The Effect of Osmotic 'Shock' on the Swelling Pattern and Respiratory Control of Rat-Liver Mitochondria

By SARA STREICHMAN AND Y. AVI-DOR

Department of Chemistry, Technion-Israel Institute of Technology, Haifa, Israel

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1. Rat-liver mitochondria suspended in 0.25M-sucrose were exposed for a few seconds to strongly hypo-osmotic conditions, and then the osmolarity of the medium was raised again to 0.25 with the aid of tris chloride (osmotic 'shock'). 2. Mitochondria after hypo-osmotic pretreatment lost their capacity for slow energy-dependent swelling in iso-osmotic tris buffer and showed no respiratory control. 3. Swelling could be induced in the 'shocked' mitochondria by ATP but not by addition of respiratory substrates. 4. It was shown that cytochrome *c* is lost from 'shocked' mitochondria when they come into contact with the tris buffer present in the assay medium, and that the changes observed in the pattern of swelling, as well as in respiratory control, are directly connected with this loss of cytochrome *c*. 5. The results of the investigation are discussed with regard to the role of cytochrome *c* in swelling and respiratory control.

Because of the highly complex nature of the swelling phenomenon, it has been difficult to correlate it with other energy-linked processes in mitochondria. One possible approach would be to induce changes in the mitochondrial structure that would eliminate swelling in a reversible manner, and then search for factors that would reverse the changes. Mitochondria modified by various treatments have been used in the past by several authors for the study of mitochondrial functions. Hunter and co-workers (McKnight, Hunter & Dehlert, 1965; McKnight & Hunter, 1966) described the physical and biochemical characteristics of the particles resulting from ferrous iron-induced lipid peroxidation of rat-liver mitochondria. Mitochondria pretreated under hypo-osmotic conditions were used for the study of phosphorylation connected with NADH oxidation (Lehninger, 1951), thyroxine-induced swelling (Glick & Bronk, 1965b) and ion translocation (Penniston, vande Zande & Green, 1966a,b). In the present investigation, mitochondria pretreated under hypo-osmotic conditions provided important information on the factors involved in swelling, especially on the role of cytochrome *c*. It was also possible to study the effect of ATP under conditions where energy derived from oxidation of respiratory substrates could not be utilized for swelling.

MATERIALS AND METHODS

Animals. Male albino rats of local strain were used.

Materials. ATP, ADP, cytochrome *c*, GSH and GSSG

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Rat-liver mitochondria. Mitochondria were isolated from homogenates prepared in 0.25M-sucrose by differential centrifugation, as described by Ernster & Löw (1955). The mitochondrial pellet was finally suspended in 0.25M-sucrose; 3.0 ml. of the final suspension corresponded to 1.0 g. wet wt. of liver.

Standard conditions for osmotic 'shock'. One vol. of the stock suspension of mitochondria in 0.25M-sucrose was diluted with 3 vol. of redistilled ice-cold water. A sample of the diluted suspension, equivalent to the amount of untreated mitochondria producing an initial E_{520} value of 0.500 (as a rule 0.12 ml.), was transferred immediately into the cells containing the assay mixture.

Preparation of 'swollen' mitochondria. To 300 ml. of 130 mM-tris-HCl buffer, pH 7.4, stock suspension of mitochondria was added in an amount producing an initial E_{520} value of 0.500. The mixture was incubated at 30°. Every 2 min. a 3.0 ml. sample was withdrawn and the extinction checked against tris-HCl buffer. Incubation was continued until spontaneous swelling reached its maximum (usually 15–30 min.). After centrifugation at 16000g for 10 min. the supernatant was decanted and the mitochondrial pellet was resuspended in the original volume of 0.25M-sucrose.

Measurement of volume changes. Swelling was studied by measuring changes in extinction with a Beckman model DB spectrophotometer equipped with a linear and logarithmic potentiometric recorder. The standard assay system used consisted of 130 mM-tris-HCl buffer, pH 7.4, and mitochondria sufficient for an initial E_{520} value between 0.500 and 0.600 in a cell of 1 cm. light-path. As a rule, 0.03 ml. of the stock suspension was used. The total volume was 3.0 ml. The blank contained all components except the

mitochondria. All other conditions are indicated in the Figure legends.

Respiration. Mitochondrial respiration was measured in a Gilson Oxygraph (model K) equipped with an oscillating platinum cathode. The standard reaction mixture contained the following components: tris-HCl buffer, pH 7.4 (25 mM); KCl (50 mM); potassium phosphate buffer, pH 7.4 (15 mM); $MgCl_2$ (8 mM); mitochondria in an amount equivalent to 2–3 mg. of protein/ml. The total volume was 2 ml. Other additions are indicated in the text.

Estimation of cytochrome c in mitochondria. Cytochrome c was estimated from the difference spectrum of the mitochondrial sample (reduced–oxidized), as described by Williams (1964). Spectra were measured in a Cary model 14 recording spectrophotometer.

Lipid peroxide determination. The thiobarbituric acid colour reaction was used as described by Hunter, Gebicki, Hoffsten, Weinstein & Scott (1963).

Protein determination. Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951).

Centrifugations. Centrifugations were carried out in a Servall model RC-2 refrigerated centrifuge (rotor SS-3).

RESULTS

Effect of temporary decrease in the osmoticity of the suspending medium on the swelling pattern in iso-osmotic tris solution. Experiments were performed in which mitochondria were exposed for a short time to hypo-osmotic conditions by diluting the stock suspension (in 0.25 M-sucrose) to different extents with ice-cold redistilled water. Pretreatment was terminated by transferring a sample of the diluted suspension into an assay medium containing tris buffer, pH 7.4, in the amount required to bring the final osmolarity back to 0.25. The volume of the sample transferred was adjusted so that the same amount of mitochondria was always present in the assay medium. Since dilution can itself affect mitochondrial functions associated with swelling (Avi-Dor, 1960) and with electron transport (Avi-Dor, Lamdin & Kaplan, 1963; McKnight & Hunter, 1966), controls were run in which the mitochondria were diluted with 0.25 M-sucrose instead of water.

The results presented in Fig. 1 show that the initial extinction of mitochondrial suspension previously exposed to hypo-osmotic conditions was always lower than that of an equal amount of untreated mitochondria, or of mitochondria diluted without change in osmolarity during pretreatment. It is also apparent that the total extent of subsequent spontaneous swelling decreased when the hypo-osmoticity of the medium was increased; when the osmoticity during pretreatment was below 0.06 osmolar (1:4 dilution), no spontaneous swelling took place.

In further work dilution of 1:4 by water was adopted as the standard procedure for inducing

Fig. 1. Effect of hypo-osmotic pretreatment on the swelling pattern of mitochondria. A suspension of mitochondria in 0.25 M-sucrose was diluted with redistilled ice-cold water as indicated below. After 30 sec. of incubation at 0°, a sample of the diluted suspension was transferred to the assay medium containing tris chloride buffer in an amount giving a final osmolarity of 0.25. In each case the volume of the sample transferred contained the same amount of mitochondria, irrespective of dilution. Curve A, untreated mitochondria; curve B, diluted eightfold with 0.25 M-sucrose. In all other curves, the diluting agent was redistilled water and the dilution was as follows: curve C, twofold; curve D, threefold; curve E, fourfold; curve F, fivefold; curve G, eightfold. The temperature of incubation during the assay was 28°.

osmotic 'shock', and the modified mitochondria obtained are referred to as 'shocked' mitochondria.

Effect of ATP and respiratory substrates on swelling in 'shocked' mitochondria. The present widely accepted view is that swelling in normal mitochondria can be caused by 'energy-rich' compounds produced either during oxidation of respiratory substrates or directly by ATP (cf. Lardy, Connelly & Johnson, 1964). On the one hand, it has been shown that 'aged' mitochondria, which did not swell in the presence of phosphate or thyroxine as a result of depletion of endogenous substrate, recovered this capacity when oxidizable substrates were added (Chappell & Greville, 1958); on the other, inhibition of spontaneous swelling caused by the presence of respiratory inhibitors could be relieved by ATP (Suranyi & Avi-Dor, 1966), whereas phosphate-induced swelling suppressed by low pH was shown to be restored both

Fig. 2. Effects of ATP and succinate on swelling in 'shocked' mitochondria. The 'shocked' mitochondria were prepared by the standard procedure described in the Materials and Methods section. The basic assay medium contained 0.25 osmolar tris chloride buffer, pH 7.4. The following additions were made to the basic assay medium: curve A, none; curve B, 66 μ M-ATP; curve C, 66 μ M-ATP + 0.66 μ g. of oligomycin/ml.; curve D, 33 mM-succinate. The temperature of incubation was 28°.

by ATP and by respiratory substrates (Connelly & Lardy, 1964). To find out which of the mechanisms for generation of 'high-energy' compounds has been impaired by the 'shock', we tested the effect of ATP and respiratory substrates on 'shocked' mitochondria. As shown in Fig. 2, ATP relieved the inhibition, but succinate was unable to induce swelling. Attempts to induce swelling by other substrates (such as β -hydroxybutyrate or malate + glutamate) were also unsuccessful. ATP-induced swelling in both 'shocked' and normal mitochondria was found to be sensitive to oligomycin. As mentioned in the preceding section, hypo-osmotic pretreatment caused a decrease in initial extinction (see Fig. 1) in addition to its effect on the slow phase of swelling. Nevertheless, with the aid of ATP it could be shown that the changes in initial extinction induced by the 'shock' are not the same as those associated with energy-dependent swelling. As shown in Fig. 3, the 'shocked' mitochondria responded to addition of ATP by a further slow decrease in extinction as described above, whereas 'swollen' mitochondria showed the well-known phenomenon of ATP-induced recontraction (cf. Lehninger, 1962).

Respiration and respiratory control in 'shocked' mitochondria. The finding that, in contrast with ATP, respiratory substrates failed to induce swelling in 'shocked' mitochondria, prompted us to test whether the hypo-osmotic pretreatment could have affected the respiratory system. Since Lehninger (1951) reported acceleration of respiration in water-treated mitochondria by cytochrome c, we measured oxygen uptake with the aid of an oxygen electrode both in the presence and absence of cytochrome c.

Fig. 3. Comparison of the effects of ATP on 'shocked' and 'swollen' mitochondria. The basic assay medium was as indicated in Fig. 2. 'Shocked' and 'swollen' mitochondria were prepared as described in the Materials and Methods section. The amount of swollen mitochondria was adjusted to give the same initial extinction as the 'shocked' mitochondria. 0.33 mM-ATP was added at the time indicated by the arrow. Other conditions were as indicated in Fig. 2. Curve A, 'shocked' mitochondria; curve B, 'swollen' mitochondria.

Fig. 4. Effect of osmotic 'shock' on the endogenous respiration of mitochondria. The assay medium contained: tris-chloride buffer, pH 7.4 (25 mM); KCl (50 mM); $MgCl_2$ (5 mM); phosphate buffer, pH 7.4 (15 mM); mitochondria in an amount equivalent to 14 mg. of protein. The total volume was 2.0 ml. The temperature of incubation was 30°. Curve A, mitochondria pretreated by diluting with 3 vol. of 0.25 M-sucrose; curve B, mitochondria pretreated by diluting with 3 vol. of ice-cold redistilled water. At the times indicated by the first and second arrows, 50 μ M-cytochrome c and 50 μ M-ADP were added respectively.

In Fig. 4, the effect of the osmotic 'shock' is shown on the endogenous respiration, and in Fig. 5 the respiratory patterns of normal and pretreated mitochondria are compared, with succinate as

oxidizable substrate. Fig. 4 shows that, as judged from the slope of the traces representing the rate of disappearance of oxygen, endogenous respiration was not appreciably affected by the 'shock', and cytochrome *c* caused no increase in rate of oxygen uptake either in normal or in 'shocked' mitochondria. Moreover, addition of ADP to the system as a potential phosphate acceptor had no effect on the respiratory rate in either of the two types of mitochondria. As shown in Fig. 5 and from the inset, 'shocked' mitochondria oxidized succinate at a decreased rate in the absence of cytochrome *c* compared with the rate measured in normal mitochondria, and the basic rate of respiration ('state 4' according to Chance & Williams, 1956) could not be increased by adding ADP (no 'state 4'-'state 3' transition). However, addition of cytochrome *c* not only restored the original respiratory rate in 'shocked' mitochondria, but also reversed the deleterious effect of the 'shock' on respiratory control. We also found in other experiments (which do not warrant detailed description) that hypo-osmotic pretreatment decreased by 30–70% the rate of oxidation of other substrates such as β -hydroxybutyrate or malate + glutamate, and that in all these cases the rate could be restored to its original level by cytochrome *c*. The effect of the 'shock' on the respiratory control was, as a rule, more pronounced than on respiration.

Cytochrome *c*-induced swelling in 'shocked' mitochondria. In view of the striking effect of cytochrome *c* on the respiratory activity of 'shocked'

Fig. 5. Effect of osmotic 'shock' on the rate of succinate oxidation by mitochondria. The assay medium contained succinate (20mM), mitochondria equivalent to 3mg. of protein and tris, KCl, $MgCl_2$ and phosphate buffer as indicated in Fig. 4. The total volume was 2.0ml. The temperature was 21°. Main Figure: 'shocked' mitochondria; curve A, no cytochrome *c* added; curve B, 50 μ M-cytochrome *c* added. Inset: intact mitochondria: curve C, no cytochrome *c* added; curve D, 50 μ M-cytochrome *c* added. At the time indicated by the arrows, 50 μ M-ADP was added.

mitochondria, we explored whether it would also restore their capacity to swell in iso-osmotic tris chloride. As shown in Fig. 6, 0.13 μ M-cytochrome *c* caused slow swelling, whereas swelling in the presence of 13 μ M-cytochrome *c* was comparable in its extent and rate with that of untreated mitochondria (cytochrome *c* had no effect on the swelling of normal mitochondria). Like some other haem compounds, cytochrome *c* is known to promote lipid peroxide formation (Tappel & Zalkin, 1959a,b). It is also known that certain agents, such as GSSG + GSH or ascorbate, which induce formation of lipid peroxide in mitochondria, cause a 'lytic' type of swelling that is much more extensive than the limited changes in extinction that occur in the presence of phosphate or thyroxine (Hoffsten, Hunter, Gebicki & Weinstein, 1962; Hunter *et al.* 1964). These findings prompted us to compare the effects of GSSG + GSH and of cytochrome *c* on swelling and on lipid peroxide formation in 'shocked' mitochondria. From the results presented in Figs. 7(a) and 7(b) it is apparent that GSSG + GSH caused a 'lytic' type of swelling also in the pretreated mitochondria and, simultaneously with the swelling, formation of lipid peroxides could be detected. In contrast with the effect of thiol compounds, cytochrome *c*-induced swelling was of the 'limited' type and was not accompanied by a significant increase in lipid peroxides. The cytochrome *c*-induced swelling in 'shocked' mitochondria resembled the 'limited' swelling of normal mitochondria also in its response to the action of inhibitors of respiration and to 2,4-dinitrophenol. As shown in Fig. 8, 13 μ M-rotenone, 1mM-Amytal or 20 μ M-2,4-dinitrophenol almost completely suppressed the cytochrome *c*-induced swelling. The above agents are also known, in this range of concentration, to inhibit phosphate- or thyroxine-induced swelling, but not that induced

Fig. 6. Effect of cytochrome *c* on the swelling of 'shocked' mitochondria. The basic assay medium and other conditions were as indicated in Fig. 2. Cytochrome *c* was added to the medium in the following concentrations: curve A, none; curve B, 0.13 μ M; curve C, 1.33 μ M; curve D, 13.0 μ M; curve E, 20.0 μ M.

Fig. 7. Effects of cytochrome *c* and of GSH + GSSG on (a) the swelling of mitochondria and on (b) the formation of lipid peroxides. The media both for swelling and lipid peroxide assay contained tris buffer, pH 7.4, and 'shocked' mitochondria as described under 'Measurement of volume changes'. The following additions were also made: curves *A*, none; curves *B*, 13 μ M-cytochrome *c*; curves *C*, 5 mM-GSSG + 1 mM-GSH. The total volume in the assay for swelling was 3.0 ml. and for formation of lipid peroxide was 9.0 ml. Incubation was conducted at room temperature (25°). For determination of lipid peroxide formation, 1.0 ml. samples of the assay mixture were removed at the times indicated and the peroxide was determined as described in the Materials and Methods section.

Fig. 8. Effects of rotenone, Amytal and 2,4-dinitrophenol on cytochrome *c*-induced swelling in 'shocked' mitochondria. Standard assay conditions were used as indicated in Fig. 2. The following additions were also made: curve *A*, none; curve *B*, 13 μ M-cytochrome *c*; curve *C*, 13 μ M-cytochrome *c* + 13 μ M-rotenone; curve *D*, 13 μ M-cytochrome *c* + 20 μ M-2,4-dinitrophenol; curve *E*, 13 μ M-cytochrome *c* + 1 mM-Amytal. At the time indicated by the arrow, 0.33 mM-ATP was added.

by GSH + GSSG (Hunter, Scott, Weinstein & Schneider, 1964). When ATP was added to 'shocked' mitochondria, after completion of the cytochrome *c*-induced swelling, it caused recontraction. Thus, in this respect as well, cytochrome *c* acted like other agents causing a 'limited' type of swelling (for references see Lehninger, 1962).

Effect of the osmotic 'shock' on the cytochrome content of mitochondria. Since all changes in the properties of mitochondria induced by osmotic 'shock' that were studied in the present work could be reversed partly or completely by cytochrome *c*, it seemed likely that the mitochondria lost cytochrome *c* either during the 'shock' or on exposure to the assay medium.

According to Jacobs & Sanadi (1960) and to Penniston *et al.* (1966a), hypo-osmotic pretreatment

in itself does not remove cytochrome *c* from the mitochondria unless followed by extraction with electrolytes. The findings of these authors suggested that, in the above case, loss of cytochrome *c* could have occurred when the 'shocked' mitochondria were exposed to the tris chloride present in the assay medium. To test this contention, we compared the cytochrome *c* contents of mitochondria pretreated by each of the following procedures: (1) diluted by 39 vol. of cold 0.25 M-sucrose; (2) diluted first by 3 vol. of cold redistilled water and then, after 2 min. of incubation at 0°, by a further 36 vol. of cold water; (3) diluted by 3 vol. of redistilled water and, after 2 min. at 0°, by a further 36 vol. of tris buffer, pH 7.4 (final concn. of tris 0.13 M). The diluted mitochondrial suspensions were then centrifuged (16000g for 30 min. at 0°) and the mitochondrial pellets were resuspended in a small volume of 0.25 M-sucrose. The cytochrome *c* and cytochrome *a* contents of each of the three samples were determined by Williams' (1964) spectrophotometric method. The results, summarized in Table 1, show that there is a loss of cytochrome *c* and, as expected, it occurred on exposure of the 'shocked' mitochondria to the electrolyte (tris chloride) and not during the hypo-osmotic pretreatment.

DISCUSSION

Mitochondria pretreated under hypo-osmotic conditions have been utilized recently in several studies for resolution and characterization of mitochondrial systems connected with swelling or ion translocation. Glick & Bronck (1965a,b) investigated thyroxine-induced swelling in rat-liver mitochondria exposed to mild osmotic 'shock' (by diluting the iso-osmotic sucrose suspension with an equal volume of water) that, as a result of the

Table 1. *Effect of various pretreatments on the cytochrome c content and on the cytochrome c/cytochrome a ratio of mitochondria*

Pretreatment		Cytochrome content (μ mole/mg. of protein)		Cytochrome c/ cytochrome a ratio
		Cytochrome c	Cytochrome a	
(1) Dilution with iso-osmotic sucrose	Expt. 1	0.310	0.325	0.97
	Expt. 2	0.281	0.281	
(2) 'Shock' followed by dilution with water	Expt. 1	0.276	0.362	0.80
	Expt. 2	0.306	0.372	
(3) 'Shock' followed by dilution with 130mm-tris buffer, pH 7.4	Expt. 1	0.074	0.262	0.30
	Expt. 2	0.079	0.252	

pretreatment, lost approximately one-third of their respiratory control. The pretreated mitochondria responded to various agents that had no apparent effect on thyroxine-induced swelling in untreated mitochondria. Penniston *et al.* (1966a,b) studied the factors involved in ion translocation in particles derived from mitochondria by prolonged swelling at 0°, followed by freezing and thawing. In such particles, ion translocation is coupled with electron transfer at the third site only, and, when the particles are extracted with dipotassium hydrogen phosphate, a requirement for external cytochrome c becomes manifest.

Separation of the two pathways leading to formation of the 'energy-rich' compounds involved in swelling, the one associated with electron transport and the other with the reversal of certain stages in ATP formation, was achieved in the past by means of respiratory inhibitors and uncouplers of oxidative phosphorylation (Azzone & Azzi, 1965; Lardy *et al.* 1964; Suranyi & Avi-Dor, 1966). In the present investigation, 'shocked' mitochondria were used for exploring some of the factors involved in energy-dependent swelling. They lost their capacity for 'spontaneous' swelling, but swelling could be induced by adding either ATP or cytochrome c. In all likelihood, the above swelling pattern is related to the observation that the mitochondria lost most of their cytochrome c on exposure to electrolytes present in the assay medium.

Still, the effect of cytochrome c cannot rest solely on its role in electron transport, since it restored swelling through the energy supplied through the endogenous respiration without having any influence on the endogenous oxygen uptake itself. Penniston *et al.* (1966a) were confronted with a similar problem while studying ion translocation. They thought it necessary to postulate 'two sites for cytochrome c in the electron transport chain, each with its unique role in coupling'. It is likely that cytochrome c, besides being a member of the respiratory chain, is also involved in coupling

between electron transport and the synthesis of the 'energy-rich' compounds necessary for swelling. The effect of cytochrome c on respiration control may be one of the manifestations of its coupling activity.

Further elucidation of the role played by cytochrome c in coupling may come from a study of other energy-linked functions in 'shocked' mitochondria.

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