The Prevention of Swelling of Liver Mitochondria *in vitro*

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Previous work has shown that mitochondria can maintain a low water content if incubated under conditions favourable to oxidative phosphorylation (Bartley & Davies, 1952, 1954; Macfarlane & Spencer, 1953; Price & Davies, 1954; Price, Fonnesu & Davies, 1956). Harman & Feigelson (1952) used the phase-contrast microscope to assess the morphological changes of isolated heart-muscle mitochondria, and concluded that oxidative phosphorylation is the essential mechanism for maintenance of mitochondrial form. They also found that uncoupling agents, such as 2:4-dinitrophenol, Janus green and usnic acid, produce swelling of isolated mitochondria. It has also been found that added adenosine triphosphate (ATP) inhibits the swelling of mitochondria (Raafaub, 1952, 1953*a, b*) and that the 'spontaneous' swelling of mitochondria left at room temperature is paralleled by a fall in the concentration of the intramitochondrial ATP (Brenner-Holzach & Raafaub, 1954).

The reciprocal relationship between water content (or swelling) and oxidative phosphorylation (or availability of 'high-energy' phosphate bonds) in mitochondria has been much emphasized, and this paper records the results of an investigation into the conditions which prevent the swelling of mitochondria. Part of this work has been communicated to the Biochemical Society (Fonnesu & Davies, 1955).

EXPERIMENTAL

Abbreviations. These are as follows: adenosine monophosphate, AMP; adenosine diphosphate, ADP; adenosine triphosphate, ATP; 2:4-dinitrophenol, DNP; inosine monophosphate, IMP; inosine diphosphate, IDP; inosine triphosphate, ITP.

Preparation of mitochondria. The method used was very similar to that of Macfarlane & Spencer (1953). All operations were carried out in a cold room at 1° with previously chilled materials.

Male albino rats were fasted for 12 hr., stunned by a blow on the head and decapitated. The liver was quickly excised and cooled for 3 min. in partially frozen 0.25 M sucrose. The chilled tissue was blotted, passed through a Fischer mincer

(Broyeur de Fischer à Main; Jouan, Paris) to remove most of the connective-tissue framework and then forced through a Craigie (1949) pressure mincer. The pulp was weighed, suspended in 9 vol. of ice-cold 0.25 M sucrose and centrifuged in a Servall angle centrifuge for 30 min. at 300 g to sediment unbroken liver cells, nuclei and red cells. The supernatant was removed with a hook-tipped pipette and centrifuged for 30 min. at 6000 g to sediment the mitochondria; the supernatant and the 'fluffy layer' (Schneider & Hogeboom, 1951) were poured off and the well-packed mitochondria resuspended in 0.25 M sucrose for washing or use. When indicated, the mitochondria were washed once or twice in a volume of 0.25 M sucrose equal to that of the original suspension and isolated by centrifuging for 30 min. at 6000 g.

Incubation. The mitochondria in 0.25 M sucrose were added to the various media in conical flasks at 28° in a Dubnoff Metabolic Shaking Incubator (Precision Instruments Co., Chicago). The gas phase was either air or N₂. Complete anaerobiosis was maintained with a stick of yellow phosphorus placed in the centre well of the flask.

Separation of the mitochondria. After incubation, the reaction mixture was pipetted or poured into a weighed Pyrex centrifuge tube and centrifuged at room temp. (approx. 20°) in the high-speed head no. 295 of an International Centrifuge, size 2, under the conditions described later. The supernatant was decanted and the inside of the tube dried with strips of filter paper. The tube was sealed with a square of Parafilm (Marathon Corporation, Menasha, Wis., U.S.A.) until it was weighed. The dry weight of the mitochondria was obtained after drying overnight at 105°.

Chromatography. Cold trichloroacetic acid [0.5 ml. of a 25% (w/v) solution] was added to a sample (3.0 ml.) of the incubation mixture. The clear supernatant obtained after centrifuging was kept frozen at -15° until it was chromatographed on paper by the method of Krebs & Hems (1953). Marker spots containing adenine, adenosine, AMP, ADP, ATP, IMP, IDP and ITP were placed on each paper.

Special chemicals. The dibarium salts of ATP and IMP were prepared by the methods of LePage (1949) and of Ostern (1932), respectively. 2:3-Dimercaptopropanol (BAL) was a gift from Dr L. A. Stocken. The following compounds were commercial preparations: AMP (free acid, Roche Products Ltd., Welwyn Garden City), adenosine 3'-phosphate (free acid, L. Light and Co., Colnbrook), ADP (dibarium salt, Schwarz Lab. Inc., New York), uridine 5'-phosphate (disodium salt, Pabst Lab., Milwaukee, Wis., U.S.A.; lot 801), flavin mononucleotide (Sigma Chemical Co., St Louis, U.S.A.; lot H 62-1) and guanosine 5'-phosphate (Sigma Chemical Co.; lot 144-608). The dibarium salts were converted into free acids by the method of Deutsch & Nilson (1953) with Amberlite Resin IR-120 (H),

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analytical grade, manufactured by the Rhom & Haas Co., Resinous Products Division, Philadelphia, U.S.A., and obtained from British Drug Houses Ltd., Poole. All the acids were neutralized to pH 7 with dilute NaOH before use.

A column containing a mixture of 10 ml. of Dowex 1 $\times$ 10 in the hydroxyl form and 10 ml. of Dowex 50 $\times$ 8 in the acid form was used, when indicated, to de-ionize 3 l. of 0.25 M A.R. sucrose (British Drug Houses Ltd.).

RESULTS

The starting point for these experiments was the work of Macfarlane & Spencer (1953) who found that the swelling of rat-liver mitochondria which occurs during incubation at 28° in the presence of oxygen, glutamate, phosphate buffer, cytochrome *c*, sucrose and Mg²⁺ ions was largely prevented by AMP. They suggested that 'the prevention of swelling is associated with the capacity of the system for oxidative phosphorylation or, more accurately, with the presence of an adenine nucleotide'.

Investigation of the action of the components of the Macfarlane & Spencer system

Cytochrome *c*. Table 1 shows that added cytochrome *c* is not necessary to prevent swelling of the mitochondria. The results obtained here with 5×10^{-6} M concentrations and in the complete absence of cytochrome *c* are identical with those of Macfarlane & Spencer who used 2×10^{-5} M cytochrome *c*.

Time of centrifuging. Macfarlane & Spencer (1953) separated the mitochondria after the incubation at 6000 *g* for 20 min. at room temperature and pointed out that the conditions of oxygenation were obviously not optimum. The same results were obtained whether the centrifuging after incubation was for 20 min. at 6000 *g* or for 4 min. at 25 000 *g* at room temperature (cf. Bartley & Davies, 1954). The results are given in detail in Table 7 of Price *et al.* (1956).

Table 1. *Effect of cytochrome c and AMP on water content of mitochondria*

Incubation for 30 min. at 28° in a Dubnoff Shaker; gas phase, air. Medium (final concentrations): 0.02 M sodium L-glutamate; 0.002 M-MgSO₄; 0.01 M potassium phosphate buffer, pH 7.4; 0.083 M sucrose (introduced with the added mitochondria) and, where indicated, 5×10^{-6} M cytochrome *c*. AMP, when present, was 0.001 M. Rat-liver mitochondria (unwashed) were incubated at a concentration of 5 mg. dry wt./ml. After incubation the mitochondria were separated at 6000 *g* for 20 min. at room temp.

Conditions	Dry wt. (%)	Relative water content (ml. of water/g. of dry solids)
Mitochondria incubated in the medium:		
(a) With cytochrome <i>c</i> { —	13.1	6.6
+ AMP	20.1	4.0
(b) Without cytochrome <i>c</i> { —	12.4	7.1
+ AMP	20.2	4.0

Table 2. *Effect of substrate and comparison of the effects of AMP and ATP on water content of mitochondria*

Incubation at 28° in a Dubnoff Shaker; gas phase, air. Medium (final concentrations): 0.002 M-MgSO₄; 0.01 M potassium phosphate buffer, pH 7.4; 0.083 M sucrose and, when indicated, 0.001 M AMP or ATP. Substrate, when present, was 0.02 M sodium L-glutamate. Rat-liver mitochondria (washed twice): 5 mg. dry wt./ml. After incubation the mitochondria were separated at 25 000 *g* for 4 min.

Conditions	Period of incubation (min.)	Dry wt. (%)	Relative water content (ml. of water/g. of dry solids)
Mitochondria in 0.25 M sucrose separated at 0°	0	30.5	2.3
Mitochondria separated at room temp. after incubation in the medium:			
(a) With substrate { —	30	15.7	5.4
+ AMP	30	23.5	3.3
+ ATP	{ 0	24.5	3.1
	{ 30	23.0	3.3
(b) Without substrate { —	30	12.4	7.1
+ AMP	30	22.8	3.4
+ ATP	{ 0	23.9	3.2
	{ 30	22.1	3.5

Table 3. *Effects of AMP, cyanide, DNP and anaerobiosis on water content of mitochondria*

Incubation for 30 min. at 28° in a Dubnoff Shaker. Medium (final concentrations): 0.002M-MgSO₄; 0.08M-NaCl; 0.01M potassium phosphate buffer, pH 7.4; 0.021M sucrose (introduced with added mitochondria) and, where indicated, 0.001M AMP. Rat-liver mitochondria (washed once): 5 mg. dry wt./ml. After incubation the mitochondria were separated at 25000 g for 4 min. at room temp.

Conditions		Dry wt. (%)	Relative water content (ml. of water/g. of dry solids)
Mitochondria in 0.25M sucrose (separated at 0° without incubation)		32.2	2.1
Mitochondria incubated in			
Gas phase	Additions to medium		
Air	— { —	15.9	5.3
	+ AMP	28.2	2.5
Air	4 mM KCN { —	23.3	3.3
	+ AMP	21.9	3.6
Air	0.01 mM DNP { —	20.8	3.8
	+ AMP	23.4	3.3
Air	0.1 mM DNP { —	24.4	3.1
	+ AMP	23.4	3.3
Air	mM DNP { —	21.1	3.7
	+ AMP	19.9	4.0
N ₂	— { —	19.6	4.1
	+ AMP	23.0	3.3

Substrate and metabolism. In the absence of added substrate and adenine nucleotides the water content of the sedimented mitochondria was higher (Table 2). This is possibly due to the reduced total osmolarity of the medium without substrate. The rapid initial swelling, which occurred when the mitochondria in 0.25M sucrose at 0° were added to any of the media at room temperature, may also be an effect of the reduction of the osmolarity (to approx. 0.12–0.16 osm). In the presence of either AMP or ATP the effect of the added substrate during the incubation was negligible. The maintenance of the water content during a 30 min. incubation without substrate suggested that oxidative metabolism is not necessary to maintain a low water content at 28°. To test this point, these experiments without substrate were repeated in the absence of oxygen or the presence of cyanide or DNP. The results show conclusively (Table 3) that aerobic metabolism or oxidative phosphorylation are not required to maintain low water contents. With no added AMP the mitochondria were actually less swollen in the presence of these inhibitors than in their absence but the protective effect of AMP in maintaining a low water content was reduced or absent. In the presence of substrate, however, DNP promoted swelling (Harman & Feigelson, 1952; Price *et al.* 1956). In the experiments recorded in Table 3 the medium contained chloride (cf. Macfarlane & Spencer, 1953). The increased osmolarity accounts for the higher values of the percentage dry weights of the mitochondria.

Inorganic ions. The remaining components of the original Macfarlane & Spencer system were found to be either advantageous or disadvantageous for the protective effect of AMP or ATP. The results in Table 4, section *a*, are the control values for the water content which were relatively high because the total osmolarity of the medium had to be reduced even further for these experiments. Section *b* shows that in the presence of Mg²⁺ ions (with the small amount of sucrose contributed by the added particles) the water content of the mitochondria was lower than in Mg²⁺ plus phosphate buffer (section *a*). AMP and ATP were also effective alone (section *d*) but the presence of Mg²⁺ ions prolonged their action (section *b*). Phosphate buffer, however, effectively antagonized Mg²⁺ ions, AMP and ATP (sections *a*, *b*, *c*). Other experiments showed that phosphate buffer alone caused the same degree of swelling at pH 6.5, 7.0 and 7.4. Added Na⁺, K⁺, Cl[−] and SO₄^{2−} ions were without significant effect in the concentration range 1–10 mM.

It was unexpected that ATP was always less effective than AMP in protecting the mitochondria from swelling. It seemed possible from the results of Table 4 that this could be due to an antagonizing effect of inorganic phosphate liberated from ATP during incubation. Table 5 shows the following descending order of effectiveness after 30 min. of incubation: AMP, ADP, ATP, 'AMP+1 phosphate', 'AMP+2 phosphates'. After 60 min. of incubation the protective action of the nucleotides has disappeared in all conditions except for AMP

Table 4. *Effect of AMP and ATP with the complete medium and with all the components separately on the water content of mitochondria*

Incubation at 28° in a Dubnoff Shaker; gas phase, air. Rat-liver mitochondria (washed twice): 5 mg. dry wt./ml. After incubation the mitochondria were separated at 25000 g for 4 min. at room temp.

Conditions	Period of incubation (min.)	Dry wt. (%)	Relative water content (ml. of water/g. of dry solids)		
Mitochondria in 0.25 M sucrose separated at 0°	0	30.9	2.2		
Mitochondria incubated in					
(a) 2 mM-MgSO ₄ ; 10 mM potassium phosphate buffer, pH 7.4; 40 mM sucrose	—	{ 0	17.8	4.6	
		{ 30	12.2	7.2	
		{ 60	11.7	7.5	
	+ mM AMP	{ 30	15.6	5.4	
		{ 60	11.9	7.4	
	+ mM ATP	{ 30	13.7	6.3	
		{ 60	12.0	7.3	
	(b) 2 mM-MgSO ₄ ; 40 mM sucrose	—	{ 0	19.9	4.0
			{ 30	14.4	5.9
			{ 60	15.3	5.5
		+ mM AMP	{ 30	17.1	4.8
			{ 60	18.3	4.5
+ mM ATP		{ 30	13.7	6.3	
		{ 60	14.0	6.1	
(c) 10 mM potassium phosphate buffer, pH 7.4; 40 mM sucrose		—	{ 0	16.3	5.1
			{ 30	10.1	8.9
			{ 60	11.6	7.6
		+ mM AMP	{ 30	11.4	7.8
			{ 60	11.5	7.7
	+ mM ATP	{ 30	10.2	8.8	
		{ 60	11.2	7.9	
	(d) 40 mM sucrose	—	{ 0	14.6	5.8
			{ 30	11.7	7.5
			{ 60	10.8	8.2
		+ mM AMP	{ 30	19.0	4.3
			{ 60	15.4	5.5
+ mM ATP		{ 30	13.6	6.4	
		{ 60	10.2	8.8	

Table 5. *Effects of AMP, 'AMP + 1 phosphate', ADP, 'AMP + 2 phosphates' and ATP on the water content of mitochondria*

Details are as in Table 4.

Conditions	Period of incubation (min.)	Dry wt. (%)	Relative water content (ml. of water/g. of dry solids)
Mitochondria in 0.25M sucrose and separated at 0°	0	30.7	2.3
Mitochondria incubated in 40 mM sucrose with addition of:			
—	{ 0	13.7	6.3
	{ 30	11.7	7.5
	{ 60	9.5	9.5
mM AMP	{ 30	17.4	4.7
	{ 60	11.6	7.6
mM AMP + mM Inorganic phosphate	{ 30	11.3	7.8
	{ 60	9.3	9.8
mM ADP	{ 30	14.4	5.9
	{ 60	9.5	9.5
mM AMP + 2 mM Inorganic phosphate	{ 30	10.0	9.0
	{ 60	9.5	9.5
mM ATP	{ 30	12.8	6.8
	{ 60	9.3	9.8

alone which has some residual activity. Table 6 shows that inorganic phosphate can inhibit large excesses of AMP.

Effects of other compounds on the water content of mitochondria

Since AMP alone was able to protect mitochondria (Tables 4 and 5) other nucleotides were tested. It was found that adenosine 3'-phosphate (1-3 mM), inosine 5'-phosphate (1-5 mM), guanosine 5'-phosphate (1-2 mM), uridine 5'-phosphate (1-2 mM) and flavin mononucleotide (mM) were completely without effect on the water content of mitochondria after 30 min. of incubation. Similarly, adenosine (mM) and inosine (mM) were ineffective.

It is known that in many systems Mn^{2+} ions have a similar effect to Mg^{2+} ions, whereas Ca^{2+} ions act as an antagonist (Lindberg & Ernster, 1954; Ernster, Lindberg & Löw, 1955; Ernster & Löw, 1955; Hunter & Ford, 1955). Table 7 shows that this relationship also applies in our system. Mn^{2+} ions are even more effective than Mg^{2+} ions in maintaining the water content of the mitochondria in the presence or absence of AMP, and both these ions and AMP are antagonized by Ca^{2+} ions. Slater & Cleland (1953) found that Ca^{2+} ions can become bound to sarcosomes during isolation and, since Ca^{2+} ions are so deleterious to liver mitochondria (Table 7), the effects of 'calcium-binding' agents were investigated. Table 8 shows that the mitochondria are protected by ethylenediaminetetraacetate, citrate and oxalate. Ethylenediaminetetra-

acetate in particular has a most powerful and prolonged effect. There was also some protection in the presence of glutathione and cysteine, but since 2:3-dimercaptopropanol (BAL) was quite ineffective it is unlikely that this protection depends simply on the presence of the sulphydryl groups.

When these experiments were almost completed it was found that, after the use of a new batch of sucrose, AMP no longer reduced swelling in the controls. Investigation showed that several new bottles of A.R. sucrose contained some impurity which could be removed by passing the sucrose solution through a mixture of resins, as described in the Experimental section. No inorganic orthophosphate was detectable in the sucrose solution by the method of Berenblum & Chain (1938), and it seemed possible that the impurities were metal ions such as Ca^{2+} , Zn^{2+} or other ions (cf. Slater & Cleland, 1953; Hunter & Ford, 1955). The results in Table 9 are in agreement with this assumption, which suggests that a routine de-ionization of A.R. sucrose would be advantageous in experiments of this kind with mitochondria.

Effect of incubation on the added nucleotides

It seemed possible that the relatively transient effects of AMP, ADP and ATP were due to their breakdown during the first 30 min. of incubation and that perhaps Mn^{2+} and Mg^{2+} ions could act by preventing the breakdown of endogenous nucleotides. The chromatograms shown in Fig. 1 were made to investigate these possibilities and were from

Table 6. *Effects of the ratio AMP/inorganic phosphate on the water content of mitochondria*

Details as in Table 4.			
Conditions	Period of incubation (min.)	Dry wt. (%)	Relative water content (ml. of water/g. of dry solids)
Mitochondria in 0.25M sucrose and separated at 0°	0	30.7	2.3
Mitochondria incubated in 40 mM sucrose with addition of			
—	{ 0	14.0	6.1
	{ 30	10.9	8.2
	{ 60	9.9	9.1
1 mM AMP	{ 30	17.1	4.9
	{ 60	12.4	7.1
5 mM AMP	{ 30	16.6	5.0
	{ 60	11.9	7.4
1 mM AMP + 1 mM Inorganic phosphate	{ 30	14.0	6.1
	{ 60	10.0	9.0
2 mM AMP + 2 mM Inorganic phosphate	{ 30	14.0	6.1
	{ 60	10.2	8.8
3 mM AMP + 3 mM Inorganic phosphate	{ 30	13.4	6.5
	{ 60	10.2	8.8
5 mM AMP + 5 mM Inorganic phosphate	{ 30	14.8	5.8
	{ 60	12.5	7.0

Table 7. *Effects of AMP, ADP, ATP, MnCl₂ and CaCl₂ on the water content of mitochondria*

Unless otherwise indicated, AMP, ADP, ATP, MnCl₂ and CaCl₂, when used either alone or in mixtures, were 0.001 M (final concn. after the addition of the mitochondria). Other details are as in Table 4.

Conditions	Period of incubation (min.)	Dry wt. (%)	Relative water content (ml. of water/g. of dry solids)
Mitochondria in 0.25 M sucrose and separated at 0°	0	30.5	2.3
Mitochondria incubated in 40 mM sucrose with addition of			
—	{ 30	10.2	8.8
	{ 60	9.8	9.2
AMP	{ 30	16.6	5.0
	{ 60	12.2	7.2
AMP (5 mM)	{ 30	16.7	5.0
	{ 60	11.8	7.5
ADP	{ 30	14.0	6.1
	{ 60	9.8	9.3
ATP	{ 30	12.3	7.1
	{ 60	9.3	9.9
MnCl ₂	{ 30	19.2	4.2
	{ 60	16.3	5.1
AMP + MnCl ₂	{ 30	22.4	3.5
	{ 60	17.8	4.6
MgCl ₂	{ 30	15.7	5.4
	{ 60	13.4	6.5
AMP + MgCl ₂	{ 30	17.1	4.8
	{ 60	13.7	6.3
CaCl ₂	{ 30	12.3	7.1
	{ 60	12.5	7.0
AMP + CaCl ₂	{ 30	11.6	7.6
	{ 60	12.3	7.1
MnCl ₂ + CaCl ₂	{ 30	14.0	6.1
	{ 60	14.8	5.8
AMP + MnCl ₂ + CaCl ₂	{ 30	13.8	6.2
	{ 60	13.4	6.5
MgCl ₂ + CaCl ₂	{ 30	13.0	6.7
	{ 60	13.4	6.5
AMP + MgCl ₂ + CaCl ₂	{ 30	12.1	7.3
	{ 60	13.5	6.4

Table 8. *Effects of AMP, glutathione, cysteine, ethylenediaminetetraacetate (EDTA), citrate and oxalate on the water content of mitochondria*

Details as in Table 4.

Conditions	Period of incubation (min.)	Dry wt. (%)	Relative water content (ml. of water/g. of dry solids)
Mitochondria in 0.25 M sucrose separated at 0°	0	30.7	2.3
Mitochondria incubated in 40 mM sucrose with addition of			
—	{ 30	10.9	8.2
	{ 60	9.9	9.1
mM AMP	{ 30	17.1	4.8
	{ 60	12.4	7.1
mM Sodium EDTA	{ 30	20.2	4.0
	{ 60	20.8	3.8
mM Sodium citrate	{ 30	15.8	5.3
	{ 60	15.7	5.4
mM Sodium oxalate	{ 30	13.7	6.3
	{ 60	11.0	8.1
mM Glutathione	{ 30	17.4	4.7
	{ 60	11.6	7.6
mM Cysteine	{ 30	16.7	5.0
	{ 60	9.7	9.3

the experiments shown in Table 7. It is clear that when the medium contained mM AMP (final concentration), AMP was largely broken down during the incubation to adenosine plus inorganic phosphate. Small amounts of adenine were also formed but deamination to inosine compounds did not occur (Fig. 1*a*). Fig. 1*b* shows that with 5 mM AMP a large fraction still remained after 60 min. incubation (the adenine formed cannot be seen in this chromatogram because only one-fifth as much medium was placed on the starting line). A similar breakdown occurred with ADP (Fig. 1*c*) and with ATP (Fig. 1*d*).

Fig. 1*e* and *f* show that Mn^{2+} , Mg^{2+} , Ca^{2+} , Mn^{2+} plus Ca^{2+} and Mg^{2+} plus Ca^{2+} ions actually stimulate the breakdown of AMP despite the prevention of swelling which occurs in the presence of AMP plus Mn^{2+} , AMP plus Mg^{2+} but not AMP plus Ca^{2+} ions (cf. Table 7).

DISCUSSION

The most surprising finding in these experiments is that mM concentrations of Mn^{2+} or Mg^{2+} ions plus AMP can largely protect non-respiring liver mitochondria from swelling even in solutions as dilute as 40 mM sucrose. The results show that neither an active state of metabolism nor the presence of 'high-energy' phosphate esters is required to maintain low water contents. It is also of interest that DNP, which causes swelling of mitochondria respiring in the presence of substrate (Harman & Feigelson, 1952; Price *et al.* 1956), actually helps to

maintain the water content of non-respiring mitochondria in the absence of substrate. Simple osmotic effects appear to be excluded as the cause of these phenomena and it seems likely that the agents which prevent swelling act on the structure of mitochondria in ways which are not yet understood.

Amongst the nucleotides tested, AMP, ADP and ATP are the only ones effective in protecting the non-respiring mitochondria from swelling, and AMP is the most powerful. It seems likely that the disappearance with time of the protection by AMP is due both to the destruction of the AMP molecule and to the appearance of inorganic orthophosphate. This loss of protective action occurs even when large amounts of AMP are added (Table 7) so that considerable quantities are still present at the end of the incubation (Fig. 1*b*). This interpretation is also supported by the finding that a low water content of non-respiring mitochondria can be maintained by nucleotides for long periods (30–60 min. at 28°) only in the presence of Mn^{2+} or Mg^{2+} ions. Although these two ions stimulate the liberation of inorganic orthophosphate from added nucleotides (cf. Fig. 1*e, f*), they antagonize the effects of the phosphate formed (Table 4). The deleterious effect of inorganic orthophosphate on the water content of mitochondria confirms the results of Raafaub (1952, 1953*a*) based on changes in optical density (see also Stanbury & Mudge, 1953), and is in agreement with the results of Hunter & Ford (1955), who found that phosphate can reversibly inhibit oxidative phosphorylation in mitochondria.

Table 9. *Effect of de-ionization of A.R. sucrose on the water content of mitochondria in the presence or absence of AMP*

The sucrose solution, when indicated, was de-ionized with a mixture of Dowex 1 $\times$ 10–Dowex 50. Other details are as in Table 4.

Conditions	Period of incubation (min.)	Dry wt. (%)	Relative water content (ml. of water/g. of dry solids)			
Mitochondria in 0.25M A.R. sucrose (separated at 0°)	0	32.8	2.1			
Mitochondria in 0.25M de-ionized A.R. sucrose (separated at 0°)	0	34.1	1.9			
Mitochondria in 0.25M de-ionized A.R. sucrose containing 0.1 mM $CaCl_2$ (separated at 0°)	0	31.4	2.2			
Mitochondria incubated in						
(a) 40 mM A.R. sucrose	{ —	{ 30	12.5	7.0		
		{ 60	12.7	6.9		
	{ + mM AMP	{ 30	11.3	7.9		
		{ 60	11.0	8.1		
		(b) 40 mM de-ionized A.R. sucrose	{ —	{ 30	14.0	6.1
				{ 60	11.1	8.0
{ + mM AMP	{ 30		19.3	4.2		
	{ 60		11.4	7.8		
	(c) 40 mM de-ionized A.R. sucrose containing 0.017 mM- $CaCl_2$		{ —	{ 30	12.0	7.3
				{ 60	12.2	7.2
{ + mM AMP		{ 30	11.6	7.6		
		{ 60	11.5	7.7		

Fig. 1

Thus the conditions are now known under which non-respiring liver mitochondria can remain contracted or swell, and respiring liver mitochondria can remain contracted, swell and recontract. The detailed mechanisms involved in these changes remain to be elucidated.

SUMMARY

1. The swelling of rat-liver mitochondria which occurs during incubation at 28° can be largely

prevented by adenosine triphosphate (ATP) and adenosine monophosphate (AMP) in the presence and absence of substrate.

2. Aerobic metabolism or oxidative phosphorylation is not required to maintain a low water content of mitochondria.

3. Mg²⁺ and Mn²⁺ ions largely protect mitochondria from swelling. Both Mg²⁺ and Mn²⁺ ions enhance and prolong the action of AMP, and both these ions and AMP are antagonized by Ca²⁺ ions

and by inorganic orthophosphate. Added Na^+ , K^+ , Cl^- and SO_4^{2-} ions in the range 1–10 mM are without effect, and citrate, oxalate and ethylenediamine-tetraacetate protect mitochondria from swelling.

4. The effects of the nucleotides, Mg^{2+} and Mn^{2+} ions on the water content of mitochondria are also appreciable in a medium consisting of only 40 mM sucrose. In this medium AMP is more effective than adenosine diphosphate (ADP), which is better than ATP, apparently because of the inhibitory effect of the inorganic phosphate liberated during incubation. All three nucleotides are broken down to adenosine and inorganic phosphate, and this breakdown is more rapid in the presence of Mg^{2+} , Mn^{2+} and Ca^{2+} ions either alone or in mixtures.

5. Adenosine 3'-phosphate, inosine 5'-phosphate, guanosine 5'-phosphate, uridine 5'-phosphate, flavin mononucleotide, adenosine and inosine were without effect on the water content of mitochondria.

6. It is concluded that the agents which prevent swelling act on the structure of the mitochondria in ways which are not yet understood.

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The Metabolism of Butylated Hydroxyanisole in the Rabbit

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This paper deals with the metabolism in the rabbit of a substance known as butylated hydroxyanisole (BHA) that is used commercially as an antioxidant in edible fats (Kraybill *et al.* 1949). The material is a mixture of two isomers, 85% of 2-*tert.*-butyl-4-methoxyphenol (I) and 15% of 3-*tert.*-butyl-4-methoxyphenol (II). Although studies of the conventional type on acute and chronic toxicity have been made (see Wilder & Kraybill, 1948; Lehman, Fitzhugh, Nelson & Woodward, 1951; Graham, Teed & Grice, 1954) no studies on metabolism have

been reported. The metabolism of the commercial mixture and of the pure isomers has been examined and the major metabolites have been isolated and characterized.

EXPERIMENTAL

Animals, diet and dosage. The rabbits used were does of the New Zealand white strain, weighing 1.6–2.3 kg. They were maintained on a standard pellet diet (wheat, 40%; pollard, 33%; bran, 27%) and were kept singly in metabolism cages designed to permit the separate collection of