The Swelling and Contraction of Isolated Rat-Liver Mitochondria

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In some pathological conditions there is an increase in the volume of the liver mitochondria. This condition, known as 'cloudy swelling', occurs in carbon tetrachloride poisoning and thyrotoxicosis (Bassi, 1960; Schulz, Löw, Ernster & Sjostrand, 1956). Similar changes can be brought about *in vitro* by physiological concentrations of thyroxine (Tapley, Cooper & Lehninger, 1955; Tapley, 1956) and also by glutathione and cysteine (Lehninger & Schneider, 1959), oxytocin and vasopressin (Lehninger & Neubert, 1961), phosphate (Raaflaub, 1953*a, b*), arsenate (Hunter & Ford, 1955) and Ca^{2+} ions (Slater, 1957). Many other substances have also been shown to cause volume changes in isolated liver mitochondria (for details see review by Lehninger, 1962). In most of the studies the change in the extinction of the mitochondria at 520 $\text{m}\mu$ (Cleland, 1952) has been used as a measure of the volume changes, and in a few cases a direct gravimetric method (Macfarlane & Spencer, 1953). The studies of Price, Fonnesu & Davies (1956) and Lehninger (1959), in which both optical and gravimetric methods were used, show that any correlation that may exist between the results obtained by the two methods is non-linear. If the results are plotted as the water content/unit dry wt. of mitochondria against the extinction a series of curves is obtained and not the expected straight line. Part of the present paper is concerned with the divergent results obtained with the two methods of measurement of mitochondrial swelling.

In most of the studies of mitochondrial swelling attention has been directed to water movements only (but see Amoores & Bartley, 1958; Bartley & Amoores, 1958; Bartley, 1961), and the accompanying movement of solutes has not been measured. The magnitude of the changes in both water and solute contents of mitochondria when they shrink or swell rules out any simple osmotic explanation, since these changes occur in response to very small alterations in the osmolarity of the extramitochondrial medium. Lehninger (1962) has suggested a coupling between energy production and utilization and swelling in mitochondria with an active process located in the mitochondrial membrane responsible for water movements. The results given below fully confirm Lehninger's (1962) experi-

mental observations about the effect of phosphate, ATP and Mg^{2+} ions on mitochondrial water content, but the solute movements and the structural changes seen under the electron microscope make it unlikely that the observations can be explained on the basis of any mechanism situated in or dependent on an intact mitochondrial membrane.

MATERIALS AND METHODS

Animals. Wistar strain rats (300–450 g.) were used.

Preparation of mitochondria. Rat-liver mitochondria were isolated from an homogenate prepared in 0.25 M-sucrose by differential centrifugation according to the method of Werkheiser & Bartley (1957). The mitochondria were finally suspended in 0.25 M-sucrose or 0.125 M-potassium chloride in 20 mM-tris hydrochloride buffer, pH 7.4.

Incubations. The mitochondria were incubated at room temperature (18–25°) in bulk in conical flasks. The concentration was usually 4 mg. wet wt. of mitochondria/ml. Samples were removed at intervals for measurement of extinction and gravimetric determination of the water content.

Isolation of the mitochondria after incubation. A concentration of about 4 mg. wet wt. of mitochondria/ml. is about the maximum that will allow transmission of sufficient light through a 2 mm. light-path. To obtain sufficient weight of mitochondria (100 mg. wet wt.) for the gravimetric determination of water content requires 30–40 ml. of suspension. Sedimentation of the mitochondria from such a suspension in the normal centrifuge tube designed to take the necessary volume of fluid results in a thin layer of sediment spread over a comparatively large area. Such pellets are difficult to free from adherent fluid and the increment in weight due to the pellet is small compared with the weight of the centrifuge tube. To overcome this problem the device shown in Fig. 1 was developed. A polypropylene centrifuge tube of 50 ml. capacity was fitted with a two-part lining. The bottom part consisted of a block of polythene into an asymmetric recess of which a small glass centrifuge tube was placed. The top part of the lining consisted of a Perspex tube with a base that sloped towards an asymmetrically placed hole. The lining was fitted so that this hole was over the top of the glass centrifuge tube. Normally 35 ml. samples of the mitochondrial suspension were put in the tube for centrifuging. The device was centrifuged in the angle head of the centrifuge with the glass tube directed towards the periphery of the head. This caused the mitochondria to collect and pack into the small glass tube, which could then be removed for weighing. A field force of 30000*g* for 5 min. at 4° was used, and this resulted in a pellet of mitochondria whose water content did not change on further centrifuging.

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Measurement of the extinction of the mitochondrial suspension. Cells of 2 mm. light-path were used and the extinctions were determined at $520\text{ m}\mu$ in a Unicam SP. 600 spectrophotometer. The extinction of freshly prepared suspensions of mitochondria containing about 100 mg. wet wt. in a final volume of 35 ml. was about 0.7 when measured with a 2 mm. light-path. From the measured amount of dry material sedimented from the 35 ml. of suspension, the value of E expected if the 35 ml. of suspension contained 100 mg. dry wt. was calculated. Over the range where it could be measured, linearity was shown between the dry weight of the suspension and the extinction for a mitochondrial suspension under any defined set of conditions. Thus the values presented in Fig. 2 are for a 2 mm. light-path, assuming that the 35 ml. of suspension contained 100 mg. dry wt.

Analysis of the mitochondrial pellets and the incubation medium. When solutes were to be determined, duplicate samples of the mitochondrial suspension were centrifuged. The supernatants were decanted and to one was added 4 ml. of 50% (w/v) trichloroacetic acid and to the other 2 ml. of 72% (w/w) perchloric acid. To determine the water content of the mitochondrial pellet the glass tube was inverted and drained and the glass surfaces were dried on filter paper. After weighing, the tube and contents were

dried at 105° for 12 hr. and then reweighed. The loss in weight is assumed to be due to evaporated water. The dried pellet was transferred to a larger tube and extracted with 4 ml. of 2N-perchloric acid at 60° for 2 hr. After centrifuging, the supernatant fluid was used for determination of the chloride content of the pellet. The duplicate mitochondrial pellet immediately after draining and wiping the tube was treated with 1.0 ml. of 10% (w/v) trichloroacetic acid, and the tube and contents were weighed. After stirring the contents the tube was allowed to stand for 3 hr. and then centrifuged. The supernatant was removed for analysis of the solutes. The pellet was washed with water and then dissolved in 4.0 ml. of 2N-sodium hydroxide for protein determination.

Determination of protein. This was the modification of the biuret method made by Professor Utter and described by Tustanoff & Bartley (1964).

Determination of sodium and potassium. A lithium-internal-standard flame photometer was used (Amoore, Parsons & Werkheiser, 1958).

Determination of acid-soluble phosphates. The determinations were made on the trichloroacetic acid extracts. Inorganic phosphate was determined by the method of Berenblum & Chain (1938) as modified by Bartley (1953). Total phosphate was determined after digestion with a sulphuric acid-perchloric acid mixture (Hanes & Isherwood, 1949). The difference in the quantity of total phosphate and the inorganic phosphate is called organic phosphate.

Determination of chloride. The method of West & Coll (1957) was used. This depends on determining the extinction at $353\text{ m}\mu$ in the presence of perchloric acid and ferric perchlorate.

RESULTS

Investigation of the optical method of measuring water content

Relationship between water content of mitochondria and extinction of suspension. The optical method depends partly on the difference in refractive index between the mitochondria and the medium. Any change in the refractive index of the mitochondria caused by a loss of soluble protein or other solutes or by penetration of the medium would give results of doubtful validity. Resuspension of mitochondria prepared in 0.25M-sucrose solution in the potassium chloride-tris hydrochloride medium caused a loss to the medium of about 10% of the mitochondrial protein. Subsequent incubation caused a variable loss of protein, depending on the incubation conditions, but amounts of protein up to 20% of the original mitochondrial content could be lost, and when cysteine was used as the swelling agent more than 70% of the dry weight of the mitochondria became solubilized in about 30 min. at room temperature. Substances absorbing at $260\text{ m}\mu$ were also lost to the medium on incubation of the mitochondria. Similar observations were also made by Bartley & Amoore (1958), and Amoore & Bartley (1958) showed that the sucrose of the

Fig. 1. Modified centrifuge tube for collecting mitochondria from a dilute suspension. A, Polypropylene centrifuge tube; B, recessed polyethylene block; C, collecting tube; D, Perspex liner.

medium passed into the interior of the mitochondria even on incubation at 0°. Consequently an investigation was made of the nature of the correlation between extinction changes and variation in the water content of mitochondrial suspensions. The results of a large number of experiments are shown in Fig. 2, in which the extinction/unit dry wt. of mitochondria is plotted against the water content/unit dry wt. Mitochondria suspended either in 0.25M-sucrose solution or in 0.125M-potassium chloride in 20 mM-tris hydrochloride buffer, pH 7.4, were caused to swell by the addition of either phosphate or cysteine. Samples were taken simultaneously from the bulk suspension of mitochondria for determination of extinction and water content. The results show a considerable scatter. This is not solely due to variations between different experiments but is also due to variations within single experiments. The variation was greatest in mitochondria with a low water content. For example, for two samples of mitochondria suspended in sucrose solution with water contents of 2.16 and 2.18/unit dry wt. the extinction/100 mg. dry wt. varied from 1.12 to 2.78. In general, at a given water content, the extinction was higher for mitochondria suspended in potassium chloride solution than in sucrose solution. There is thus no clear relationship between the changes in extinction of a mitochondrial suspension and the changes in the water content of the mitochondria.

Artifacts of the measurement of changes in extinction in mitochondria. Mitochondrial suspensions

Fig. 2. Relationship between the extinction of a mitochondrial suspension and the water content of the mitochondria. ○, Mitochondria suspended in 0.125M-potassium chloride in 20 mM-tris hydrochloride buffer, pH 7.4; ●, mitochondria suspended in 0.25M-sucrose. The quantities of mitochondria ranged from about 50 to 150 mg. wet wt. of mitochondria/35 ml. of suspension. The values of E presented are calculated for a nominal suspension containing 100 mg. dry wt./35 ml. The water content is given as the weight of water/unit dry wt. of mitochondria (I/M).

often showed rapid diminutions in extinction that began a few minutes after the mitochondria had been placed in the cuvette and reached a maximum after about 15–20 min. The change in extinction could be as much as 0.7 and was usually above 0.3. Such changes could be taken as the optical indication of mitochondrial swelling. However, when fresh samples were removed from the bulk suspension and the extinction again measured it was found that little or no change had occurred. Gravimetric determination of the water content of the mitochondria in the bulk sample confirmed that no change had occurred. Fig. 3 illustrates the changes that occurred with mitochondria suspended in 0.25M-sucrose solution. Fresh samples of mitochondria were put in the cuvette at times 0, 18, 39 and 72 min. The extinction of the first sample fell rapidly after 5 min., but no change occurred in the bulk suspension as shown by the extinction of the fresh sample at 18 min. However, the rapid change in extinction was repeated by this sample. At 39 min. the value shows that the bulk suspension had begun to show changes in extinction. Fig. 4 illustrates the same phenomenon with the mitochondria suspended in 0.125M-potassium chloride in 20 mM-tris hydrochloride buffer, pH 7.4, and treated with orthophosphate (1 mM). Fresh samples

Fig. 3. Optical changes of a mitochondrial suspension induced by transfer to the cuvette. The mitochondria were suspended in 0.25M-sucrose solution. The ΔE was determined at 520 m μ and fresh samples were taken from the bulk suspension at 18, 39 and 72 min. Initially the mitochondrial suspension contained about 110 mg. wet wt./35 ml. of suspension, with a dry wt. of about 40 mg.

were placed in the cuvette at 2, 35, 40, 45, 60 and 90 min. The anomalous changes in the extinction of the suspension in the cuvette occurred only once. Changes in extinction brought about by transfer to the cuvette occurred more readily in the presence of phosphate and could be partially prevented by rinsing the cuvette thoroughly with the mitochondrial suspension. None of the conventional procedures for cleaning the cuvettes abolished the effect. This effect must therefore be kept in mind when interpreting optical measured changes in mitochondrial suspension.

Contraction of phosphate-swollen mitochondria

Because of the lack of precision of the optical method for measuring mitochondrial volume changes some re-examination of mitochondrial swelling and shrinking by making use of non-optical methods appeared justified. The simplest system, already studied by Lehninger and others (see review by Lehninger, 1962), was the swelling of mitochondria brought about by the addition of orthophosphate and a subsequent contraction induced by the addition of ATP and Mg^{2+} ions. Both

the swelling and the shrinking agents are involved in oxidative phosphorylation, and Lehninger (1962) has suggested that swelling and shrinking is a function of this involvement. A typical experiment is as follows. Mitochondria were suspended in 0.125M-potassium chloride in 20 mM-tris hydrochloride buffer, pH 7.4, at 100 mg. wet wt./35 ml. and the suspension was divided into two batches. To one batch was added potassium phosphate buffer, pH 7.4, so that the final concentration of phosphate was 1.0 mM. Samples were taken immediately after mixing for electron microscopy and for optical and gravimetric studies. After 60 min. further samples were taken. Then 0.01 vol. of a solution of ATP and Mg^{2+} ions was added to the phosphate-treated mitochondria to give final concentrations of 5 and 3 mM respectively. Samples were taken for analysis and for electron microscopy immediately after mixing and again 30 min. later. The changes in extinction are shown in Fig. 5. The rapid change in extinction in the mitochondria treated with phosphate is partly due to the anomalous changes already described, since a fresh sample tested at 35 min. did not show such a large

Fig. 4. Changes in extinction of a mitochondrial suspension induced by transfer to the cuvette. The mitochondria were suspended in 0.125M-potassium chloride in 20 mM-tris hydrochloride buffer, pH 7.4, and treated with orthophosphate (1 mM). The ΔE was determined at 520 m μ and fresh samples were taken at 2, 35, 40, 45, 60 and 90 min. Initially the mitochondrial suspension contained about 90 mg. wet wt./35 ml. of suspension, with a dry wt. of about 30 mg.

Fig. 5. Changes in extinction during phosphate-induced swelling and ATP-stimulated contraction of mitochondria. Mitochondria (100 mg. wet wt./35 ml.) were suspended in 0.125M-potassium chloride in 20 mM-tris hydrochloride buffer, pH 7.4. The ΔE was measured at 520 m μ and fresh samples were taken at 35 min. Δ , Spontaneous changes; $\circ$, mitochondria treated with 1 mM-potassium phosphate; $\bullet$, phosphate-swollen mitochondria treated with 5 mM-ATP and 3 mM- Mg^{2+} ion at 60 min.

change. The spontaneous changes in the absence of phosphate showed no anomalous behaviour, but the extinction decreased at an accelerating rate. By 60 min. the phosphate-treated suspension of mitochondria had almost reached the limit of its optical changes. When ATP and Mg^{2+} ions were added there was a rapid increase in extinction which was almost complete within 5 min. Subsequently up to 105 min. there was only a slight increase in the extinction.

The gravimetrically determined changes in the water content of the mitochondria treated first with phosphate and then subsequently with ATP and Mg^{2+} ions are shown in Table 1. Although the initial sample and that taken immediately after the addition of phosphate were centrifuged at the same time, there was already a change in the water content of the mitochondria. The water changes follow the optical changes, and the final water content after treatment with ATP was only slightly higher than the initial level. Thus the pattern of changes was qualitatively similar both by optical and by gravimetric criteria.

Solute changes in swelling and shrinking. Table 1 also shows the quantities of some of the solutes in the mitochondrial pellets during the stages of swelling with phosphate and shrinking with ATP and Mg^{2+} ions. Table 2 gives the changes in the

nominal concentrations of the solutes, assuming they are freely soluble in the water of the mitochondria. The total millimolal concentration of the medium is also given for comparison. It is clear that swelling is not simply an uptake of water but of solution somewhat similar in composition to that of the medium. This swelling is thus similar to that described by Leaf (1956) for the whole cell. Table 2 shows that the nominal osmolarity of the mitochondrial fluid is considerably greater than that of the medium. However, since there is no spontaneous uptake of water, it must be assumed that the interior fluid of the mitochondria and the external medium are at osmotic equilibrium. Initially the nominal concentration of K^+ ions in the mitochondria was twice that of the Cl^- ions, and, during swelling, in spite of the net uptake of K^+ ions, there was a fall in the nominal concentration of K^+ ions. Even when maximally swollen there was still a nominal osmotic differential between mitochondrial fluid and the medium. This was largely due to the higher nominal concentration of potassium chloride within the mitochondria, and presumably this fraction of potassium chloride must be considered unable to equilibrate with the medium. During contraction the potassium chloride was lost, but Cl^- ions were lost more readily than K^+ ions. However, the K^+ ion concen-

Table 1. *Changes in amounts of solutes in mitochondria during swelling and shrinking*

Experimental details are given in the text. Amounts of solutes are given as μ moles/100 mg. dry wt. Water content is given as the weight of water/unit dry wt. of mitochondria (I/M).

	Amount of material in mitochondria				
	Initial	Phosphate (1 mM) added		ATP and Mg^{2+} ions added	
		Initial	After 60 min.	After 60 min.	After 90 min.
Water content (I/M)	2	2.25	6.62	4.0	2.6
Na^+	11.3	10.1	14.4	19.5	14.9
K^+	44.6	50	121	78.2	46.8
Cl^-	21.9	26	106	68	37.4
P_i	2.24	4.68	1.05	4.44	3.97
Organic phosphate	6.16	5.52	2.11	3.18	3.38

Table 2. *Nominal concentration of solutes in mitochondria during swelling and shrinking*

Experimental details are given in the text. Concentrations are given as millimolal.

Solute	Nominal concn. of solutes in mitochondria				
	Initial	Phosphate (1 mM) added		ATP and Mg^{2+} ions added	
		Initial	After 60 min.	After 60 min.	After 90 min.
Na^+	51.0	43.5	20.6	50.2	58.2
K^+	201	214	176	179	182
Cl^-	111	120	161	170	144
P_i	10.7	19.9	1.47	10.2	15.4
Organic phosphate	27.2	24.0	4.6	15.8	24.3
Total ...	401	421	364	425	424
Total in medium...	264	274	283	310	294

tration failed to reach its initial nominal concentration.

The quantity of Na^+ ions in the mitochondria showed little change although the concentration fell during the swelling.

Initially the orthophosphate of the mitochondria was present at a considerable nominal concentration differential. In spite of this, when the concentration of orthophosphate in the medium was increased by 1 mM the nominal concentration of orthophosphate in the mitochondria increased by 9 mM. However, when swelling was maximal there was a fall in the total quantity of orthophosphate in the mitochondria and the concentration fell to a level not greatly in excess of that in the medium. The addition of ATP and Mg^{2+} ions raised the orthophosphate concentration to that present when phosphate was first added. After 30 min. the concentration of phosphate was 50 % higher than that present initially and the total quantity had increased in roughly the same proportion.

Organic phosphate was lost rapidly by the mitochondria when phosphate was added. The addition of ATP only increased the quantity of organic phosphate to half its initial amount. The values for organic phosphate were somewhat variable, since in some other experiments the organic phosphate concentration was restored to the initial levels during the contraction.

Morphological changes. Electron micrographs of mitochondria taken at different stages during the swelling and contraction cycle are shown in Plate 1. Initially (Plate 1a) the mitochondria contained small vesicles and no limiting membrane was visible. Clearly resuspension in 0.125M-potassium chloride had caused some morphological changes. However, such mitochondrial preparations could carry out normal oxidative phosphorylation. The original preparation of mitochondria suspended in 0.25M-sucrose solution showed good morphological preservation. Immediately after the addition of phosphate the mitochondria were more electron-dense (Plate 1b) and slightly swollen, although the structure was very similar. After 60 min., when the mitochondria had the largest water content, there were very few discrete mitochondria visible, but a large amount of disorganized material (Plate 1c). Immediately after the addition of ATP and Mg^{2+} ions there was little visible change in the ultra-structure, but at this time there were already changes in the water and solute content of the sedimented material. When contraction ceased 30 min. after the addition of ATP and Mg^{2+} ions discrete structures were present (Plate 1d). Although some of these were very similar to the initial mitochondria there was a much greater range in size and shape. Again no limiting membrane was visible.

Effect of mitochondrial concentration on the swelling and contraction of mitochondria

The spontaneous swelling of mitochondria was the same at between 50 and 150 mg. wet wt. of mitochondria/35 ml. of suspension medium. However, the phosphate-induced swelling and the ATP-induced contraction of mitochondria were influenced by the concentration of mitochondria in the suspending medium. Fig. 6 shows the changes in extinction of four mitochondrial suspensions in 0.125M-potassium chloride in 20 mM-tris hydrochloride buffer, pH 7.4. In two batches the mitochondrial concentration was 150 mg. wet wt./35 ml. and in the other two 100 mg. wet wt./35 ml. One of each pair was treated with phosphate to give a final concentration of 1 mM. There was little difference in the spontaneous swelling, but in the presence of phosphate the change in extinction/100 mg. dry wt. was much greater in the more dilute suspension. After 30 min. the change in extinction/100 mg. dry wt. was six times as great in the 100 mg. sample as that in the samples showing spontaneous

Fig. 6. Changes in extinction of mitochondria during swelling and contraction at different concentrations. Mitochondria were suspended in 0.125M-potassium chloride in 20 mM-tris hydrochloride buffer, pH 7.4, at concentrations of approx. 100 mg. wet wt. (●, ○) and 150 mg. wet wt. (▲, △) of mitochondria/35 ml. of medium. ○ and ▲, Mitochondrial suspensions treated initially with potassium phosphate to give a concentration of 1 mM. After 30 min. ATP and Mg^{2+} ions were added to all samples to give concentrations of 5 and 3 mM respectively.

swelling. However, the extinction of the phosphate-treated 150 mg. sample was only twice as great as that in the samples that had swollen spontaneously.

When ATP was added the change in extinction was greatest in the more dilute suspensions, especially the phosphate-treated sample. The extinction of the two 150 mg. samples did not return to the original values, but the spontaneously swollen 100 mg. sample reached an extinction greater than its starting value.

The gravimetrically measured water content of the mitochondria showed the same pattern of changes as the extinctions (Fig. 7). The largest changes occurred in the 100 mg. sample treated with phosphate.

Fig. 7. Effect of mitochondrial concentration on the water content during swelling and contraction. The water content is expressed as the weight of water/unit dry wt. of mitochondria (I/M). Mitochondria were suspended in 0.125 M-potassium chloride in 20 mM-tris hydrochloride buffer, pH 7.4, at concentrations of approx. 100 mg. wet wt. (○, □) and 150 mg. wet wt. (▲, ●) of mitochondria/35 ml. of medium. ● and □, Mitochondrial suspensions treated initially with potassium phosphate to give a concentration of 1 mM. After 30 min. ATP and Mg^{2+} ions were added to all samples to give concentrations of 0.5 mM and 3 mM respectively.

DISCUSSION

Use of optical measurements in measuring water movements in mitochondria. The results presented illustrate some of the difficulties inherent in the use of light-scattering as a measure of mitochondrial swelling. Thus we confirm the results of Lehninger (1959) and of Price *et al.* (1956), who showed that there was no linear relationship between water content measured gravimetrically and extinction. Our experiments have shown the scatter of results and also that, although the optical method gives qualitatively the same results as the gravimetric method, it can often be unsatisfactory for quantitative studies. In most studies where the optical method is used the initial mitochondrial suspension is made up to give a convenient extinction without regard to the absolute quantity of the mitochondria in the suspension. The present studies have shown that there is considerable variation in the extinction of a mitochondrial suspension even if the water content is constant. It is unusual for authors to state the water content of their mitochondrial suspension, and in some cases where this value is given (Lehninger, 1959) the initial water content/unit dry wt. of mitochondria varied from 2.3 to 3.1, and our experience has shown that larger variations than this may occur. Thus the concentration of mitochondria and hence the results may vary. It is one of the basic premises in the use of the optical method for studying swelling that water is the only component that is gained or lost by the mitochondria. The present study and those of Werkheiser & Bartley (1957), Amoores & Bartley (1958) and Bartley & Amoores (1958) have shown that there may be considerable changes in the solute contents of mitochondria under various conditions. Often there is an accompanying water movement but this does not necessarily occur. The loss of soluble protein from mitochondria as noted above and by the other workers will be critical in determining the difference in refractive index between

EXPLANATION OF PLATE 1

(a) Electron micrograph of initial state of mitochondria suspended in 0.125 M-potassium chloride in 20 mM-tris hydrochloride buffer, pH 7.4. (b) Electron micrograph of mitochondria immediately after the addition of phosphate to the suspension in 0.125 M-potassium chloride in 20 mM-tris hydrochloride buffer, pH 7.4. (c) Electron micrograph of swollen mitochondria 60 min. after the addition of phosphate. Other conditions were as in (a) and (b). (d) Electron micrograph of contracted mitochondria 30 min. after the addition of ATP and Mg^{2+} ions to the phosphate-swollen mitochondria. The final concentrations of ATP and Mg^{2+} ions were 5 mM and 3 mM respectively. Magnification $\times 23000$.

mitochondria and medium and hence the light-scattering of the mitochondria.

Another difficulty in the use of the optical method is the catalytic effect glass surfaces apparently have on swelling. The nature of the 'glass effect' is unknown. The cuvettes were always thoroughly cleaned before use and the prevention of the effect by washing the cuvettes with mitochondrial suspension suggests that a protein film deposited on the glass is protective. The enhancement of the glass effect in the presence of phosphate might be caused by the adsorption of the phosphate on the glass to produce a local high phosphate concentration. The glass effect was not studied gravimetrically, so that it is not known if this change in extinction was caused by uptake of water, although this seems likely.

In spite of the deficiencies of the optical method of measuring swelling it has the advantages of speed and of affording a continuous time record, and thus it will continue to be used. What is important is that extensive theories of the control of mitochondrial metabolism should not be built on optical changes alone but that ancillary measurements should be made to support the inferences from the optical changes.

Nature of the swelling process. The swelling of mitochondria induced by phosphate is correlated with an uptake of phosphate by the mitochondria. The quantity of inorganic phosphate in the mitochondria doubles within a few minutes of the addition of phosphate to the suspension. However, whereas the presence of 1.0 mM-phosphate causes mitochondria at a concentration of 100 mg. wet wt./35 ml. to swell almost completely, the extent of swelling induced by the same concentration of phosphate on mitochondria of 150 mg. wet wt./35 ml. is much less. Thus the extent of swelling depends on the ratio of phosphate to mitochondria, but it is not a direct relationship since the phosphate/mg. dry wt. of mitochondria ratio in the 100 mg. sample compared with the 150 mg. sample is 1.49 but the ratio of the water content after swelling is 2.3. The explanation could, however, be that some mitochondria swell completely and others do not change. This is supported by the electron micrographs, in which some mitochondria appear more swollen than others. Also, once swelling has taken place the phosphate taken up initially is lost together with some endogenous phosphate. After centrifugation of the swollen mitochondria a layer of apparently unswollen mitochondria was often seen at the bottom of the pellet. This is in agreement with the variations in the behaviour of mitochondria found by Amoore & Bartley (1958). However, this can only partially explain the effect of phosphate and would depend on the uptake of phosphate by the mitochondria that did not swell. The swelling of

mitochondria under the conditions used in the present study is not a simple osmotic phenomenon. The nominal concentration of the measured solutes in the mitochondria was almost twice that in the medium and yet there was no immediate uptake of water unless phosphate was added. There is no evidence that mitochondria are in any way impermeable to water and therefore we must assume that the activity of water in the medium is the same as the activity of the water in the mitochondria. Evidence presented by Amoore (1960) shows that not all the mitochondrial K^+ ions are readily exchangeable until the mitochondria start to swell. Werkheiser & Bartley (1957) and Bartley (1961) have also considered that a fraction of the solutes are segregated from those that rapidly exchange with those of the medium. It is possible that in hydrophobic regions of the mitochondria proteins, lipoproteins and lipids contain bound within themselves the excess of solutes isolated from contact with the free water of the main solute solution. Changes in the conformation of the macromolecular complexes of the mitochondria could result in access by the medium to hitherto hydrophobic regions. This would account for the increase in water and solute content of the mitochondria on swelling and development of rapid exchange between all solutes of the mitochondria. At areas of macromolecules where a hydrophobic region changes to a region where water is not excluded it is probable that local orientation of the hydrophobic side chains of the macromolecules will depend greatly on the possibility of hydrophobic bonding (Nemethy & Scheraga, 1962*a, b, c*) between the chains, with consequent changes in the structure and entropy of the adjacent water. Such a region of the macromolecule might well be critically and catalytically sensitive to small changes in the local concentration of solutes such as phosphate, which, by opening key hydrophobic bonds, would allow major re-orientation of the macromolecules. The overall energy change need not be large since, although a number of hydrophobic bonds may be broken, some new bonds could be established within the areas of the molecules previously inaccessible to the water. Such a process should be easily reversible and, although it is not easy to visualize the role of ATP in reversing the process, it must be remembered that the adsorption, for example, of ATP at a specific site on the macromolecules would undoubtedly alter the structure of the surrounding water and thus the hydrophobic bonding between adjacent side chains. Extensive changes in conformation might well result, leading to formation of hydrophobic regions within the macromolecule and extrusion of water. Provided that the ensuing changes in conformation result in the formation of as many hydrophobic bonds as are broken, there

should be no energy barrier to such changes. Lehninger (1959) has already suggested that a contractile protein similar to actin may exist in the mitochondria, and this could utilize the energy of splitting ATP to change its conformation. Whether a specific contractile protein is concerned in the extrusion of water from mitochondria is uncertain, but it would seem likely that, if such a protein does exist, it would act catalytically in initiating changes in conformation, not in supplying the total energy for physically squeezing out the water from the rest of the macromolecules. Although our experimental observations are entirely consistent with those reviewed by Lehninger (1962), we feel that the electron-microscope studies are not consistent with the location of any coupled system in the mitochondrial membrane responsible for mitochondrial swelling and contraction and the simultaneous production of ATP. The complete disruption of the mitochondria by phosphate and the altered size and shape of the particles resulting from the treatment with ATP suggest that the swelling and shrinking is more likely to be a general property of many of the macromolecules making up the mitochondria and is not the reflection of a membrane mechanism. It is also doubtful whether our observations (or those of others) on mitochondrial swelling *in vitro* have any relevance to the behaviour of mitochondria *in vivo* except to illustrate how macromolecular shape and hydration can be influenced by the presence of chemicals of physiological importance.

SUMMARY

1. The swelling and contraction of isolated rat-liver mitochondria have been studied by optical and gravimetric methods.

2. The results of optical studies and gravimetric determinations of the water content of mitochondria are qualitatively similar. There is, however, no direct relationship between water content and extinction.

3. The concentration of mitochondria has no effect on spontaneous swelling, studied by either method. However, the concentration does affect the changes in extinction during phosphate-induced swelling and ATP-stimulated contraction. The change in extinction is not directly proportional to the ratio of phosphate to mitochondria.

4. The changes in mitochondrial solutes during phosphate-induced swelling and ATP-stimulated contraction are reversible.

5. Electron micrographs of mitochondria during

swelling and contraction indicate that the process is not truly reversible. Some fragmentation of the mitochondria occurs, and these fragments contract.

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REFERENCES

- Amoore, J. E. (1960). *Biochem. J.* **76**, 438.
 Amoore, J. E. & Bartley, W. (1958). *Biochem. J.* **69**, 223.
 Amoore, J. E., Parsons, D. S. & Werkheiser, W. C. (1958). *Biochem. J.* **69**, 236.
 Bartley, W. (1953). *Biochem. J.* **54**, 677.
 Bartley, W. (1961). *Biochem. J.* **80**, 46.
 Bartley, W. & Amoore, J. E. (1958). *Biochem. J.* **69**, 348.
 Bassi, M. (1960). *Exp. Cell Res.* **20**, 313.
 Berenblum, I. & Chain, E. (1938). *Biochem. J.* **32**, 295.
 Cleland, K. W. (1952). *Nature, Lond.*, **170**, 497.
 Hanes, C. S. & Isherwood, F. A. (1949). *Nature, Lond.*, **164**, 1107.
 Hunter, F. E., jun. & Ford, L. (1955). *J. biol. Chem.* **216**, 357.
 Leaf, A. (1956). *Biochem. J.* **62**, 241.
 Lehninger, A. L. (1959). *J. biol. Chem.* **234**, 2465.
 Lehninger, A. L. (1962). *Physiol. Rev.* **42**, 467.
 Lehninger, A. L. & Neubert, D. (1961). *Proc. nat. Acad. Sci., Wash.*, **47**, 1929.
 Lehninger, A. L. & Schneider, M. (1959). *J. biophys. biochem. Cytol.* **5**, 109.
 Macfarlane, M. G. & Spencer, A. C. (1953). *Biochem. J.* **54**, 569.
 Nemethy, G. & Sheraga, H. A. (1962a). *J. chem. Phys.* **36**, 3382.
 Nemethy, G. & Sheraga, H. A. (1962b). *J. chem. Phys.* **36**, 3401.
 Nemethy, G. & Sheraga, H. A. (1962c). *J. phys. Chem.* **66**, 1773.
 Price, C. A., Fonnesu, A. & Davies, R. E. (1956). *Biochem. J.* **64**, 754.
 Raaflaub, J. (1953a). *Helv. physiol. pharmacol. acta*, **11**, 142.
 Raaflaub, J. (1953b). *Helv. physiol. pharmacol. acta*, **11**, 157.
 Schulz, H., Löw, H., Ernster, L. & Sjostrand, F. S. (1956). *Proc. Stockholm Conf. Electron Microscopy*, p. 134.
 Slater, E. C. (1957). *Symp. Soc. exp. Biol.* **10**, 110.
 Tapley, D. F. (1956). *J. biol. Chem.* **222**, 325.
 Tapley, D. F., Cooper, C. & Lehninger, A. L. (1955). *Biochim. biophys. Acta*, **18**, 597.
 Tustanoff, E. R. & Bartley, W. (1964). *Biochem. J.* **91**, 595.
 Werkheiser, W. C. & Bartley, W. (1957). *Biochem. J.* **66**, 79.
 West, P. W. & Coll, H. (1957). *J. Amer. Wat. Wks Ass.* **49**, 1485.