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Movements of Water and Ions in Mitochondria

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The osmotic environment can affect the metabolic activity of mitochondria (Kielley & Kielley, 1951; Potter & Recknagel, 1951; Raaflaub, 1953*b*; Lehninger & Kennedy, 1948). Harman & Feigelson (1952*a*, *b*) concluded from visual comparisons that the morphological state of swelling not only influenced, but was in turn influenced by, the rates of oxidation and oxidative phosphorylation. Another relation, that oxidative phosphorylation in isolated mitochondria can be coupled to active transport, was shown by Bartley & Davies (1952, 1954) and Macfarlane & Spencer (1953), who also found that these particles maintained a low water content in the presence but not the absence of an adenosine phosphate.

This paper describes experiments on the nature of the water and ion movements in respiring rat-liver mitochondria (Macfarlane & Spencer, 1953). One finding of the present study, the extrusion of water by mitochondria as distinct from the maintenance of the initial water content, has been obtained by Chappell & Perry (1954) with non-respiring suspensions of sarcosomes under conditions widely different from ours. Part of this work has been communicated to the Biochemical Society (Price & Davies, 1954).

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EXPERIMENTAL

Abbreviations. These are as follows: adenosine monophosphate, AMP; adenosine diphosphate, ADP; adenosine triphosphate, ATP; aminotrishydroxymethylmethane, tris; 2:4-dinitrophenol, DNP; diphosphopyridine nucleotide, DPN; ethylenediaminetetraacetic acid, EDTA.

Preparation of mitochondria and sarcosomes. Albino rats were made unconscious by stunning and then their throats were cut. The livers were removed, cooled for several minutes in partially frozen 0·25 M sucrose or partially frozen 0·9% KCl, weighed and passed through a Fischer mincer (Broyeur de Fischer à Main; Jouan, Paris) and then a Craigie pressure mincer with a finely corrugated plug (Craigie, 1949). The homogenate formed was suspended in

9 vol. of 0·25 M sucrose and centrifuged in an International Refrigerated Centrifuge Model PR-1 for 10 min. at 900 *g*. The supernatant was collected and centrifuged for 10 min. at 20000 *g*. The sediment from the high-speed centrifuging included both mitochondria and a major part of the 'fluffy layer' (Schneider & Hogeboom, 1951). This fraction was either used directly or washed in 2 ml. of 0·25 M sucrose/g. wet wt. of original tissue, centrifuged again for 10 min. at 20000 *g*, and the sediment resuspended in approx. 1 ml. of 0·25 M sucrose/g. wet wt. of original tissue. The washed mitochondria were macroscopically free from the 'fluffy layer'.

The method of preparation of sarcosomes from pigeon-breast muscle was similar to that recommended by Slater & Cleland (1953): the excised muscle was quickly cut into small pieces and placed in a mixture of 0·25 M sucrose and 0·01 M EDTA (adjusted to pH 7·4 with NaOH); the chilled tissue was then passed through an ice-cold Latapie mincer, mixed with sufficient sucrose–EDTA to make a 10% (w/v) suspension and ground for 30 sec. in a homogenizer of the Potter–Elvehjem type with a nylon pestle. The suspension was then centrifuged for 5 min. at 700 *g* and the resulting supernatant spun at 3000 *g* for 15 min. The sediment from this centrifuging was taken up in approx. 2 ml. of the sucrose–EDTA mixture/g. wet wt. of original muscle, transferred to the high-speed head of the International Refrigerated Centrifuge, spun at 10000 *g* for 5 min., and resuspended in the desired volume.

Incubation media. Unless otherwise indicated, the standard medium for respiring particles was similar to that employed by Macfarlane & Spencer (1953): 0·02 M substrate (usually succinic acid neutralized with NaOH); 0·01 M potassium phosphate buffer, pH 7·4; 0·005 M-$MgSO_4$; 0·03 M tris buffer adjusted to pH 7·4 with HCl; 0·083 M sucrose (contributed from the added mitochondria) and, where indicated, 0·001 M AMP or ATP. Mitochondria were present in a final concentration of between 4 and 10 mg. dry wt./ml. in a final volume of 3 ml. In the standard procedure the suspensions were shaken at 35° in a Dubnoff Metabolic Shaking Incubator (Precision Scientific Co., Chicago, U.S.A.). The gas phase was air.

The medium for the non-respiring system was similar to that of Cleland (1952), namely 0·1 M-KCl, 0·05 M tris buffer (pH 7·4), 0·005 M-$MgSO_4$, approx. 0·01 M sucrose, 5×10^{-4} M EDTA (contributed from the added sarcosomes) and, where indicated, 0·001 M ATP. Sarcosomes were present in a final concentration of about 0·5 mg. dry wt./ml. The final volume varied, but samples of 20 ml. were required for determinations involving centrifuging. In the non-respiring system the suspensions were not shaken but allowed to stand in flasks.

Estimation of water content. (*a*) Gravimetric method. The suspensions in the Dubnoff vessels were drained into tared glass centrifuge tubes and spun in the high-speed head of an International Centrifuge at room temperature (20°). The centrifuge was accelerated to a maximum velocity equivalent to 25000 *g* in 0·5 min., maintained for 2 or 4 min. as indicated, and stopped within a further 2 min. The supernatants were decanted and the interiors of the tubes blotted with filter-paper strips. To prevent evaporation a small sheet of Parafilm (Marathon Corp., Menasha, Wis.) was pressed over the centrifuge tubes until their wet weights had been determined. After drying overnight at 105°, the tubes plus mitochondria were reweighed. A comparison of the two determinations and the known tare weights of the centrifuge tubes provided measurements of wet weight, percentage dry weight of the mitochondrial sediment, etc.

(*b*) Optical method. The method of Cleland (1952) was found to offer a rapid and essentially continuous estimation of mitochondrial water. It consists simply in measuring the optical density of the suspension, but a very dilute suspension (approx. 0·3 mg. dry wt./ml.) is required for optical densities low enough to be significant. In our studies several variations of the basic principle were employed for measurements of suspensions sufficiently dense for simultaneous determination of respiration, water content and ion content. The following were advantageous modifications: samples from a suspension (5–15 mg. dry wt./ml.) were removed serially from the incubating vessels in a Pasteur pipette calibrated at 0·3 ml., and placed in thin cuvettes (1 mm. light path; Unicam Instruments Ltd., Cambridge). Within 30 sec. the optical density was determined at 950 mμ.; the sample was then withdrawn with the same pipette and discarded. A decrease in the light-scattering and hence optical density corresponded to swelling due to the entrance of water.

Since different preparations showed different lag times before the onset of swelling, the optical method was particularly useful as a guide for the removal of samples for centrifuging in the gravimetric method.

Estimation of sodium and potassium. An amount of 16 N-HNO_3 (known to be free of Na and K) approximately equal to the original wet weight of mitochondria was added to the dried mitochondria in the glass centrifuge tubes obtained in the course of estimation of water content. The samples were digested either for 18 hr. at room temperature or for 30 min. at 100° and made up to 10 ml. with water. Two 5 ml. samples were then diluted to 10 ml. with KCl and NaCl respectively, so that the final concentration of KCl was 0·0382% (w/v) for estimations of Na and the final concentration of NaCl was 0·84% (w/v) for estimations of K (Whittam & Davies, 1953). The supernatant samples were treated in a parallel manner, except that no digestion was required. The two cations were then estimated by flame photometry.

Phosphate analyses. The general method of phosphate analysis was that of Krebs & Hems (1953). Where measurements were required on the unfractionated incubation mixtures, the procedure was as follows: 0·5 ml. samples were removed at various times and simultaneously chilled in cracked ice and stirred with 0·05 ml. of 50% trichloroacetic acid.

Where separate measurements were required on the isolated mitochondria, 3 ml. samples (the entire contents of a Dubnoff vessel) were centrifuged at high speed, as described for the gravimetric method of estimating water content. Immediately after the rotor had stopped, the supernatant was poured into another centrifuge tube and deproteinized by the addition of 0·25 ml. of 50% trichloroacetic acid. Simultaneously 0·5 ml. of 5% trichloroacetic acid was stirred vigorously into the mitochondrial sediment. At the same time, the glass centrifuge tubes were cooled in ice.

After the protein in the several fractions had been denatured, the extracts were clarified by centrifuging and samples of the trichloroacetic acid extracts spotted on paper chromatograms which were developed and analysed for phosphate compounds by the methods of Krebs & Hems (1953).

RESULTS

Correlation of methods. A primary problem was to correlate the three methods that have been used to measure swelling. Visual observation of mitochondria under a phase-contrast microscope is specific, but only qualitative (Harman & Feigelson, 1952*a*; Watanabe & Williams, 1953; Cleland & Slater, 1953). It was employed in the present study as a check on the validity of other methods. High-speed centrifuging followed by wet- and dry-weight determinations is the most direct method. The optical method for measuring swelling (Cleland, 1952) is convenient, but no quantitative information was available to indicate the relation between changes in optical density and swelling of mitochondria. This relation was investigated in the following way.

Samples (3 ml.) of an incubation mixture containing washed rat-liver mitochondria were placed in Dubnoff vessels, half of which contained 0·001 M ATP, and shaking was begun. Samples were removed from one pair at regular intervals for determination of optical density; the remaining pairs were centrifuged at 10 min. intervals. In the absence of ATP both optical density and percentage dry weight fell within a 30 min. interval to a low level. The presence of ATP stabilized both the high optical density and the percentage dry weight (Fig. 1). It was found that optical-density changes reflected changes in mitochondrial water, except in one detail; mitochondria frequently lost up to 10 % of their dry weight to the solution during the initial minutes of an incubation (not shown in Fig. 1) regardless of whether adenosine phosphates were present or not. In these circumstances the optical density fell without the occurrence of corresponding changes in the water content of the mitochondria.

The decrease in optical density, presumably caused by swelling, of a sarcosome suspension under rather different conditions was ascribed by Cleland (1952) to the entry of ions. The rate of change of optical density was, in fact, taken as a measure of the permeability of the particles to various ions. Measurements of the potassium and sodium contents of the mitochondria isolated by centrifuging are recorded in Fig. 1*c*, *d*. It was found invariably that the concentration of these cations was less in the swollen particles than in those with lower water content. The potassium concentration tended to fall during swelling, although in the presence of ATP the initially high potassium level was maintained. Similarly, the gradient of Na^+ ions is greater in the presence of ATP. In both cases the movement was opposite to that postulated by Cleland as the cause of swelling. However, Tables 3 and 5 show that under some conditions sodium and potassium can enter mitochondria during swelling.

With sarcosomes from pigeon-breast muscle in a system corresponding more precisely to that employed by Cleland (1952; cf. Chappell & Perry, 1954), a fall in optical density was again associated

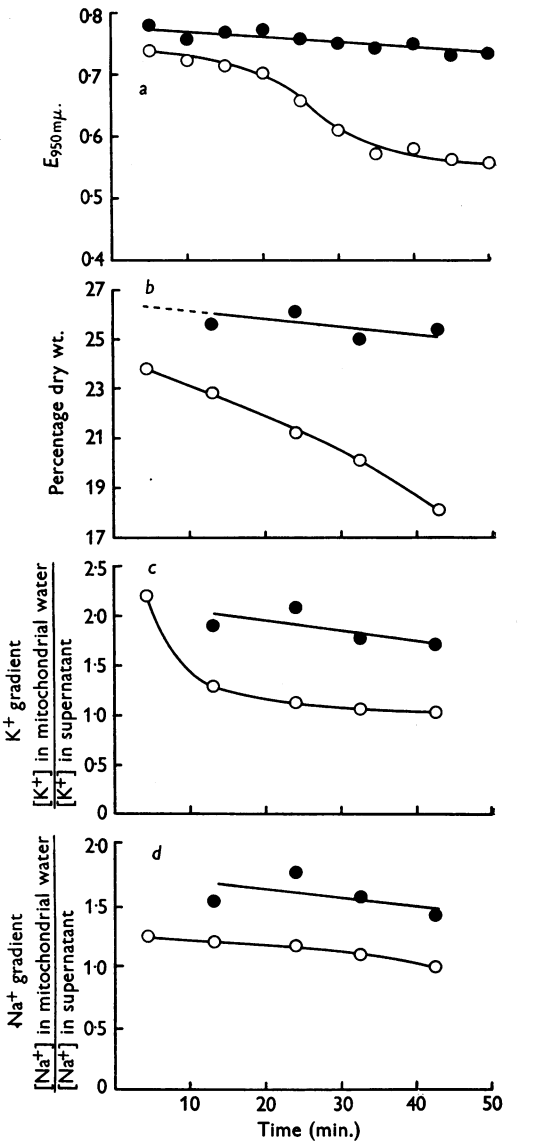


Fig. 1. Course of swelling of rat-liver mitochondria estimated by optical and gravimetric methods, with concurrent cation changes in similar samples. All samples contain about 7 mg. dry wt. of washed mitochondria in the standard stock solution including 20 mM succinate; shaken in air at 35°; ●, mM ATP; ○, no additions. *a* shows the extinction at 950 mμ. of the suspension in cuvettes of 1 mm. light path; *b*, percentage dry wt., determined gravimetrically; *c*, potassium gradients; *d*, sodium gradients.

with an increase in water content (Fig. 2*a*, *b*). This system differed from the one above in several respects; most important was that no substrate was added and there was no shaking. The direction of the changes in the potassium gradients in this system were similar to those of the respiring mitochondria, but the magnitudes were much lower and in the absence of ATP the potassium concentration of the sarcosomes was nearly at equilibrium with the external solution throughout the course of swelling.

Thus in two types of particle systems, differing markedly in composition, dilution and physiological state, the optical density changes associated with swelling parallel changes in water content rather than salt concentration.

Conditions for maintenance of low water content. Macfarlane & Spencer's (1953) system for the maintenance of a low water content consisted of unwashed rat-liver mitochondria oxidizing glutamate and fortified with phosphate, magnesium and cytochrome *c*; it was shaken at 28° and was specifically dependent upon the addition of AMP. Experiments were conducted to determine in more detail the factors required for water maintenance in this system.

No consistent differences were found between mitochondria from recently fed and from fasting rats and the following factors were found to have only small effects: washing the mitochondria once or twice in sucrose; varying the temperature from 20° to 40°; dilution of the mitochondria from 15 to 3 mg. dry wt./ml.; substitution of succinate for glutamate; substitution of ATP for AMP and omission of cytochrome *c* (cf. Fonnesu & Davies, 1956).

Magnesium ions, however, had a strong influence on the rate of swelling. They were included in most systems at 0·005 M concentration and were found to decrease the water content of samples with and

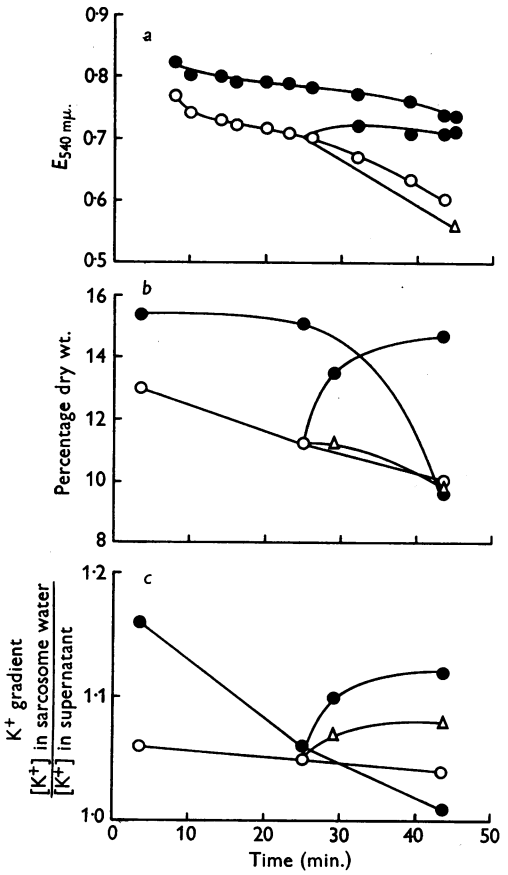


Fig. 2. Course of swelling of non-respiring sarcosomes of pigeon-breast muscle, and reversal by ATP. Sarcosomes present at about 0·5 mg. dry wt./ml. of suspension medium, which consisted of 0·1 M-KCl, 0·05 M tris buffer (pH 7·4) and traces of sucrose and EDTA contributed from the sarcosome stock suspensions; allowed to stand without shaking at 23·6°; ○, no additions; ●, mM ATP; △, mM-NaCl. *a*, the extinction of the suspension at 540 mμ. in cuvettes of 10 mm. light path; *b*, percentage dry wt. determined gravimetrically; *c*, potassium gradient.

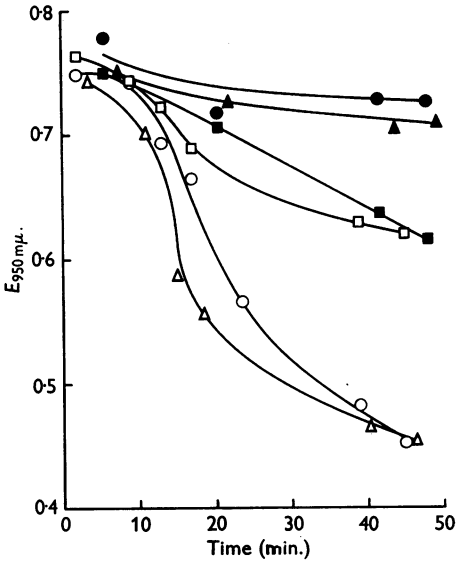


Fig. 3. Effect of inorganic phosphate on the swelling of rat-liver mitochondria. Swelling was estimated by the optical method, extinction at 950 mμ. of suspension in 1 mm. cuvettes; washed mitochondria were suspended in standard stock solution including 20 mM succinate shaken in air at 35°; solid symbols (●, ▲, ■) indicate mM AMP present; open symbols (○, △, □), no AMP; ● and ○, 10 mM inorganic phosphate; ▲ and △, 30 mM inorganic phosphate; ■ and □, no added inorganic phosphate.

without AMP but were most effective in the presence of AMP. They were effective at 0·001 and 0·002 M but much more so at 0·020 M (Tables 1 and 6). Inorganic phosphate (0·01 and 0·03 M) had an opposite effect and normally increased the rate of entry of water in the absence of AMP (Fig. 3). Similarly, bovine-plasma albumin (3 mg./ml.) increased the rate of entry of water.

Aeration also influenced swelling. When the suspensions were allowed to stand in open Dubnoff vessels, so that oxygen could enter by diffusion only, swelling was delayed in the absence of 0·001 M ATP (Fig. 4).

It seems unlikely that differences in the rate of oxygen uptake in the presence and absence of AMP can account for differences in dry weight. The respiration of washed mitochondria, which showed greater differences in water content than unwashed mitochondria, may be greater in the absence of AMP with succinate as substrate (Fig. 5), but with glutamate as substrate the oxygen uptake was greater in the presence of AMP. Neither with succinate nor glutamate did the rate of oxygen uptake fall to zero before 30 min. (cf. Macfarlane & Spencer, 1953) although swelling in the absence of AMP usually occurred during the first 20 min. of incubation. The concentration of magnesium in the present experiments was 5 mM rather than 2 mM, as used by Macfarlane & Spencer (1953).

In preliminary work tris buffer (0·03 M) was found to be advantageous in maintaining the water content of mitochondria and was used in most experiments.

Reversal of swelling. Although Macfarlane & Spencer (1953) had shown that AMP prevents the swelling of respiring mitochondria, it was not known if the swelling process was reversible. To investigate this, 3 ml. of mitochondrial suspension was incubated in Dubnoff vessels under conditions closely approximating those of Macfarlane & Spencer. After swelling had proceeded for 15 min., AMP was added and the resulting changes in water content were determined by the gravimetric method (Table 1). There were two sets of vessels corresponding to two levels (2 and 5 mM) of magnesium. At the lower level, no net reversal was detected although the addition of AMP appeared to reduce further swelling. At the higher level there was a transitory and incomplete reversal. At 2 mM-Mg^{2+} ions concentration, there was no marked difference between AMP and ATP (Table 2) but at 5 mM-Mg^{2+} ions concentration nearly complete reversal was obtained on some but not all occasions upon the addition of ATP (Fig. 6).

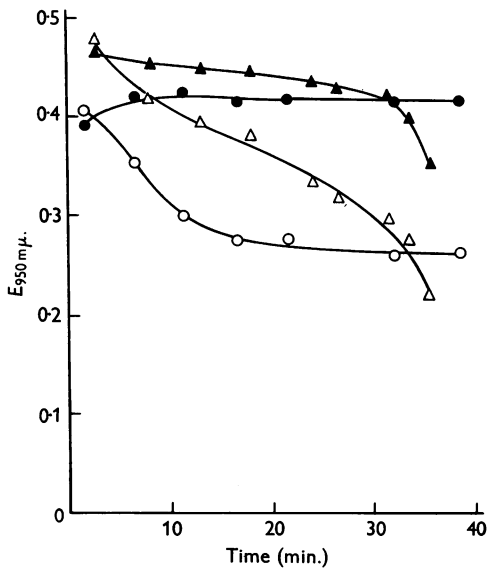


Fig. 4. Effect of aeration on the rate of swelling of rat-liver mitochondria at 35°; estimated by optical method; washed mitochondria were suspended in standard stock solution including 20 mM succinate; ● and ○, suspensions shaken in open Dubnoff vessels; ▲ and △, suspensions not shaken and oxygenation by diffusion only; solid symbols (● and ▲), mM ATP added; open symbols (○ and △), no additions.

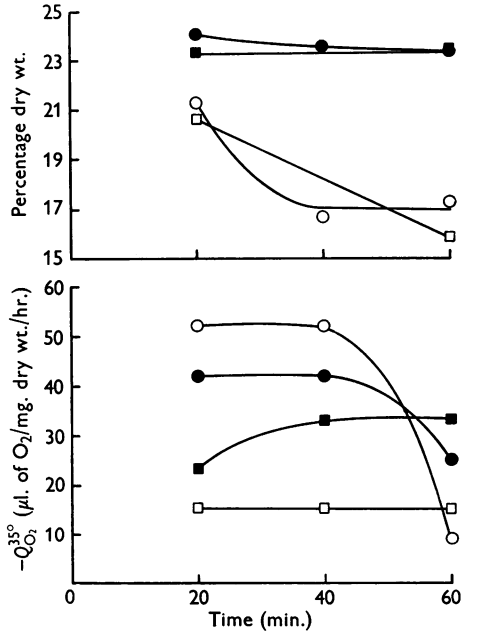


Fig. 5. Effects of succinate, glutamate, and AMP on the rates of oxygen uptake of washed mitochondria in standard stock solution, relative to rates of swelling; ● and ○, 20 mM succinate; ■ and □, 20 mM glutamate; solid symbols (● and ■), mM AMP; open symbols (○ and □), no AMP added.

EDTA has been widely reported to stabilize particle preparations (Slater & Cleland, 1953; Price & Thimann, 1954) and the possibility that it might promote the reversal of swelling was therefore tested. Although 3 mM EDTA completely prevented swelling in the presence or absence of ATP, its effect on reversal was inhibitory (Fig. 6). A slower rate of swelling was noted upon the addition of mM EDTA (Fig. 6), but subsequent reversal of swelling on addition of ATP was not promoted (not shown in Fig. 6).

With the system of non-respiring muscle sarcosomes, swelling was reversed regularly upon the addition of ATP (Chappell & Perry, 1954, and Fig. 2). For the respiring liver mitochondria, an essential factor was clearly indicated. Hunter & Ford (1954) at this time reported that with phosphate-induced inactivation of oxidative phosphorylation, DPN was essential for recovery. This coenzyme was also found to favour the reversal of swelling (Fig. 7). (Further parallels between the two processes are discussed below.)

Dubnoff vessels containing 3 ml. of mitochondrial suspensions without added adenosine phosphates were shaken until swelling had commenced as indicated by the optical method. A few minutes

Table 1. *Effect of magnesium concentration on swelling and the effect of delayed addition of AMP*

Conditions of incubation were similar to those of Macfarlane & Spencer (1953); each sample contained about 20 mg. dry wt. of unwashed mitochondria from the liver of a fasted rat in 3 ml. of medium containing 20 mM sodium glutamate, 10 mM potassium phosphate, pH 7·4, and $MgSO_4$ at two concentrations; it was shaken in Dubnoff vessels at 35°; AMP was added to final concentration of mM either initially or after 15 min.; mitochondria were collected by high-speed centrifuging at times as indicated and percentage dry wt. was determined by gravimetric method.

Time of addition of AMP (min.)	Time of collection of mitochondria (min.)	Percentage dry wt. 2 mM Mg^{2+}	Percentage dry wt. 5 mM Mg^{2+}
0	15	26	26
No AMP	15	15·5	16·5
15	20	15·5	17·5
15	25	13·5	17
15	30	14	16·5
No AMP	30	13	15

Table 2. *Comparison of AMP and ATP on the reversal of swelling at low magnesium concentration*

Conditions as in Table 1, except that $MgSO_4$ was 2 mM throughout, and both AMP and ATP were mM, where added.

Time of addition of adenosine phosphate (min.)	Time of collection of mitochondria (min.)	Percentage dry wt. No adenosine phosphate	mM AMP	mM ATP
0	21	13·9	22·3	21·4
20	21	—	12·6	12·9
20	30	12·4	12·9	13·0
0	30	—	21·5	21·0

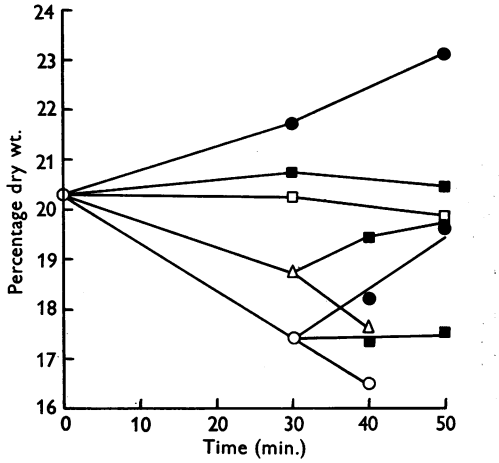


Fig. 6. Reversal of swelling in respiring mitochondria by the addition of ATP, and the inhibition of swelling by EDTA. Standard suspension of mitochondria shaken with 20 mM succinate in air at 35°; open symbols (○, △, □), no ATP; closed symbols (● and ■), mM ATP; ○ and ●, no EDTA; △, mM EDTA; □ and ■, 3 mM EDTA.

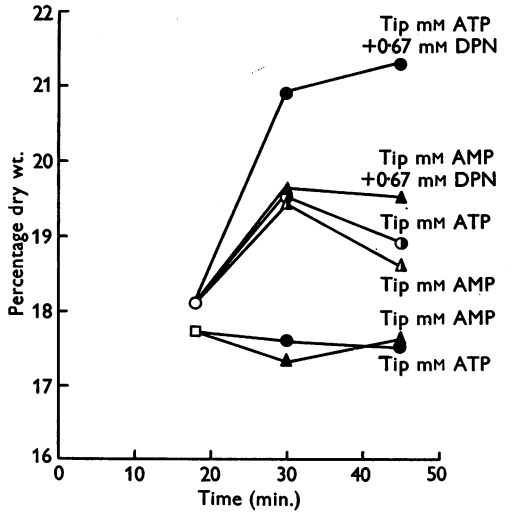


Fig. 7. Effect of DPN on the reversal of swelling by ATP and AMP. Standard suspension of mitochondria shaken with 20 mM succinate in air at 35°; two sets of vessels without ATP or AMP; ○, without DPN initially, and □, with 0·67 mM DPN; combinations of AMP, ATP, and DPN added at 18 min. to final concentrations as indicated.

Table 3. *Cation gradients as affected by swelling and the reversal of swelling*

Washed rat-liver mitochondria were suspended in standard medium including 20 mM succinate and shaken in air at 35°. To vessels initially without adenosine phosphates or DPN, AMP, ATP and DPN were added to final concentrations of mM at 36 min. and the effect on the state of swelling was determined gravimetrically on sediments obtained by high-speed centrifuging at subsequent times. Control values were by (1) centrifuging of mitochondria in the original suspension (0·25 M sucrose) and (2) centrifuging of mitochondria in vessels in which mM AMP was present initially; after determination of wet and dry weights, the sodium and potassium content of the mitochondria was determined by flame photometry (Methods) and compared with those of the corresponding supernatant media.

Time of collection of mitochondria (min.)	Additions at 36 min.	Percentage dry wt.	Potassium content: m-moles/kg. dry wt.	Potassium content: m-moles/l. of mitochondrial water	Potassium content: $\frac{[K^+]\ (M)^*}{[K^+]\ (S)^\dagger}$	Sodium content: m-moles/kg. dry wt.	Sodium content: m-moles/l. of mitochondrial water	Sodium content: $\frac{[Na^+]\ (M)^*}{[Na^+]\ (S)^\dagger}$
0 (sucrose control)	—	29·6	113	47·6	54	24·4	10·3	11·9
36 (AMP initially)	—	25·4	110	37·5	1·71	135	45·7	1·13
55 (AMP initially)	—	25·4	107	36·4	1·66	146	49·8	1·25
0	—	25·5	154	52·6	2·48	130	44·5	1·11
36 } duplicate	—	19·0	100	23·4	1·12	188	44·0	1·10
36 } duplicate	—	19·4	98·3	23·7	0·98	179	43·3	1·08
45	AMP	15·6	116	21·4	1·0	176	32·6	0·83
55	AMP	19·9	94·4	23·4	1·07	168	41·6	1·05
45	ATP	20·7	93·5	24·6	1·14	206	54·3	1·17
55	ATP	21·7	101	27·9	1·29	214	59·0	1·27
45	DPN	16·7	113	22·4	1·03	211	42·1	1·06
55	DPN	16·3	114	22·2	1·04	212	41·1	1·04
45	ATP + DPN	21·5	96·3	26·4	1·2	197	53·8	1·16
55	ATP + DPN	22·1	90·5	25·6	1·18	185	52·5	1·14

* M = mitochondria. † S = supernatant.

Table 4. *Effect of DPN on the course of swelling and cation gradients upon delayed addition of ATP*

Conditions of incubation were those of the standard procedure (Methods): approx. 20 mg. dry wt. of washed mitochondria from the liver of fed rats, in 3 ml. of medium, including 20 mM succinate, were shaken in Dubnoff vessels at 35°; there were three sets of vessels, none of which initially contained ATP. To set *A*, mM ATP was added at indicated time, to set *B*, mM ATP + mM DPN and to set *C* (initially containing mM DPN) mM ATP; percentage dry weight of mitochondrial pellets collected by high-speed centrifuging was determined gravimetrically; cation estimations were performed on identical samples.

Set	Time of collection of mitochondria (min.)	Percentage dry wt.	Cation concentrations in supernatant (mM): Na^+	Cation concentrations in supernatant (mM): K^+	Cation gradients: $\frac{[Na^+]\ in\ MW^*}{[Na^+]\ in\ SS^\dagger}$	Cation gradients: $\frac{[K^+]\ in\ MW^*}{[K^+]\ in\ SS^\dagger}$
A	0	28·8	45	19	1·72	3·78
	10	18·6	45	20	1·27	1·34
Tip ATP →						
	20	19·9	55	20	1·28	1·32
	30	19·7	55	20	1·05	2·37
	40	21·5	55	20	1·11	1·36
	50	21	55	19	1·21	1·28
	60	19·1	55	19	1·33	1·20
	70	17·8	55	19	0·96	1·14
B	0	27·6	45	19	1·82	3·40
	10	19·5	45	21	1·13	1·12
Tip ATP + DPN →						
	20	20·0	55	17·5	1·11	1·35
	30	21·3	55	17·5	1·20	1·46
	40	19·8	55	17·5	1·25	1·44
	50	19·5	55	17·5	1·19	1·46
	60	17	55	17·5	1·24	1·51
	70	16·1	55	17·5	1·13	1·18
C (+DPN)	0	29·3	45	17	1·61	3·80
	10	21·2	45	18·5	1·28	1·43
Tip ATP →						
	20	23·8	57	17·5	1·0	1·66
	30	24·5	57	17·5	1·0	1·59
	40	23·0	57	17·5	1·0	1·47
	50	21·5	57	17·5	1·0	1·47
	60	18·8	57	17·5	1·13	1·47
	70	17	57	19	1·11	1·33

* MW = mitochondrial water. † SS = supernatant solution.

later AMP and ATP were added with and without DPN. Analysis at subsequent intervals showed that the largest decreases in water content were obtained with ATP or AMP plus DPN. The coenzyme alone, either initially or upon delayed addition, however, affected the rate of swelling only slightly (Fig. 7, Tables 3 and 4).

Ion gradients in reversible swelling. In the course of swelling, mitochondria lose in large part their initially high concentration of potassium and somewhat lower concentration of sodium (Fig. 1*c*, *d*). The question arises whether the original cation gradients are restored in the reversal of swelling. With the non-respiring sarcosome system a recovery of the potassium gradient was observed upon the addition of ATP (Fig. 2*c*), but in this case the initial gradient was already very low and the differences involved were small.

Rat-liver mitochondria, when prepared in sucrose, have a high potassium content (Table 3). In the presence of the incubation medium the potassium gradients were initially high and remained high when AMP or ATP was included. During swelling the mitochondria not only lost their potassium gradients but the absolute amount of potassium decreased (Tables 3 and 5). With more extensive swelling the gradients approached unity, but the potassium/kg. dry weight increased, indicating the net entry of external ions in addition to water (Table 5). When swelling was reversed, the potassium gradients usually increased slightly but not nearly to the initial level (Tables 3 and 4). Although the differences were small, it is none the less evident that the combination of ATP and DPN, which caused the greatest reversal of swelling, was not superior (with respect to potassium gradient) to ATP alone (Table 3). Moreover, the peaks of reversal of the two quantities in time did not coincide (Table 4).

The same conclusions may be drawn for sodium, save that the sodium content of the freshly prepared mitochondria was low. The particles took up Na^+ ions from the incubation medium to the extent of a positive gradient not exceeding 2. The gradients were then lost and partially restored in the same manner as potassium. The small increases in cation gradients coincident with the reversal of swelling were equivalent (regardless of the actual mechanism) to an extrusion of water.

EDTA, which was shown above to stabilize the water content of mitochondria, did not maintain high ion gradients to any comparable degree (Table 5). Although all of the gradients in this experiment were unusually low, it is clear that with 3 mM EDTA both the sodium and potassium gradients fell toward the control level and there was no synergistic effect on ATP in the maintenance or recovery of ion gradients.

The interaction between phosphate and magnesium on cation gradients was also investigated. In this particular experiment (Table 6), increasing phosphate led to a decrease rather than the usual increase in the extent of swelling, but had no strong effect on the cation gradients.

The high potassium gradients at the lowest phosphate level are due to the low concentration of potassium in the medium, since the mitochondria themselves retain a large amount of this cation.

A discrepancy was noted at this point between the present findings and those of Macfarlane & Spencer (1953). They observed (their Table 2) that the potassium and sodium contents of mitochondria isolated after 10 min. exposure to the incubation medium containing AMP were low, only to double in the succeeding 20 min. This establishment of moderately high ion gradients with AMP is of greater importance physiologically than the maintenance of pre-existing gradients, but the closest attention to detail in the repetition of these experiments failed to duplicate the results. It was thought in particular that the long centrifuging time (20 min.) employed by Macfarlane & Spencer in their final separation of the particles for analysis might result in a loss of pre-existing gradients, particularly after the early minutes of the incubation when ATP might be limiting.

A comparison of short and long centrifuging with equal acceleration-time integrals (cf. Duve & Berthet, 1954) of samples removed after various times of incubation failed to show any increase of ion gradients, or indeed of any substantial difference between short and long centrifuging. Many experiments were conducted, one of which is reported here (Table 7).

Gradients of phosphate fractions during swelling. Mitochondria, freshly prepared in sucrose, contained appreciable amounts of inorganic phosphate. Upon exposure to the incubation medium phosphate was absorbed and a positive gradient established (Fig. 8). In the presence of AMP, this

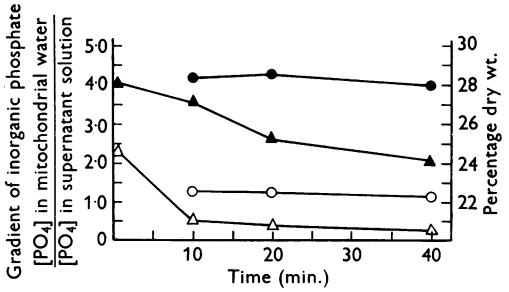


Fig. 8. Effect of AMP on the course of swelling and inorganic phosphate gradients. Standard suspension of mitochondria shaken with 20 mM succinate in air at 35°; ▲ and ●, % dry wt. and phosphate gradient respectively, with 2 mM AMP present; △ and ○, % dry wt. and phosphate gradient respectively, without AMP.

Table 5. *Reversal and prevention of swelling of mitochondria: effects of ATP and EDTA on swelling and cation content*

Suspension of mitochondria in standard medium including 20 mM succinate; shaken in air at 35°; 3 mM or mM EDTA and mM or no ATP was present initially; after 30 min. of incubation combinations of ATP and EDTA were tipped and the mitochondria collected after 10 and 20 min. intervals by high-speed centrifuging.

Initial additions to suspension	Additions at 30 min.	Time of collection of mitochondria (min.)	Percentage dry wt.	Potassium content: m-moles/kg. dry wt.	Potassium content: m-moles/l. of mitochondrial water	Potassium content: [K+] in MW* / [K+] in SS†	Sodium content: m-moles/kg. dry wt.	Sodium content: m-moles/l. of mitochondrial water	Sodium content: [Na+] in MW* / [Na+] in SS†
—	—	0	20·3	147	37·8	1·30	246	63·2	1·45
—	—	30	17·4	110	23·2	0·80	338	71·3	1·64
—	—	40	16·5	120	23·8	0·82	316	62·5	1·44
mM ATP	—	30	21·7	117	32·4	1·13	278	77·0	1·52
mM ATP	—	50	23·1	107	31·8	1·11	269	80·6	1·59
mM EDTA	—	30	18·7	104	24·0	0·81	264	65·5	1·43
mM EDTA	—	40	17·6	106	22·8	0·77	291	62·4	1·36
3 mM EDTA	—	30	20·2	119	30·4	1·04	280	71·5	1·56
3 mM EDTA	—	50	19·8	104	25·6	0·88	286	70·6	1·55
mM ATP + 3 mM EDTA	—	30	20·7	120	31·6	1·11	272	71·8	1·35
mM ATP + 3 mM EDTA	—	50	20·4	—	—	—	—	—	—
—	mM ATP	40	18·2	110	24·4	0·85	318	70·4	1·39
—	mM ATP	50	19·6	100	24·2	0·85	269	80·4	1·58
mM EDTA	mM ATP + 2 mM EDTA‡	40	19·4	103	24·8	0·87	293	70·8	1·34
mM EDTA	mM ATP + 2 mM EDTA‡	50	19·8	100	24·6	0·86	302	81·4	1·40
—	mM ATP + 3 mM EDTA	40	17·3	109	22·8	0·80	363	75·7	1·43
—	mM ATP + 3 mM EDTA	50	17·5	99	20·9	0·73	358	75·5	1·42

* MW = mitochondrial water. † SS = supernatant solution. ‡ To final concn. of EDTA = 3 mM.

Table 6. *Effects of phosphate and magnesium levels on swelling and cation gradients*

Conditions of incubation similar to those of Macfarlane & Spencer (1953), but liver mitochondria from well-fed rats were used and no cytochrome *c* was added; AMP = mM, where present; incubation period, 30 min.; percentage dry wt. was determined gravimetrically and cation analyses were performed on identical samples.

Concn. of phosphate buffer (mM)		Percentage dry wt.	Concentration of Na^+ (mM): Mitochondrial water	Supernatant soln.	Excess	Na^+ gradient	Concentration of K^+ (mM): Mitochondrial water	Supernatant soln.	Excess	K^+ gradient
20 mM Mg^{2+}	Low* +AMP	20	32	22	10	1·5	18·5	2·2	16·3	8·4
	1 −AMP	18	28	22	6	1·3	5·0	2·8	2·2	1·8
	Normal +AMP	21·3	34	22	12	1·5	30·5	19	11·5	1·6
	10 −AMP	18	30	21	9	1·4	20	19	1	1·0
	High +AMP	25	47	24	23	2·0	106	65	41	1·6
	30 −AMP	21·6	37	21	16	1·8	75	56	19	1·3
2 mM Mg^{2+}	Low* +AMP	20	33	22	11	1·5	17·5	2·9	14·6	6·0
	1 −AMP	13·2	26	21	5	1·2	4·5	3·3	1·2	1·4
	Normal +AMP	21·6	41	22	19	1·9	41	20	21	2
	10 −AMP	12·8	26	21	5	1·2	20·5	20	0·5	1
	High +AMP	24·2	46	22	24	2·1	80	59	21	1·4
	30 −AMP	17·5	30	21	9	1·4	53	54	−1	1

* pH = 6·6–6·8 at end of experiment.

Table 7. *Comparison of short and long centrifuging on the swelling of washed rat-liver mitochondria*

Conditions were chosen to duplicate closely the procedure of Macfarlane & Spencer (1953): 2 mM-$MgSO_4$, 10 mM potassium phosphate buffer, pH 7·4, mM AMP, 20 mM sodium glutamate, etc.; long centrifuging: 6000 *g* for 20 min.; short centrifuging: 25000 *g* for 4 min.; the two force–time integrals were adjusted to be equal.

		Time of incubation (min.)													
		0				10				30				30+15*	
Centrifuging interval ...		Short		Long		Short		Long		Short		Long		Short	
mM AMP		+	−	+	−	+	−	+	−	+	−	+	−	+	−
Percentage dry wt.		20·6	19·3	20·8	19·7	23·2	16·4	24·3	16·4	26·5	13·3	20	12·8	20·5	13·2
		20·7	20·1	21	20·1	20·9	16·6	—	—	—	—	—	—	—	—
Na^+ (mM)	/kg. of mitochondrial water	31	26	28	26	32	25	33	27	32	23	30	21	27	24
		44	30	40	30	30	24	—	—	—	—	—	—	—	—
	/l. of supernatant soln.	22	20	22	20	21	19	23	22	22	19	22	20	22	19
		21	18	22	20	21	19	—	—	—	—	—	—	—	—
	excess in mitochondrial water	9	6	6	6	11	6	10	5	10	4	8	1	5	5
		23	12	18	10	9	5	—	—	—	—	—	—	—	—
	Na^+ gradient	1·4	1·3	1·3	1·3	1·5	1·3	1·4	1·2	1·5	1·2	1·4	1	1·2	1·2
		2·1	1·7	1·8	1·5	1·4	1·3	—	—	—	—	—	—	—	—
K^+ (mM)	/kg. of mitochondrial water	38	37	38	24	37	26	44	27	38	22	35	23	38	23
		44	43	43	38	39	30	—	—	—	—	—	—	—	—
	/l. of supernatant soln.	19	19	19	19	19	19	21	21	20	20	21	19	18	19
		19	18	19	19	18	19	—	—	—	—	—	—	—	—
	excess in mitochondrial water	19	18	19	15	18	7	23	6	18	2	14	4	20	4
		25	25	24	20	21	11	—	—	—	—	—	—	—	—
	K^+ gradient	2·0	1·9	2·0	1·8	1·9	1·4	2·1	1·3	1·9	1·1	1·7	1·2	2·1	1·2
		2·3	2·4	2·3	2·1	2·2	1·6	—	—	—	—	—	—	—	—

* 30 min. shaking followed by 15 min. without shaking to simulate conditions of aeration during the centrifuging.

Table 8. *Effect of AMP on the content of phosphate fractions during incubation*

Suspension of washed mitochondria, prepared by the standard procedure, was divided into two sets; one was analysed for phosphate (see Methods) and the other for water content, gravimetrically; standard conditions of incubation were used except that AMP = 2 mM, where added.

			Time of incubation (min.)			
			0·5	10	20	40
Dry wt. (%)		+AMP	—	26·6	25·2	24·1
		−AMP	24·6	21·0	20·8	20·5
			28·1*	—	—	—
P in adenosine phosphates	m-moles of P/kg. dry wt. of mitochondria	+AMP	—	26·8	25·5	22·6
		−AMP	24	13·8	15·2	12·3
			23·5*	—	—	—
	m-moles of P/l. of mitochondrial water	+AMP	—	9·70	8·59	7·15
		−AMP	7·81	3·66	3·98	3·17
			9·23*	—	—	—
	m-moles of P/l. of supernatant soln.	+AMP	—	3·77	3·89	4·24
		−AMP	0·135	0	0	0
Inorganic P	m-moles of P/kg. dry wt. of mitochondria	+AMP	—	61·2	68·5	68·1
		−AMP	70·6	37·4	41·1	37·2
			19·9*	—	—	—
	m-moles of P/l. of mitochondrial water	+AMP	—	22·2	23·1	21·6
		−AMP	23·0	9·94	10·8	9·6
			7·8*	—	—	—
	m-moles of P/l. of supernatant soln.	+AMP	—	5·32	5·43	5·42
		−AMP	7·83	7·96	8·41	8·65

* Centrifuged in 0·25 M sucrose without incubation.

gradient was maintained at a level of about 4; in the absence of AMP, it fell within 10 min. to about 1.

The adenosine phosphates were similarly present at concentrations in excess of 2 mM-P (Table 8). In the absence of added AMP they fell rapidly to a level one-third of that initially. Separation by paper chromatography showed that most of the adenosine phosphate was then AMP. After the first 30 sec. no trace of adenosine phosphate could be detected in the extra-mitochondrial fluid.

In the presence of 2 mM AMP the initial adenosine phosphate was augmented and maintained at a high level. These compounds also appeared in the supernatant fluid at a total concentration about one-half of that in the mitochondrial water.

Swelling and oxidative phosphorylation. The requirement for AMP in the prevention of swelling led Macfarlane & Spencer (1953) to expect a relation between swelling and oxidative phosphorylation. Also, Chappell & Perry (1954) showed that the reversal of swelling of non-respiring sarcosomes (cf. Fig. 2) could be brought about by the addition of ATP to the swollen particles. With the respiring liver mitochondria, however, the former authors found no appreciable effect of 10^{-5} M DNP on the water content. The optical method showed that the particles in the present experiments failed to respond to 3×10^{-5} M DNP in the presence of mM AMP, but with 10^{-4} M DNP + mM AMP swelling was accelerated beyond the rate of the control (Fig. 9). Oxidative phosphorylation is normally arrested at lower concentrations and it was observed (but not shown on Fig. 9) that 3×10^{-5} M DNP prevented the reversal of swelling otherwise obtained in the presence of mM ATP.

If the reversal of swelling is dependent upon oxidative phosphorylation, one would expect the synthesis of ATP to coincide with the increase in percentage dry weight, so experiments were designed to test this prediction.

Samples (3 ml.) of a suspension of mitochondria were shaken in Dubnoff vessels. At zero time radioactive inorganic phosphate was added to one set of vessels containing also DPN and ATP. Samples

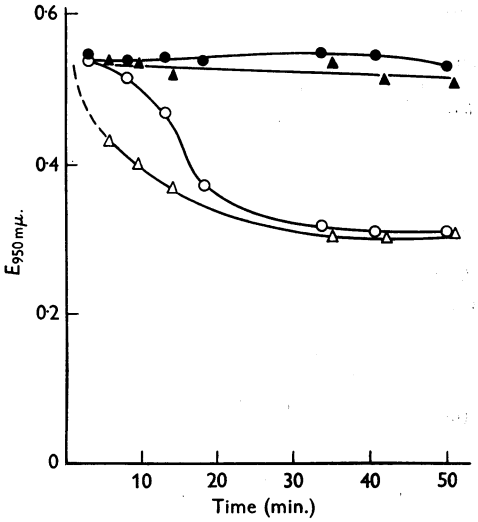


Fig. 9. Effect of DNP on swelling as estimated by the optical method. All samples contained 20 mM succinate in standard stock solution and were shaken in air at 35°; ○, no addition; ●, mM AMP; ▲, mM AMP + 3×10^{-5} M DNP; △, mM AMP + 10^{-4} M DNP.

were taken at various intervals for phosphate analysis and others centrifuged for determination of water content. Whilst swelling was progressing, as indicated by the optical method, radioactive inorganic phosphate and DPN + AMP or ATP were added to another set of the vessels and samples taken for phosphate- and water-content determination as indicated (Fig. 10). With AMP, extrusion of water began after a 10 min. delay, and proceeded vigorously (Fig. 10*a*). The onset of extrusion was correlated with the build-up of ATP to a level equal to that of the samples fortified with ATP from the beginning (Fig. 10*b*). With ATP, reversal of swelling was immediate, but fell off rapidly after 20 min.; this, too, was correlated closely with the concentration of ATP in the suspension (Fig. 10*b*).

The phosphate fractions separated chromatographically were also examined for radioactivity (Fig. 10*c*, *d*). Whether ATP was present initially or

Fig. 10. Oxidative phosphorylation coincident with the reversal of swelling. Standard suspensions of mitochondria in standard stock solution shaken with 20 mM succinate in air at 35°; separate but identical samples were taken for estimations of phosphate content, water content, and oxygen uptake estimations. Phosphate compounds were separated chromatographically by the method of Krebs & Hems (1953). *a*, Percentage dry weight determined gravimetrically; two sets of vessels, with (●) and without (○) mM DPN and 2 mM ATP; to certain of those initially without, mM DPN and 2 mM ATP (▲) or AMP (■) were added at 36 min. *b*, ATP content in μmoles/vessel (3·15 ml.); ●, vessels with 2 mM ATP initially; ▲, ATP added at 36 min.; ■, AMP added at 36 min.; all vessels include mM DPN. *c*, Incorporation of $^{32}PO_4$ into ATP occurring initially and after delayed addition of ATP; ● and ▲, total radioactivity in ATP/vessel (counts/min. in ATP/vessel $\times 10^{-4}$); ◑ and △, specific activity in ATP (counts/min./μmole of P in ATP $\times 10^{-4}$). *d*, Incorporation of $^{32}PO_4$ into ADP and ATP upon delayed addition of AMP. ○ and ●, total radioactivity/vessel in ATP and ADP respectively (counts/min./vessel $\times 10^{-5}$); △ and ▲, specific activity in ATP and ADP respectively (counts/min./μmole of P $\times 10^{-4}$). *e*, Oxygen uptake; ○, no additions; ●, mM DPN and 2 mM ATP initially; ■, tip 2 mM ATP and mM DPN; ▲, tip 2 mM AMP and mM DPN at 26 min.

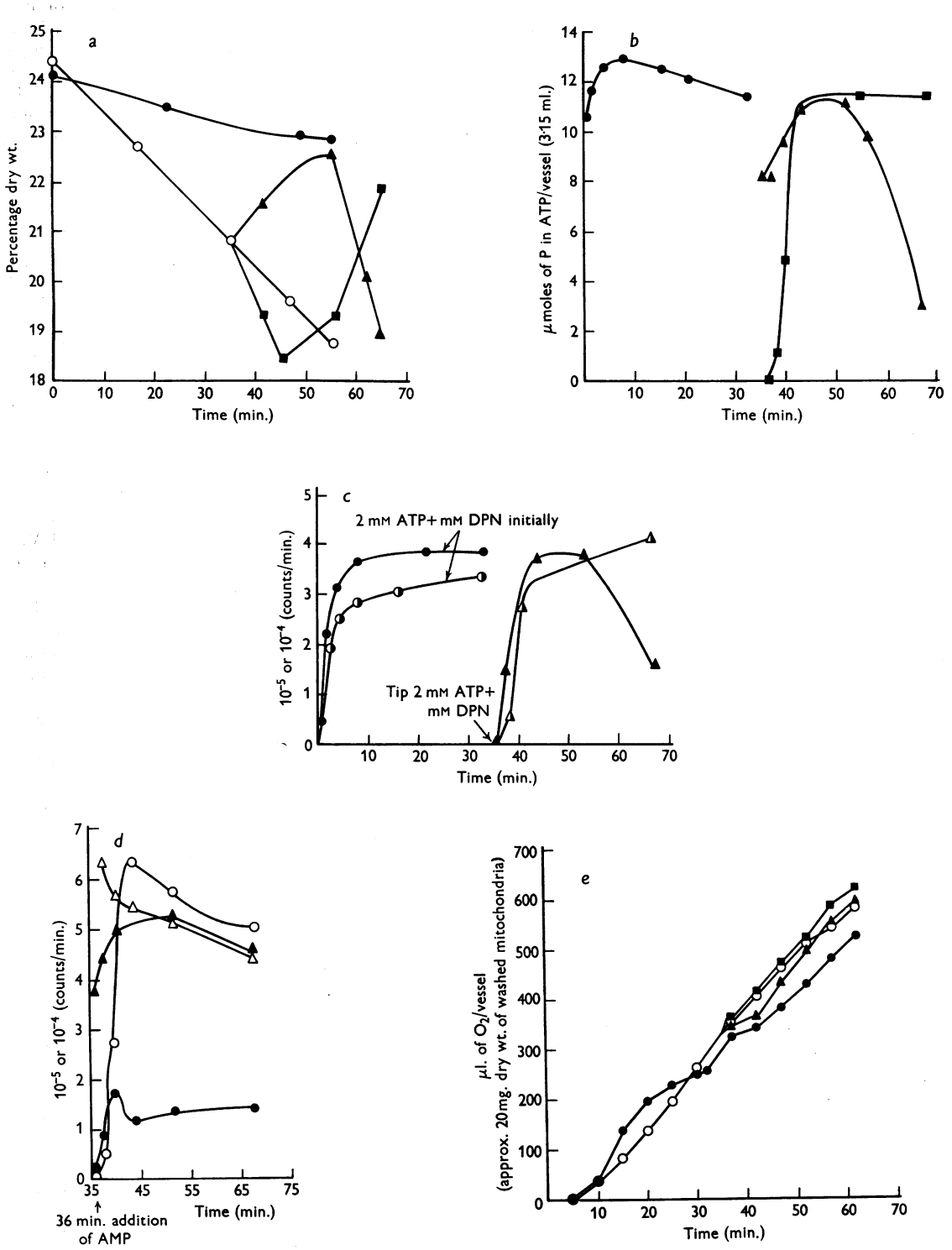


Fig. 10 *a–e*. For legend see p. 764.

added after swelling had proceeded, the initial rate of phosphorylation was essentially the same. The final specific radioactivity of the ATP was greater with delayed addition owing to decreased dilution from endogenous ATP. With the delayed addition of AMP (Fig. 10*d*), the total activity of ADP was first increased and was subsequently outstripped by ATP. The initially higher specific radioactivity of ATP shows, however, that the primary phosphorylation occurs in the synthesis of ATP. The oxygen uptakes of identical samples were followed in Warburg vessels, shaking at the same rate as the Dubnoff flasks (Fig. 10*e*). They show that the swelling and reversals of swelling were not mirrored in the rates of respiration. The positive correlations appeared rather with the levels of ATP.

DISCUSSION

Causes of swelling. Before the studies that indicated an 'active' component in the swelling of mitochondria (Bartley & Davies, 1952, 1954; Raaflaub, 1953*a*, *b*; Macfarlane & Spencer, 1953) it was generally assumed that 'mitochondria behave as fairly typical osmotic systems' (Duve & Berthet, 1954). The present work shows that typical osmotic behaviour (i.e. hypotonic swelling) of respiring liver mitochondria is observed only under detrimental conditions, e.g. deficiency of magnesium or phosphate acceptors, presence of inorganic phosphate, and sarcosomes swell under relatively simple conditions in the absence of substrate or phosphate and without aeration (Cleland, 1952; Chappell & Perry, 1954; Fig. 2 above). Fresh liver mitochondria remain stable to hypotonic media under these conditions (Raaflaub, 1953*a*; Fonnesu & Davies, 1956). With both particle systems adenosine phosphates have important effects. Cleland (1952) observed the greatly decreased swelling of sarcosomes treated with ATP, but interpreted the phenomenon in terms of permeability. Raaflaub (1953*a*) realized that the action was more complex and wrote: '...wir (sind) geneigt, die Ursache der nicht-osmotischen Schwellung im Vorhandensein kontraktiler und dilatationsfähiger Strukturelemente zu suchen. Ein Vergleich mit dem trotz enormen Arbeitsaufwandes immer noch ungelösten Problem der Muskelkontraktion drängt sich auf, indem bei beiden Vorgängen ATP der im Zentrum des Interesses stehende Stoff ist.'

Effects on the ATP level and swelling are difficult to separate. Whether Mg^{2+} ions affect the contractile elements directly or the supply of ATP is not known, but they are known to be essential for oxidative phosphorylation. The effect of oxygen may be related to an increase in adenosine triphosphatase; Pressman (personal communication) finds that the adenosine triphosphatase activity of respiring mitochondria is about twice as great as that of non-respiring preparations. It appears possible, however, that not simply the concentration of oxygen but the rate of consumption might be the significant factor in swelling. Raaflaub (1953*a*), for example, found that in the absence of added adenosine phosphates, succinate promoted swelling and malonate prevented this promotion.

With several substrates, but not succinate, the rate of oxygen uptake and swelling are directly proportional (Harman & Feigelson, 1952*a*; Raaflaub, 1953*b*; Fig. 5 above). The relation for substrates other than succinate is explained by the diffusion of coenzymes into the medium during swelling (Brenner-Holzach & Raaflaub, 1954; Hunter & Ford, 1955). Where oxidative phosphorylation is necessary to maintain respiring mitochondria in a contracted state, an interdependency exists: ATP maintains the contracted state and this state is essential for the preservation of coenzymes necessary for the generation of ATP.

That the concentration of ATP controls the onset of swelling was determined directly by Brenner-Holzach & Raaflaub (1954); they found that liver mitochondria began to swell when the internal ATP fell from a normal value of about 0·05 μmole/mg. of N to less than 0·01 μmole/mg. of N.

The effect of inorganic phosphate in promoting swelling is clearly related to the finding of Hunter & Ford (1955) that phosphate reversibly inactivates oxidative phosphorylation; but the mechanism remains obscure. In their system magnesium, oxygen, and inorganic phosphate have effects on oxidative phosphorylation exactly parallel to those in the present study. In fact, their discovery that DPN uniquely reverses inactivation by phosphate led to the successful use of this substance in the reversal of swelling (Fig. 7 above).

Swelling, contraction and ion gradients. The non-osmotic contraction or reversal of swelling of mitochondria is brought about by a supply of ATP and is greatly promoted by DPN. Fig. 10 shows that oxidative phosphorylation serves only to restore ATP to a high level; for while the rate of incorporation of $^{32}PO_4$ into ATP starts at a high level immediately upon the addition of AMP or ATP, contraction does not begin until the ATP has accumulated to a level equivalent to that of fresh mitochondria. Indeed, there is an initial acceleration of swelling upon the addition of AMP which suggests that endogenous ATP is consumed in a myokinase reaction.

From the data of Hunter & Ford (1955) it is clear that DPN is important in oxidative phosphorylation and may be identical with the factor of Ernster, Löw, Nordenbrand & Ernster (1955). An effect of DPN on succinate oxidation, however, is unexpected; for at the time in question only about half

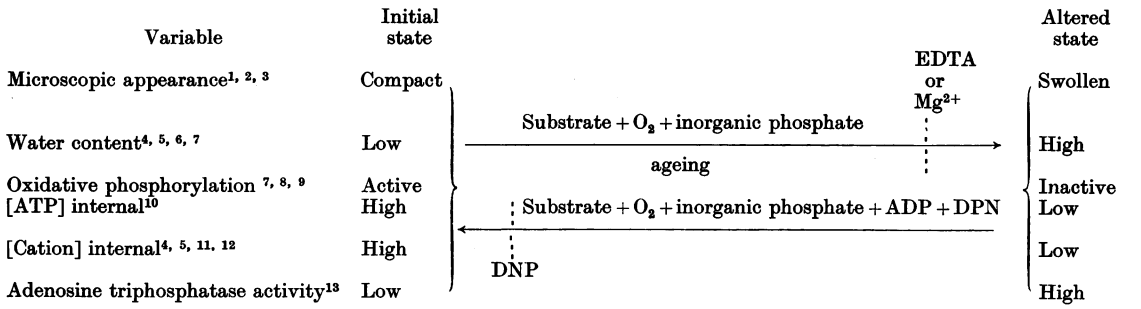
of the succinate has been consumed in a single-step reaction to malate (Krebs, Ruffo, Johnson, Eggleston & Hems, 1953; Price & Thimann, 1954; Whittam, Bartley & Weber, 1955). It is therefore possible, but unlikely, that DPN is promoting phosphorylation by accelerating malic dehydrogenase or other oxidations at the substrate level.

The apparent necessity for DPN in oxidative phosphorylation suggests an explanation of the role of oxygen in swelling, namely, the enhanced lability of the oxidized relative to the reduced nucleotide.

DPN has also been cited as reversing the impaired oxidation of mitochondria from liver treated with carbon tetrachloride (Christie & Judah, 1954; Dianzani, 1955). These and other observations suggest that 'cloudy swelling' of the liver is directly related to mitochondrial swelling (Fonnesu, 1952; Robinson, 1953; Fonnesu & Severi, 1954, 1956).

in a manner analogous to swelling (Spector, 1953; Stanbury & Mudge, 1953).

Among swelling, ion gradients, oxidative phosphorylation and adenosine triphosphatase activity a number of correlations are to be found. As may be inferred from many studies (e.g. Harman & Feigelson, 1952*b*; Macfarlane & Spencer, 1953; Slater & Cleland, 1953; Hunter & Ford, 1955; Beyer, Ernster, Löw & Beyer, 1955) oxidative phosphorylation and the state of swelling are reversibly interdependent; likewise swelling and adenosine triphosphatase (Potter & Recknagel, 1951; Slater & Cleland, 1953; Chappell & Perry, 1954; Kaltenbach & Harman, 1955). The interlinking of these phenomena is illustrated in the scheme shown below, and it may be that this has physical reality in that the enzymes of oxidative phosphorylation and adenosine triphosphatase may be identical with the contractile elements of the mitochondrial structure.



([1] Harman & Feigelson, 1952*a*; [2] Watanabe & Williams, 1953; [3] Cleland & Slater, 1953; [4] Bartley & Davies, 1952, 1954; [5] Macfarlane & Spencer, 1953; [6] Raaflaub, 1953*a*, *b*; [7] Ernster *et al.* 1955; [8] Harman & Feigelson, 1952*b*; [9] Hunter & Ford, 1955; [10] Brenner-Holzach & Raaflaub, 1954; [11] Spector, 1953; [12] Stanbury & Mudge, 1953; [13] Kaltenbach & Harman, 1955.)

Swelling and contraction are clearly more than the movement of mitochondrial protein under the influence of ATP. The particles contain K^+ and Na^+ ions in excess of those in the ambient fluid and their concentrations are affected by swelling and contraction. These ions, moreover, are for the most part rapidly exchangeable (Bartley & Davies, 1954), although a small residue exchanges slowly with ions of the medium (Spector, 1953; Stanbury & Mudge, 1953). During swelling these gradients of cations and also of inorganic phosphate are nearly lost. Upon contraction the gradients are recovered but not to the same degree as the decrease in water content. It is significant that even when adenosine phosphates are present initially, the high potassium gradient is not maintained for as long as the low water content.

The means by which ions are held at relatively high internal concentrations are not known, but whatever the mechanism and sites of accumulation, the ion gradients and even the slowly exchangeable potassium are affected by phosphate and respiration

Possible physiological significance. Even though it cannot be assumed that present techniques with mitochondrial preparations allow even a near approach *in vitro* to true physiological conditions (Slater & Cleland, 1953; Cleland & Slater, 1953) reversible swelling of mitochondria has been observed under conditions simulating cellular respiration. Transport of water and ions is a general physiological process, and the results of Tables 3–5 suggest that the mitochondria may perform such a function (see also Bartley & Davies, 1952, 1954; Macfarlane & Spencer, 1953). In terms of the measured water contents the range of reversible swelling (about 18–23 % dry weight) is quite the same as that found with intact kidney tissue (Whittam & Davies, 1953). The *in vivo* ion contents of mitochondria and the cytoplasms are unknown, but the ion gradients developed by these isolated metabolizing mitochondria are much less than those maintained by metabolizing cells (cf. Whittam & Davies, 1953). Since the turnover of ions in mitochondria is high (Bartley & Davies, 1954) it is

possible that these particles are the pumps which enable the cells to do osmotic work (cf. Robertson, Wilkins, Hope & Nesztel, 1955).

SUMMARY

1. Investigations have been made of the conditions in which respiring rat-liver mitochondria can swell and contract in solutions of constant osmotic pressure.

2. An essentially continuous estimation of mitochondrial water was obtained from measurements of the optical density of suspensions at 950 mμ. with a 1 mm. light path.

3. When suspensions of rat-liver mitochondria are shaken in air at 35° with 20 mM sodium succinate, 10 mM potassium phosphate buffer, pH 7·4, 5 mM magnesium sulphate, 30 mM tris buffer, 83 mM sucrose and mM ATP or ADP, the rate of oxygen uptake is high (Q_{O_2} approx. -40 μl./mg. dry wt./hr.) and the particles retain their initially low water content (20–25 % dry wt.).

4. In the absence of adenosine phosphates the oxygen uptake is essentially unchanged in the first 30 min., but the mitochondria take up water (15 % dry wt.) and the sodium and potassium concentrations decrease. If swelling has not proceeded too far, the subsequent addition of mM AMP or ATP alone, or, much better, together with mM DPN, will cause the extrusion of water (17–21 % dry wt.) and partial recovery of the sodium and potassium gradients. The time courses of these processes are not identical.

5. This recovery is coincident with the resumption of oxidative phosphorylation and is abolished by 10^{-4} M DNP.

6. These results indicate that 'high-energy' phosphate esters can be used by mitochondria to move water and ions.

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