PROTECTIVE EFFECT OF AMMONIA ON MITOCHONDRIAL STRUCTURE AND RESPIRATION

J.POPINIGIS, J.ŚWIERCZYŃSKI and T.WRZOLKOWA

Department of Biochemistry and Laboratory of Electron Microscopy, Medical School, Gdańsk, Poland

Received 2 July 1970

1. Introduction

The mechanism of the irreversible inhibitory action of protamine and gramicidin on succinate (+ rotenone) oxidation in isolated rat liver mitochondria has been studied. Samples of mitochondrial suspensions taken from experiments on respiration were also examined by electron microscopy.

The data obtained indicate that the irreversible inhibition of respiration is accompanied by severe damage to the mitochondrial structure. The observed protective effect of ammonia on mitochondrial structure and respiration confirms our previous suggestion that, in the presence of protamine + gramicidin, the inside of the mitochondrion becomes highly alkaline [1].

2. Materials and methods

Rat liver mitochondria were prepared and suspended in 0.25 M sucrose with 3 mM tris-chloride, pH 7.3 [2]. Respiration was measured with a Clark oxygen electrode at 25° in 3.5 ml of medium (composition shown in table 1), after addition of 0.1 ml of a suspension of freshly prepared rat liver mitochondria, corresponding to 8 mg of mitochondrial protein. The protein content of the suspension was estimated by the biuret method [3]. Protamine sulphate, ex herring (Koch-Light, pure), was used after adjusting to pH 7.3 with tris.

Samples (3 ml) were removed from the oxygen electrode chamber for electron microscopy. They were centrifuged for 40 sec at 10,000 *g* and the pellets fixed in 2% osmium tetroxide for 1 hr. The fixative was brought to pH 7.4 with 0.21 M sodium phosphate in 50 mM sucrose. After fixation the pellets were stained for 4 min with 1% uranyl acetate in 25% ethanol at 0°, dehydrated with ethanol, and embedded in Epon 812. Sections were cut with a glass knife and post-stained with uranyl acetate and lead citrate. Specimens were examined in a JEM 7A electron microscope at 80 kV.

3. Results

Table 1 presents the effects of some agents on protamine-inhibited succinate (+ rotenone) oxidation in intact rat liver mitochondria. Inhibition of respiration was partially reversed by addition of DNP (exp. 3) but not by gramicidin (exp. 4). When NH_4Cl was added prior to gramicidin, the latter acted as a reversing agent (exp. 9) and oligomycin did not prevent this effect (exp. 10). On the other hand, addition of NH_4Cl without gramicidin (exp. 7) or after gramicidin (exp. 6) did not affect inhibition by protamine. Comparison of exp. 3 and 8 indicates that NH_4Cl partially diminished the reversing effect of DNP.

Samples of mitochondrial suspension were collected for electron microscopy at the points indicated by the letters A through E in table 1 and the pictures obtained are shown in fig. 1, A–E.

4. Discussion

We have previously suggested [1] that the basic

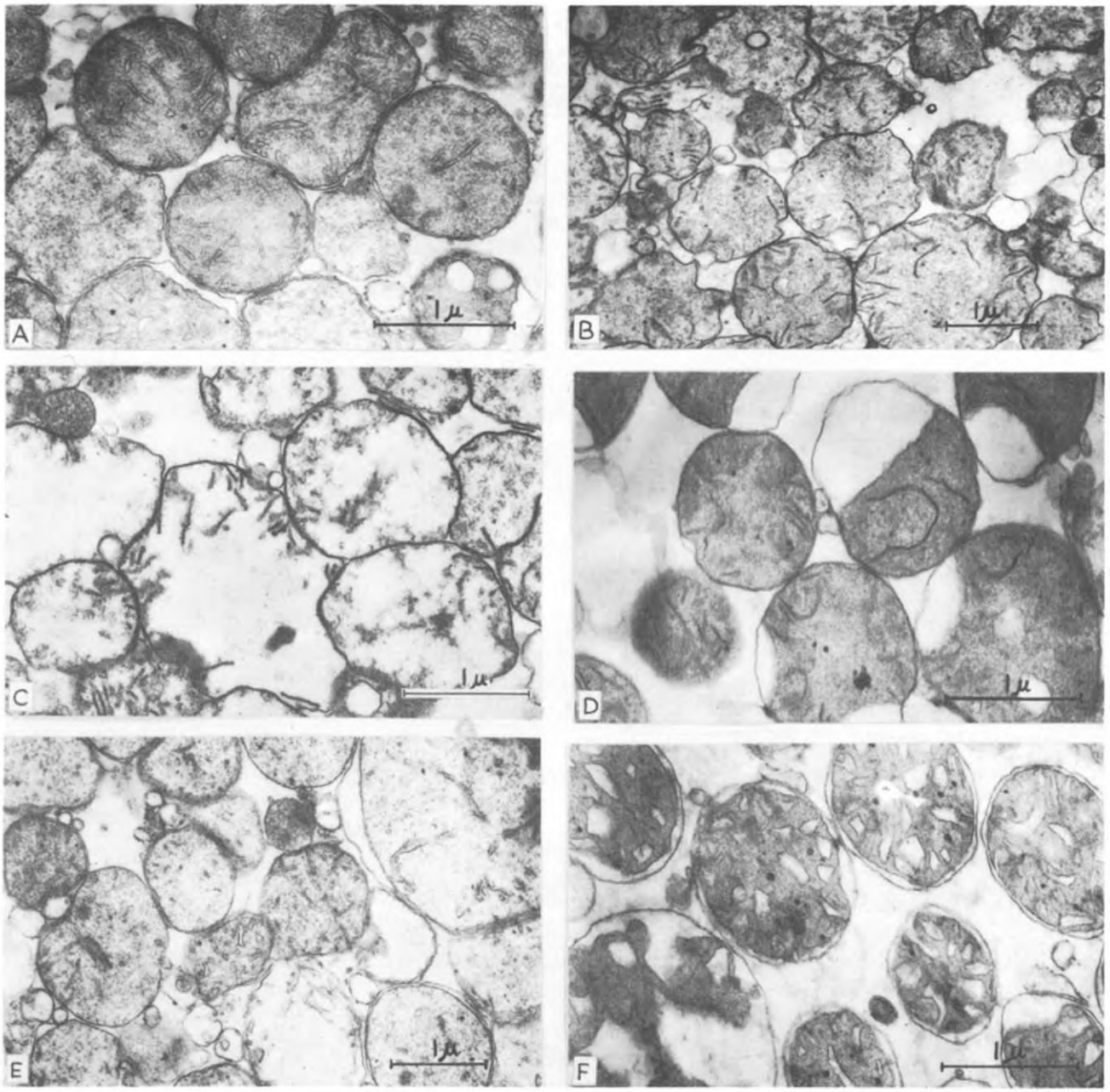

Fig. 1. Electronmicrographs of rat liver mitochondria from experiments presented in table 1. A: succinate, state 4 respiration; B: succinate + ADP + protamine; C: succinate + ADP + protamine + gramicidin; D: succinate + ADP + protamine + NH_4Cl + gramicidin; E: succinate + ADP + gramicidin. F: freshly prepared mitochondria suspension. For experimental conditions see text.

Table 1
The protective effect of ammonia on mitochondrial respiration.

Exp. no.	Additions and oxygen uptake							
	0 min	1 min	2 min	3 min	4 min	5 min	6 min	7 min
1	succ.	20	20	20	20	20	20	A
2	succ.	20 ADP	70 prot.	12	12	12	12	B
3	succ.	20 ADP	70 prot.	12 DNP	35	35	35	35
4	succ.	23 ADP	75 prot.	12	12 gra	12	12	C
5	succ.	20 ADP	70 prot.	10 gra.	10 DNP	10	10	10
6	succ.	22 ADP	75 prot.	12 gra.	12 NH_4Cl	12	12	12
7	succ.	22 ADP	75 prot.	10 NH_4Cl	10	10	10	10
8	succ.	22 ADP	75 prot.	12 NH_4Cl	12 DNP	20	20	20
9	succ.	21 ADP	75 prot.	12 NH_4Cl	12 gra.	40	40	D
10	succ.	20 ADP	70 prot.	12 NH_4Cl	12 olig.	12 gra.	40	40
11	succ.	20 ADP	70	70	75 gra.	75	75	E

The respiration was measured with a Clark oxygen electrode in 3.5 ml of medium, pH 7.3, containing: 15 mM KCl, 50 mM tris-chloride, 5 mM potassium phosphate and 4 μg of rotenone. Other additions: 10 mM potassium succinate (succ.), 3 μmoles of ADP, 300 μg of protamine (prot.), 10 μg of oligomycin (olig.), 3 μg of gramicidin (gra.), 0.1 mM DNP and 10 mM NH_4Cl, as indicated.

Data in the table present oxygen consumption as n-atoms O/min/mg of protein. The letters A through E indicate stages of the experiment at which aliquots of the mitochondrial suspension were aerated, centrifuged and fixed for electron microscopy. (The whole procedure from the removal of the aliquot to the beginning of fixation lasted 3½ min).

protein protamine, a non-penetrant polycation, reacts with the mitochondrial membrane when the inner part of the mitochondrion is negatively charged by the action of the respiratory chain and inhibits respiration by affecting back-diffusion of protons. It was also suggested that this inhibition may be reversed by weak acids which conduct protons by the A^-/H^+ symport. These conclusions were based on observations that protamine did not affect succinate oxidase in sonic particles but did inhibit succinate (+ rotenone) oxidation in intact mitochondria and that this inhibition could be partially reversed by the addition of DNP, citrate, or isocitrate but not by gramicidin. When protamine was used together with gramicidin, irreversible inhibition of oxidation was observed.

Slater [4] has calculated that the pH of the cristae space according to Mitchell's hypothesis would be 13.5, which is incompatible with the operation of the intercristae enzymic systems. It cannot, however, be excluded that in the conditions of our experiments, when irreversible inhibition is observed (exp. 4–6), that such a large pH difference between phases "R" and "L" occurs, probably caused by the inhibitory effect of protamine on proton back-diffusion into the mitochondria added to the extra ejection of protons from the mitochondria caused by gramicidin.

In these circumstances very high alkalinisation occurs of the inside of the mitochondria which causes serious damage to the mitochondrial structure, particularly changes in the cristae, which are observable by electron microscopy (fig. 1C).

The possibility that this damage may be due to pH changes is supported by experiments in which NH_4Cl was added prior to gramicidin when the structural changes are reduced (fig. 1D). A proposed explanation of the protective effect of ammonium ions on both mitochondrial structure and respiration is presented in fig. 2.

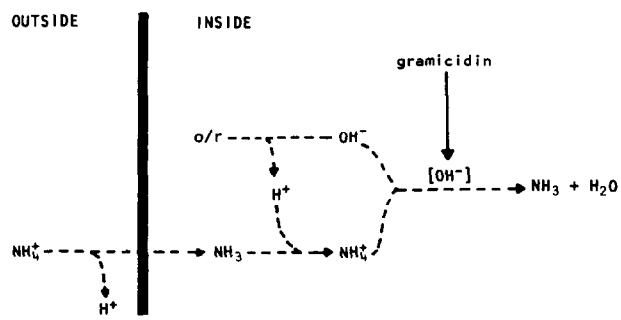

Fig. 2. Proposed mechanism for ammonia transport in mitochondria in the presence of gramicidin.

Ammonia as an electroneutral agent passes across the mitochondrial membrane despite the presence of protamine. Inside the mitochondria NH_3 can form NH_4^+ ions, probably by reacting with protons from the respiratory chain. Exp. 8, in which the reversing effect of DNP, a proton conducting agent, on protamine-inhibited respiration is diminished by the prior addition of NH_4Cl suggests that intramitochondrially formed NH_4^+ ions can react with metabolically generated OH^- ions only when the pH inside the mitochondria increases. Addition of gramicidin causes an increase of OH^- ion concentration inside the mitochondria by ejection of extra protons. In these conditions the excess OH^- ions produced by the action of the respiratory chain can react with NH_4^+ ions to form NH_3 and H_2O. Other mechanisms are also possible and have been proposed [5].

We can only speculate on the physiological significance of the observed protective effect of ammonium ions on mitochondrial structure and function but it is interesting that ammonia-forming enzymes in mitochondria are activated by externally applied or metabolically generated alkaline conditions [6, 7].

Even though the results obtained by electron microscopy may always be disputed, in this particular instance they fit reasonably well with the chemiosmotic hypothesis, as do the pH-induced changes in the ultrastructure of submitochondrial particles [8].

References

[1] J.Popinigis, W.Rzeczycki and J.Świerczyński, FEBS Letters 8 (1970) 149.
[2] E.C.Weinbach, Anal. Biochem. 2 (1961) 335.
[3] E.Layne, Methods of Enzymology, Vol. III (Academic Press New York, 1957) p. 447.
[4] E.C.Slater, European J. Biochem. 1 (1967) 317.
[5] J.B.Chappell and A.R.Crofts, Biochem. J. 95 (1965) 393.
[6] H.B.Le'John, J. Biol. Chem. 243 (1968) 5126.
[7] Z.Kovačevič, J.D.McGivan and J.B.Chappell, Biochem. J. 116 (1970) 21 p.
[8] J.M.Wrigglesworth and L.Packer, Arch. Biochem. Biophys. 133 (1969) 194.