RAT LIVER MITOCHONDRIA

Expt.	$MgSO_4$ μM/ml	No addition	Aminosidine 50 μg/ml	Inhibition (%)
		P:O	P:O	
No. 1	7·5	2·6	1·64	37
	15	2·9	2·35	21
No. 2	7·5	2·43	1·45	40
	22·5	2·42	2·39	0·8

Substrate: ketoglutarate.

dinitrophenol, does not stimulate ATPase activity of fresh mitochondria. It does not either influence that of aged mitochondria (Table 5). The uncoupling activity of aminosidine is prevented by an excess of Mg^{2+} ions (Table 6). The analysis by titration, according to Albert,[10] did not show formation of complexes of aminosidine with Mg^{2+}.

DISCUSSION

Among the oligosaccharidic antibiotics tested, those having both a tri- or tetrasaccharidic molecule and the character of polyvalent base, act as uncouplers of mitochondrial oxidative phosphorylations. As concerns aminosidine, we could prove, after a comparison with the methanesulphonate derivatives and the products of methanolysis, that both the presence of free amino groups and a certain structural complexity are required for the occurrence of an inhibiting activity on the processes of oxidative phosphorylation. The mechanism of the action of aminosidine differs from that of DNP as demonstrated by the absence of any effect on ATPase activity and by lack of QO_2 stimulation in phosphate, or phosphate acceptor deficient, systems. The same behaviour was observed in the antibiotics belonging to tetracyclines.[13, 14] These antibiotics, too, induce a decrease of the P/O ratio in mitochondria and do not stimulate O_2 uptake in phosphate or ADP-deficient media. Their action is antagonized by Mg^{2+} at concentrations similar to those antagonizing aminosidine.

From the comparison between aminosidine and DNP it may be calculated that while the latter would act previously to the introduction of Pi, thus favouring the hydrolysis of a non-phosphorylated intermediate ($X \sim I$),[15] aminosidine would exert its influence on a reaction in which both ADP and Pi are involved. According to the formulation of Slater and Hulsmann[15] it is possible that this reaction be: $X \sim P + ADP \rightleftharpoons ATP + X$. $X \sim P$ is formed by a Pi requiring reaction: $X \sim I + Pi \rightleftharpoons X \sim P + I$. It is known that this reaction requires Mg^{2+} as coenzyme: therefore we may suppose that aminosidine, in its quality of polyvalent base, acts by removing the Mg^{2+} ions from the enzyme surface. We observe, however, that aminosidine has no effect on the action of hexokinase, a further Mg^{2+} requiring enzyme.

We hope to draw further conclusions from the study of the influence of aminosidine on the partial reactions catalysed by submitochondrial particles.

REFERENCES

1. F. ARCAMONE, C. BERTAZZOLI, M. GHIONE and T. SCOTTI, *G. Microbiol.* **7**, 251 (1959).
2. F. ARCAMONE and F. BIZIOLI, *Gazz. Chim. Ital.* **87**, 896 (1957).
3. F. ARCAMONE. Personal communication.
4. T. H. HASKEL, J. C. FRENCH, Q. R. BARTZ, *J. Amer. Chem. Soc.* **81**, 3480 (1959).
5. G. HEGEMANN, F. NOMINE and L. PENASSE, *Ann. Pharm. Franc.* **16**, 585 (1958).
6. S. UMEZAWA, V. ITO, S. FUKATSU and S. H. UMEZAWA, *J. Antibiot.* A, **12**, 114 (1959).
7. W. C. SCHNEIDER, *J. Biol. Chem.* **176**, 259 (1948).
8. B. B. MARSH, *Biochem. Biophys. Acta* **32**, 357 (1959).
9. H. A. LARDY and H. WELLMAN, *J. Biol. Chem.* **195**, 215 (1952).
10. D. K. MYERS and E. C. SLATER, *Biochem. J.* **67**, 558 (1957).
11. S. P. COLOWICK and H. M. KALCKAR, *J. Biol. Chem.* **148**, 117 (1943).
12. A. ALBERT, *Biochem. J.* **47**, 531 (1950).
13. T. M. BRODY, *Proc. Soc. Exp. Biol., N.Y.* **80**, 533 (1952).
14. T. M. BRODY, R. HARWITZ, J. A. BAIN, *Antibiot. and Chemother* **4**, 864 (1954).
15. E. C. SLATER, W. C. HÜLSMAN, *Ciba Foundation Symposium on the Regulation of Cell Metabolism* (Edited by G. E. W. WOLSTENHOLME and CECILIA M. O'CONNOR, J. & A. Churchill Ltd, London p. 48) (1959)