ROLE OF C-FACTOR IN WATER UPTAKE AND EXTRUSION BY MITOCHONDRIA AND INTERFERENCE BY VARIOUS DRUGS

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WATER movements through mitochondrial membranes are of some interest because they involve the enzymes of oxidative phosphorylation and they are strikingly affected by certain hormones and other chemical agents. Mitochondria isolated from rat liver or kidney can take up significant amounts of water from an *in vitro* incubation medium, leading to doubling or tripling of mitochondrial volume. This process of spontaneous swelling can be markedly enhanced by adding any one of a number of so-called swelling agents, which include thyroxine, phosphate, calcium, inorganic and organic mercurials, thiols, and disulfides, phlorizin and others. Such a change in mitochondrial volume is not a passive process but is geared to the action of the respiratory chains in the mitochondrial membrane since swelling does not occur when respiration is blocked or under anaerobic conditions[1,2].

Mitochondrial swelling produced in this manner *in vitro* can be reversed again by the addition of ATP + Mg^{++}, with easily measured extrusion of water. It is of interest that ATP-dependent water extrusion from thyroxine-swollen mitochondria takes place independently of the nature of the ionic environment and does not require or depend on movements of potassium or sodium ions[3]. "Contraction" is specifically linked to ATP and is inhibited by uncoupling agents like azide or sucrose, but not by 2,4-dinitrophenol[4]. However mitochondrial water uptake induced by reduced glutathione (GSH) is an exception, since it is not reversed on addition of ATP + Mg^{++}. More recently Lehninger and Gotterer[5] have demonstrated that mitochondrial swelling induced

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Abbreviations used are:
ATP, adenosine triphosphate
BSA, bovine serum albumin
GSH, reduced glutathione
GSSG, oxidized glutathione
PCMB, p-chloromercuribenzoate

by glutathione can also be reversed with extrusion of water, if a mitochondrial factor is added to the swelling medium. This factor, called contraction factor or C-factor, appears to leak from the mitochondria on exposure to glutathione. C-factor apparently becomes limiting in the medium during GSH-induced swelling. Its action appears to be stoichiometric, since increasing concentrations of C-factor produce increasing levels of contraction in the presence of excess ATP.

In this paper we wish to report the purification, properties, and distribution of C-factor, as well as the action of some drugs and enzyme inhibitors on mitochondrial water uptake and ATP-dependent water extrusion. We measured the change in mitochondrial volume in rat liver

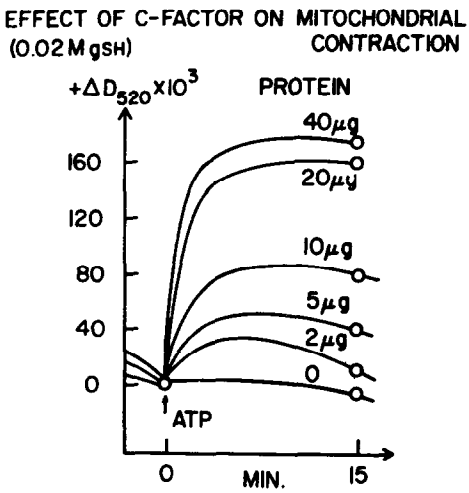

FIG. 1. Graded response of GSH-swollen rat liver mitochondria to C-factor in the presence of ATP. Mitochondria corresponding to about 0·5 mg of protein were allowed to swell in 5·0 ml, of a 0·125 M KCl — 0·02 M Tris-HCl-medium (pH 7·7) containing 0·02 M GSH at 21°. At the point shown, a mixture of ATP, $MgCl_2$, and BSA was added to give a final concentration of 0·005 M ATP, 0·003 M Mg^{++} and 2 mg/ml. BSA. C-factor was added to the swelling medium in the amounts indicated.

mitochondria by following the change in absorbancy at 520 mμ, a method which has been standardized against direct gravimetric measurements of mitochondrial water content. The general experimental conditions are demonstrated in Fig. 1: ATP, Mg^{++} and bovine serum albumin (BSA) alone are not able to reverse the GSH-induced swelling of rat liver mitochondria. But on the addition of a mitochondrial extract, containing C-factor, together with these agents, there is an increasing extrusion of water depending on the amount of C-factor added. This graded response of GSH-swollen mitochondria to C-factor is the basis of a bioassay for C-factor[6] and it has functioned satisfactorily under the precise conditions defined.

TABLE I

Specificity of C-factor activity

Examples of some of the proteins tested for C-factor activity. One unit of activity is defined as the amount producing halfmaximal ATP-driven water extrusion from rat liver mitochondria swollen by 0·02 M GSH. Experimental conditions as indicated in Fig. 1.

	Units activity per mg protein
Crude mitochondrial extract	2-5
Purified C-factor	250-500
Human serum	< 0·2
Cytochrome-c	< 0·3
Malic dehydrogenase	< 0·2
Glutamic dehydrogenase	< 0·2
Hexokinase	< 0 2
Ribonuclease	< 0·2

The activity of C-factor in initiating water extrusion in the presence of ATP is rather specific since as the data in Table I show, a variety of other proteins including some purified enzymes and blood serum proteins have no measurable C-factor activity.

C-factor present in mitochondria can be obtained in soluble form not only by incubation of the mitochondria with 0·01-0·02 M GSH but also by extracting them following sonic oscillation or acetone dehydration, or alternatively by extraction with salt solutions at high ionic strength.

C-factor is definitely a protein or tightly associated with a protein: the activity does not pass through a dialysis bag. It can be precipitated from aqueous solutions by high concentrations of ammonium sulfate or acetone. It is inactivated by temperatures exceeding 55° and is

TABLE II

Purification of C- factor from rat liver mitochondria

	Spec. activity (units/mg prot.)	Relative activity	Yield
1. Sonicated mitochondria spernatant	3	1·0	100%
2. 10 min 52° C.	6	2·0	98%
3. pH 5·5 precipitation	10	3·3	96%
4. Ethanol fractionation	55	18	84%
5. Sephadex G 75	114	47	79%
6. DEAE-Cellulose	410	136	7%

evidently a high molecular weight substance which is excluded from Sephadex.

Although our first efforts to purify C-factor using conventional methods like ammonium sulfate fractionation and isoelectric precipitation failed to give significant purification, we now have been able to purify C-factor activity reproducibly 50 to 100-fold and in some cases well over 100-fold. Table II gives an outline of one of the purification methods used which has given us the best results. It includes selective heat denaturation at 52° and isoelectric precipitation at pH 5·5. Alcohol fractionation then gives an excellent separation from inactive protein. This is followed by chromatography on Sephadex G 75 to remove small molecular weight compounds, and the final step involves chromatography on DEAE-cellulose. The preparation obtained in this way is virtually free of respiratory chain electron carriers like cytochrome-*c*,-*b*, and -*a* and contains only a very small amount of flavoprotein. Whether this flavoprotein is a component of the activity or an impurity we cannot say for certain. The purified C-factor contains only very little if any ATP-ADP exchange activity or ATP-ase activity.

In order to give an indication of the amount of C-factor required to bring about mitochondrial water extrusion in the presence of ATP, it has been found that half a microgram of the purified C-factor protein per milliliter of test medium will produce half maximal stimulation of ATP-induced water extrusion from GSH swollen mitochondria. The biophysical characteristics of the water extrusion are now being investigated.

TABLE III

Distribution of C-factor activity in subcellular fractions of rat liver and other rat tissues

Fractionation of tissue homogenates by the method of Hogeboom, Schneider and Palade[12]. The activity of the soluble fraction was assayed directly; that of the mitochondrial fraction after sonic treatment and clarification by centrifugation (100,000 × *g* for 60 min).

	Units C-factor/mg soluble protein	
	Mitochondria	Hyaloplasm (soluble)
Liver	4·5	6·0
Kidney	4·5	3·0
Heart	1·5	2·5
Skeletal muscle	1·5	0·0
Brain	1·0	0·0

Studies on the distribution of C-factor activity in various tissues of different species, performed together with T. H.. Rose, showed that

C-factor activity could be detected in sonic extracts from kidney, heart, testis, skeletal muscle and brain mitochondria (Table III). Rat liver mitochondria contain the largest amount of sonic-extractable activity and brain mitochondria least. But C-factor activity was also found in the soluble extra-mitochondrial fraction of rat liver, kidney, heart and other tissues. While some of this extra-mitochondrial C-factor may have arisen by loss from the mitochondria, the rather high specific acitvity and the large amounts present suggest much of it is normally present in the hyaloplasm.

A significant activity could be detected in hemolysates of erythrocytes. We have been able to show that C-factor activity can also be liberated from the washed erythrocyte membranes, and brought to a high state of purity. These findings suggest that C-factor is located in other membranes than those of mitochondria and may play some unique role in such structures.

TABLE IV

Effect of glutathione and disulfide hormones on mitochondrial water uptake

Water uptake is expressed as decline of optical density at 50 min, corrected for control.

	Concentration mM	Water uptake ($\Delta OD \times 10^3$)
GSH	1·0	10
GSSG	1·0	20
GSH + GSSG	Combined	250
Insulin	0·008	0
GSH	1·0	15
Insulin + GSH	Combined	130
Vasopressin	0·02	10
GSH	0·002	0
Vasopressin + GSH	Combined	120
Oxytocin	0·02	10
GSH	0·3	5
Oxytocin + GSH	Combined	115

The effect of some enzyme inhibitors and drugs on mitochondrial water uptake and extrusion may now be surveyed. Certain thiols as well as certain disulfides may cause mitochondrial swelling. But we have found that *combinations* of a thiol and a disulfide, such as GSH + GSSG, are far more effective in causing swelling than either tested singly, as is shown in Table IV. Furthermore, water uptake and extrusion depend critically on the ratio of — SH and — S — S —[7,8]. A thiol-disulfide interchange reaction in the mitochondrial membrane,

initiated by the added thiol-disulfide system, may take place, leading to increased permeability.

It is of interest that the uptake of water by the mitochondria is not only induced by simple disulfides such as GSSG, but also occurs in the presence of some high molecular weight peptide hormones containing disulfide groups, such as insulin, and highly purified vasopressin and oxytocin (Table IV). The action of these hormones can also be greatly potentiated by the presence of thiols such as GSH[9]. Although the mitochondrial membranes are not necessarily the normal physiological site of action for all these hormones, and the concentrations necessary to produce swelling are rather high, these findings are nevertheless interesting, since the action of each of these hormones is currently thought to be located at certain membrane sites. Actually recent work by Fong *et al.*[10] has shown that vasopressin might act by forming a mixed disulfide with the receptor membrane. The mitochondrial membranes may prove to be a convenient model for studying such interactions between certain drugs or hormones and membranes.

TABLE V

Effect of some drugs on mitochondrial water uptake and extrusion

	Conc. (mM)	Water uptake	Water extrusion
Hg^{++}	0·01	Promotion	Inhibition
PCMB	0·1	Promotion	Inhibition
NEMI	0·1	Promotion	Inhibition
Oxophenarsine	0·05	Promotion	Inhibition
DOCA	0·02	Promotion	No effect
Prednisolon	0·02	Promotion	No effect
Chlorothiazide	3·0	No effect	No effect

The water extrusion through the mitochondrial membrane is also inhibited by sulfhydryl reagents (Table V). Both inorganic mercury ions and a large variety of organic mercury compounds show this inhibition. This fact is interesting since some of these substances are effective diuretics. However we have found that mercury compounds with diuretic activity are not more effective in our test system than non-diuretics such as PCMB[11]. Other substances which combine with sulfhydrol groups, such as N-ethylmaleimide, are also very potent swelling agents and inhibit the ATP-induced water extrusion from mitochondria[11]. There was on the other hand no inhibitory effect with some other classes of diuretics tested, such as chlorothiazide, and no significant effects with steroid hormones such as DOCA or prednisolon. We are now attempting to localize the action of the active compounds, especially

TABLE VI

Effect of respiratory chain inhibitors and uncoupling agents on mitochondria water uptake and extrusion

The inhibition of the ATP-dependent water extrusion was tested with GSH-swollen mitochondria in the presence of C-factor.

	Conc. (mM)	Water uptake	Water extrusion
Antimycin A	1 μg/ml	Inhibition	No effect
Cyanide	1.0	Inhibition	No effect
2,4-Dinitrophenol	0.02	Promotion	No effect
Dicumarol	0.02	Promotion	No effect
Azide	0.5	Promotion	Inhibition
Gramicidin	0.001	Promotion	Inhibition

those inhibiting the ATP-induced water extrusion, which is apparently brought about by enzymes of oxidative phosphorylation, one of which may be C-factor.

For the uptake of water by the mitochondria, electron transport through the respiratory chains in the membranes is necessary. On the other hand there seems to be no need for electron transport during the ATP-driven water extrusion from the mitochondria, but reversal of the water uptake is strongly inhibited by azide and gramicidin, as shown in Table VI. Azide and gramicidin are known to block one of the terminal steps of oxidative phosphorylation. On the other hand the point

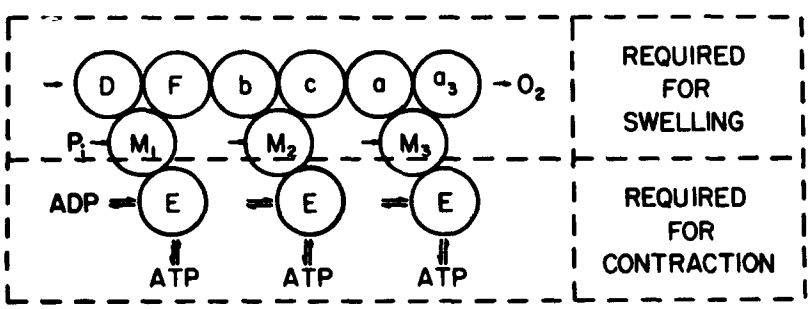

FIG. 2. Hypothetical scheme showing the parts of the respiratory and phosphorylating chain involved in mitochondrial water uptake and in ATP-driven water extrusion ("contraction").

of action of 2,4-dinitrophenol, a compound which exhibits no inhibitory effect on ATP-linked water extrusion, is currently belived to be located at or near the respiratory chain carriers in the coupling sequence. These relationships are shown in Fig. 2.

It has been proposed by Lehninger[4] that one or more of the enzymes involved in the energy-trapping process of oxidative phosphorylation

may act as a "mechonoenzyme" and may alter the structure of the membrane depending on the dynamic state of the respiratory chain. C-factor could be such an enzyme. In agreement with this view are experiments performed together with Dr. C. T. Gregg in which we have been able to stimulate the P/O ratio of a damaged mitochondrial fragment preparation quite considerably by addition of C-factor. Further studies will show how specific this effect is. On the other hand the occurrence of large amounts of C-factor activity in the hyaloplasm suggests the alternative possibility that C-factor may be located on a side path of oxidative phosphorylation.

Isolation of C-factor in a fairly pure state should make it possible to determine more directly its significance in mitochondrial and cellular physiology, and to examine more fully the pharmacologic action of certain drugs and hormones on water balance in mitochondria.

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