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The Impairment of Mitochondrial Function by Triton X-100. A Study of Mitochondrial Respiration and Energy-Dependent Swelling

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Coupled and uncoupled respiration, and energy-dependent phosphate swelling have been studied in rat liver mitochondria in the presence of various concentrations of Triton X-100. Detergent concentrations up to 10^{-5} M do not affect any of the processes under study. At 10^{-5} M, Triton X-100 produces a slight decrease of coupled respiration and a considerable inhibition of mersalyl-induced shrinking in swollen mitochondria. Increasing the surfactant concentration to 10^{-4}, coupled as well as uncoupled O_2 consumption is decreased, succinate-dependent phosphate swelling is inhibited and an energy-dependent phosphate swelling in the absence of valinomycin is observed. At 2×10^{-4} M. Triton X-100, ATP- dependent phosphate swelling is abolished, and passive swelling may be induced by various ions. Higher detergent concentrations do not allow observation of any of these events. On the basis of these results, a model of membrane-detergent interaction is proposed.

Detergents are widely used in membrane research. However their mechanism of action in the solubilization of membrane lipids and proteins is not fully understood yet (9). A better knowledge of this mechanism would open possibilities for the synthesis of more efficient or more selective surfactant molecules. It would also help to understand biological phenomena in which amphipathic molecules are probably involved, such as membrane fusion (11) or cell lysis by melittin (6) or similar peptides.

Much of the effort in this area has been devoted to elucidate the effect of detergents on the structure of cell membranes, and the solubilization of membrane components (9). Previous work from this laboratory, especially on mito-

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chondria and model membranes, has pointed in that direction (1, 7). However, we consider that the study of alteration of membrane function as a result of detergent treatment could give a further insight into the interaction of biomembranes with soluble amphiphiles, each particular function acting as an independent «probe» of detergent action.

In the present paper we study the effect of the non-ionic detergent Triton X-100 on mitochondrial respiration and energy-dependent swelling. This particular detergent was chosen because of its mild effects on enzyme activity, and also because of our previous studies of its effects on mitochondrial structure (2, 4). On the other hand, respiration and swelling are two easily detectable manifestations of mitochondrial physiology.

Materials and Methods

Male Wistar albino rats (weighing about 100 g) were allowed to starve overnight, with water *ad libitum*. They were killed by decapitation, and their livers quickly excised. Mitochondria were obtained by the method of HOGEBOOM (10), and washed twice in a buffer containing bovine serum albumin (0.2 mg/ml), 0.25 M sucrose, 0.1 mM EDTA, 10 mM Tris-HCl, pH 7.6, after which they were resuspended in the same buffer at a concentration of about 20 mg protein/ml.

For oxygen electrode measurements, the mitochondrial suspension was diluted when required to a final concentration of 1 mg protein/ml in an assay medium containing 75 mM KCl, 10 mM Tris-HCl (pH 7.0), 2 mM potassium phosphate, 3 mM $MgCl_2$, 0.5 mM EDTA, 1 μM rotenone, and bovine serum albumin (1 mg/ml). Mitochondrial respiration was measured in a Clark-type oxygen electrode (Rank Bros., Bottisham, U.K.) at 37° C, with 10 mM succinate as a substrate. When required, Triton X-100 was added to the mitochondrial suspension and allowed to equilibrate for 1 min before performing the experiments. Other reagents were also added when required: CCCP (2 μM), antimycin A (3 μg/ml), oligomycin (3 μg/ml), mersalyl (50 μM), 2-mercapthoetanol (0.5 mM), ADP (150 M), CN^- (2.5 mM) and sodium azide (5 mM).

Changes in mitochondrial volume were monitored as changes in turbidity (absorbance at 546 nm) at 37° C against a cell placed in the reference path with its ground-glass surface facing the light beam. Measurements were carried out in a UV 5260 Beckman spectrophotometer. A dilute mitochondrial suspension was used as above, except that the 75 mM KCl substituted by 230 mM sucrose and 2 mM KCl, and that the incubation medium contained also 250 ng/ml valinomycin. Triton X-100 and the other detergents were used as indicated above. When required, ATP (sodium salt) was added to a 1 mM final concentration.

Results

The polarographic record of our mitochondrial preparations during succinate-maintained respiration is shown in figure 1. Under these conditions, respiratory control indexes had values around 8. The effects of Triton X-100 on oxygen consumption by coupled and uncoupled mitochondria are also shown in figure 1. Surfactant concentrations below 10^{-5} M did have not any effect on the process: 10^{-5} M Triton X-100 produces a slight decrease in coupled respiration (about 20 %); while oxygen consumption in the presence of CCCP does not seem to be affected at this stage. However, when Triton X-100 concentration is raised to 10^{-4} M, oxygen consumption of coupled as well as of uncoupled mitochondria is greatly decreased, and becomes virtually zero at 2×10^{-4} M detergent. The same

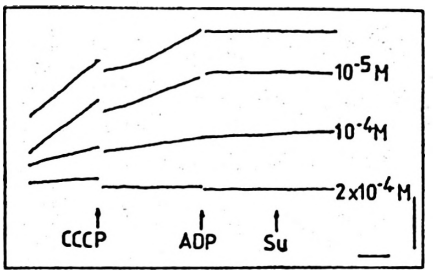

Fig. 1. *Polarographic record of mitochondrial coupled and uncoupled oxygen consumption, and the effect of increasing concentrations of Triton X-100.*
The horizontal and vertical bars correspond respectively to 20 s and 0.12 μmoles O_2.

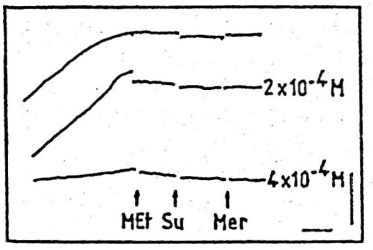

Fig. 3. *Polarographic record of mitochondrial oxygen consumption in the presence of mersalyl and mercaptoethanol, and the effect of increasing concentrations of Triton X-100.*
Scales as in figure 1.

phenomenon of suppression of O_2 uptake by uncoupled mitochondria at surfactant concentration around $10^{-4} - 2 \times 10^{-4}$ M is observed in the presence of oligomycin (fig. 2). Triton X-100 did not affect in any way the suppression of succinate-dependent respiration by inhibitors such as azide, cyanide, antimycin and mersalyl (data not shown). The inhibitory effect of mersalyl may be reversed by mercapthoethanol, which in turn seems to act also as uncoupler. Oxygen consumption is uncoupled mitochondria in the presence of mersalyl and mercapthoethanol is abruptly suppressed by Triton X-100 concentrations higher than 2×10^{-4} M (figure 3).

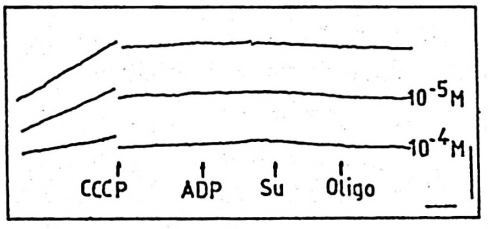

Fig. 2. *Polarographic record of mitochondrial uncoupled oxygen consumption in the presence of oligomycin, and the effect of increasing concentrations of Triton X-100.*
Scales as in figure 1.

The variation of suspension turbidity as a function of Triton X-100 concentration is shown in figure 4. It can be seen that concentrations lower than 10^{-4} M scarcely produce any decrease in turbidity whereas 4×10^{-4} M surfactant causes the mitochondrial suspension to become nearly transparent.

Phosphate-dependent swelling requires an energy source and an ionophore to facilitate the diffusion of the counterion (in this case K^+). In our experiment, the ionophore was valinomycin, while the energy source was succinate. Mitochon-

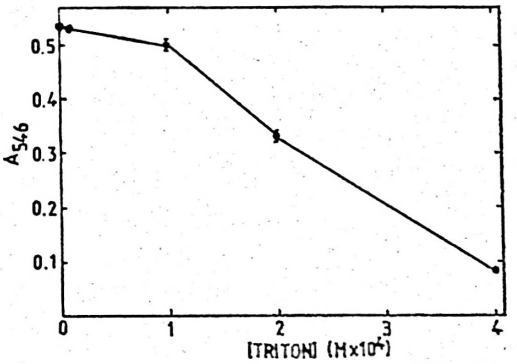

Fig. 4. *The turbidity (A_{546}) of a mitochondrial suspension (1 mg protein/ml) as a function of detergent concentration.*
Points correspond to mean values ± S.E.M. (n = 5).

dria were resuspended in the assay buffer, as described under Methods, and valinomycin was added to the suspension. Preliminary experiments showed that valinomycin addition did not produce any change in turbidity, so that recording was usually started immediately before succinate addition. Figure 5 depicts mitochondrial swelling induced by succinate, and its reversal by the uncoupler CCCP, as well as the effects of Triton X-100 on this process. Both swelling and contraction are affected by 10^{-5} M surfactant, and nearly totally suppressed at 10^{-4} M. It must be said in this context that although in the particular experiment reported in figure 5 an inhibition of swelling is already seen at 10^{-5} M Triton X-100, this is not a constant feature of our preparations; on the other hand, 10^{-4} M detergent always inhibits phosphate swelling. The reason of the variations observed with 10^{-5} M detergent is not clear at present.

As expected, phosphate-dependent swelling was inhibited by mersalyl, and this inhibition reversed by mercaptho-

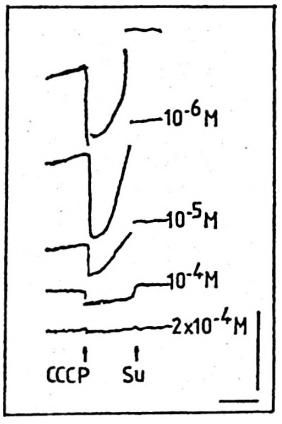

Fig. 5. *Succinate-dependent phosphate swelling and its reversal by CCCP. Effect of increasing concentration of Triton X-100.*
The horizontal and vertical bars correspond respectively to 30 s and 0.05 absorbance units.

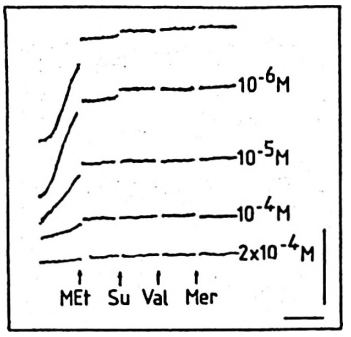

Fig. 6. *Inhibition of phosphate swelling by mersalyl and its reversal by mercapthoethanol. Effect of increasing concentrations of Triton X-100.*
Scales as in figure 5.

ethanol. It can be seen (fig. 6) that phosphate swelling in the presence of mersalyl and mercapthoethanol is affected by 10^{-5} M and totally suppressed above 10^{-4} M Triton X-100. (Note that, in these experiments, mersalyl was added prior to valinomycin). Being phosphate swelling an energy dependent process, it is totally suppressed by electron-transport inhibitors, such as azide or cyanide. This inhibition was not released by the detergent at any of the concentrations studied (data not shown).

Mersalyl does not only inhibit phosphate swelling, but also induces contraction when added to swollen mitochondria. As shown in figure 7, swelling may again be produced in these mersalyl-treated mitochondria by uncouplers, such as CCCP. In the same figure it may be seen that mersalyl concentration and subsequent CCCP swelling are severely inhibited by 10^{-5} Triton X-100, and totally suppressed at higher surfactant concentrations; this particular phenomenon is thus more sensitive to the detergent than swelling primarily induced by phosphate. On the other hand, energy for the swelling process can be provided by ATP, even in uncoupled mitochondria. As shown also

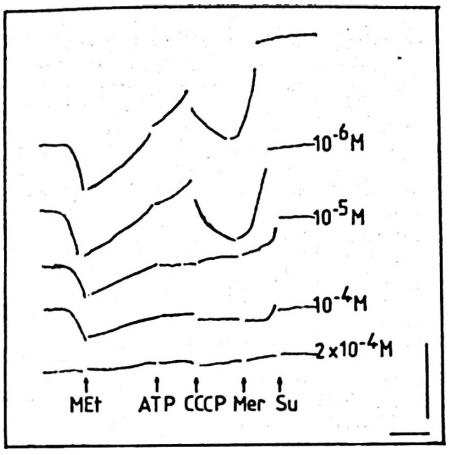

Fig. 7. *Reversal of succinate-dependent phosphate swelling by mersalyl, and ATP-dependent phosphate swelling. Effect of increasing concentrations of Triton X-100.*
Scales as in figure 5.

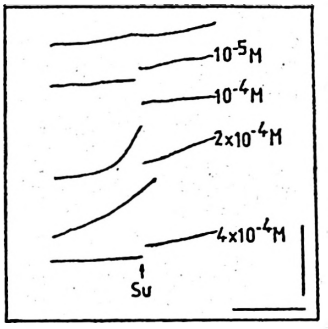

Fig. 8. *Succinate-induced phosphate swelling in the absence of valinomycin. Effect of increasing concentrations of Triton X-100.*
Scales as in figure 5.

in figure 7, ATP induces further swelling in mersalyl-CCCP swollen mitochondria, and its effect is reversed by mercaptoethanol. ATP-dependent swelling and its reversal seem to be quite resistant to detergent action since they are severely inhibited at Triton X-100 concentrations up to 10^{-4} M.

Apart from the inhibitory effects that have been stressed up to now, Triton X-100 could have some facilitating effects on swelling, due to its ionophoric properties. In order to explore this possibility, we repeated some of the previously mentioned phosphate swelling experiments in the absence of valinomycin. The corresponding results are summarized in figure 8. It can be seen that, in the absence of the ionophore, the addition of succinate does not elicit any swelling response unless in the presence of 10^{-4} or 2×10^{-4} M Triton X-100. Higher surfactant concentrations do not allow the observation of any swelling process. Note that, in this case, CCCP does not reverse the phosphate swelling in the presence of succinate.

Phosphate-swelling with Triton X-100 instead of valinomycin could still be an energy-dependent process or just a passive phenomenon due to the increased permeability of the detergent-containing membranes. This possibility was checked by performing the above experiments in the

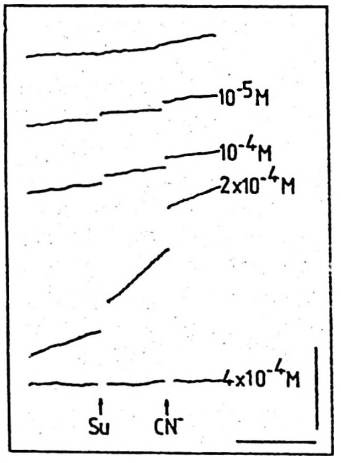

Fig. 9. *Effect of cyanide on succinate-induced phosphate swelling in the absence of valinomycin. Effect of increasing concentrations of Triton X-100.*
Scales as in figure 5.

Table I. *Summary of the main effects of Triton X-100 on mitochondria, including membrane solubilization and impairment of mitochondrial function.*
Data of percent solubilization of protein and lipid phosphorus are taken from (2).

Triton X-100	% solubilized		Detergent effect
	Protein	Lipid P	
Control	15	n.d.[a]	—
10^{-6} M	16	n.d.	n.d.
10^{-5} M	23	n.d.	— slight decrease of coupled respiration — decrease of succinate-dependent phosphate swelling — great inhibition of mersalyl shrinking in phosphate swollen mitochondria
10^{-4} M	25	n.d.	— decreased coupled and uncoupled O_2 consumption — great inhibition of succinate-dependent phosphate swelling — partial inhibition of ATP-dependent phosphate swelling — induction of energy-dependent phosphate swelling in the absence of valinomycin
2×10^{-4} M	27	8	— great inhibition of ATP-dependent phosphate swelling — induction of passive ion diffusion and swelling
4×10^{-4} M	46	16	— no manifestation of mitochondrial activity is seen.

[a] None detected.

presence of electron-transport inhibitors. The result obtained in the presence of cyanide can be seen in figure 9. Similar observations could be made in the presence of sodium azide (data not shown). With such inhibitors, phosphate swelling in the presence of 10^{-4} M Triton X-100 was entirely inhibited, whereas the effects observed at 2×10^{-4} M remained unchanged. This shows that, in the first case, but not in the second, energy-dependent swelling took place.

In the absence of valinomycin, mitochondria treated with mersalyl and CCCP can undergo a swelling cycle, respectively in the presence of ATP and mercaptoethanol, exactly as they did when valinomycin was added (fig. 7). As in the previous case, this swelling and contraction are not inhibited by the presence of at least 2×10^{-4} M Triton X-100 (data no shown).

Discussion

From the results sumarized in table I, it is apparent that the effects of Triton X-100 on mitochondrial respiration and swelling take place in the range of detergent concentrations between 10^{-5} M and 2×10^{-4} M; these concentrations correspond roughly to detergent:lipid molar ratios of 1:500 and 1:25 respectively. Below 10^{-4} M, although the detergent will most probably bind to the membrane (9), no structural or functional effect is observed: the amount of mitochondrial protein «solubilized» is the same in the absence of detergent, and corresponds probably to matrix or peripheral proteins. On the other hand, above 2×10^{-4} M, the mitochondrial suspension becomes optically clear (fig. 4), a substantial proportion of the membrane phospholipids are solubilized, the bilayer structure is severely altered (2), and no manifestation of mitochondrial activity is apparent.

Between these two extremes, each of the three detergent concentration studied constitutes a step towards functional impairment. At 10^{-5} M surfactant, there is some inhibition of coupled respiration, whereas in the presence of CCCP, O_2

consumption is not altered; this shows that one of the first effects of the detergent takes place at the F_1-ATPase, thereby inhibiting coupled respiration (fig. 1). The ATP-phosphohydrolase activity of the enzyme is not affected at those detergent concentrations (7). But the most notorius effect of 10^{-5} M Triton X-100 is the inhibition of mersalyl shrinking in phosphate-swollen mitochondria (fig. 7). Swollen mitochondria are known to contract in the presence of CCCP, due to the lack of energetic support caused by the uncoupler; CCCP shrinking is diminished albeit not dramatically, by 10^{-5} M Triton X-100. Mersalyl shrinking, on the other hand, is probably due to an increase in K^+/H^+ exchange between the mitochondrial matrix and the medium (3), the K^+ outflow being accompanied by phosphate and water. The precise molecular mechanism of mersalyl stimulated K^+/H^+ exchange is not known (2). Some authors have pointed out that, in addition to its effect of phosphate carriers, mersalyl could act through other ways (5). Our results indicate that mersalyl shrinking is differently affected by detergents than CCCP shrinking. If this implies than both shrinking processes operate through different mechanisms, it would give support to the hypothesis of DIWAN *et al.* (5).

Among the most noteworthy events taking place in the presence of 10^{-4} M Triton X-100, we can note three; the decrease in uncoupled O_2 consumption (figure 1), the inhibition of succinate-dependent phosphate swelling (fig. 5), and the induction of phosphate swelling in the absence of valinomycin (fig. 8). All these observations may be rationalized as the result of a single effect of the detergent on the mitochondrial membrane lipid bilayer, namely the conversion of part of the lipids into lipid-detergent mixed micelles. Note, however, that these micelles remain embedded in the bilayer, since no phospholipid solubilization is observed. Micelle formation would be expected from the presence of detergent at sub-lytic concentrations (12). As a result of this reduction in bilayer area, the lateral and rotational diffusion of the electron transport chain functional complexes in the inner membrane (8) would be reduced, and consequently the mitochondrial consumption of O_2 would be decreased. The partial inhibition of the respiratory chain would lead to a decrease in the energy-dependent phosphate swelling. Finally, the presence of micelles in the lipid bilayer would increase the ionic permeability of the membrane, and thus phosphate swelling would occur even in the absence of valinomycin. The «ionophoric» properties of Triton X-100 have been described previously (13). This effect of valinomycin-independent phosphate swelling requires the operation of the electron transport chain (fig. 9), that is still working, though partially inhibited by the detergent. This also shows that the ionophoric properties of Triton X-100 exhibit a certain degree of selectivity: DE ROBERTIS *et al.* had already shown that K^+ was one of the most easily translocated cations (13).

When the concentration of Triton X-100 is raised from 10^{-4} M to 2×10^{-4} M, a significant event happens, i.e., the solubilization of some membrane phospholipids. This means the disruption of the bilayer architecture. Electron micrographs confirm (2) that, although the overall bilayer structure is preserved, the «trilaminar image» is seriously altered. This agrees with our observations of passive swelling induced by ions under these conditions (fig. 8 and 9); the mitochondrial membranes constitute still a barrier for water permeation, but succinate and other normally impermeant ions (e.g. CN^-) can move freely across it. On the other hand, ATP-dependent phosphate swelling is also severely inhibited at this surfactant concentration (fig. 7). This can also be explained by the collapse of electrochemical potential across the membrane bar-

rier revealed by the passive diffusion of ions (fig. 8 and 9). However, ATP-dependent phosphate swelling appears to be more resistant to surfactant action than its succinate-dependent counterpart, obviously because the ATP-dependent phenomenon does not rely on the integrity of the electron transport chain for the creation of an electrochemical gradient.

Acknowledgements

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Resumen

Se estudia la respiración acoplada y desacoplada y la tumefacción por fosfato dependiente de energía en mitocondrias de hígado de rata, en presencia de distintas concentraciones de Triton X-100. Las concentraciones de detergente menores de 10^{-5} M no afectan a ninguno de los procesos estudiados. El Triton X-100 10^{-5} M produce un ligero desacoplamiento y una considerable inhibición de la contracción inducida por el mersalil en mitocondrias tumefactas. Al aumentar la concentración de surfactante hasta 10^{-4} M desciende el consumo de O_2 tanto en mitocondrias acopladas como en desacopladas, se inhibe la tumefacción por fosfato dependiente de succinato y se observa una tumefacción por fosfato no dependiente de energía en ausencia de valinomicina. Con Triton X-100 2×10^{-4} M se suprime la tumefacción por fosfato dependiente de ATP, y se observa tumefacción pasiva inducible por diversos iones. Concentraciones más elevadas de detergente no permiten la observación de ninguno de estos fenómenos. Basándose en estos resultados, se propone un modelo de interacción membrana-detergente.

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