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ON THE IMPERMEABILITY OF THE OUTER MITOCHONDRIAL MEMBRANE TO CYTOCHROME *c*

I. STUDIES ON WHOLE MITOCHONDRIA

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SUMMARY

The effect of digitonin on the release from rat-liver mitochondria of adenylate kinase (EC 2.7.4.3) and malate dehydrogenase (EC 1.1.1.37) and on the unmasking of activities of cytochrome oxidase (EC 1.9.3.1) and succinate–cytochrome *c* reductase with external cytochrome *c* was studied. Electron micrographs of digiton-in-treated mitochondria were also taken.

The results indicate that the unmasking of mitochondrial enzyme activities with exogenous cytochrome *c* goes parallelly with the release of adenylate kinase and the detachment of the outer mitochondrial membrane but does not require the opening of the inner membrane.

INTRODUCTION

It has been postulated (*cf.* ref. 1) that the outer mitochondrial membrane is freely permeable to small molecules but is impermeable to compounds of high molecular weight. This assumption was supported more recently by studies on the accessibility of the mitochondrial space to substances of various molecular weight[2,3]. The present investigation provides some more direct evidence for the impermeability of the outer mitochondrial membrane to cytochrome *c*. It is based on the study of the effect of various concentrations of digitonin on the unmasking of cytochrome oxidase (EC 1.9.3.1) and of the succinate–cytochrome *c* reductase system measured with external cytochrome *c*, as correlated with the removal of the outer mitochondrial membrane. The use of digitonin to detach the outer mitochondrial membrane has been described by LÉVY *et al.*[4,5] and SCHNAITMAN *et al.*[6] and SCHNAITMAN AND GREENAWALT[7]. The present paper also gives some additional information on the effect of digitonin on the structure of mitochondrial membranes.

MATERIAL AND METHODS

Rat-liver mitochondria were obtained by the standard procedure[8]. Digitonin treatment was carried out according to SCHNAITMAN AND GREENAWALT[7] with small modifications. Mitochondria were suspended in 250 mM sucrose containing 2 mM Tris–

HCl buffer (pH 7.4) and 0.05 % serum albumin. To this suspension, containing 30–50 mg mitochondrial protein per ml, various amounts of a 2 % solution of digitonin in the same medium were added, and the resulting mixtures were incubated for 15 min at 0°. Then, the suspensions were diluted with 3 vol. of the suspension medium (not containing digitonin), and aliquots were taken for the determination of cytochrome oxidase and succinate–cytochrome *c* reductase. The remainder was centrifuged for 15 min at 15000 × *g*, and the resulting supernatants were used for the determination of adenylate kinase (EC 2.7.4.3) and malate dehydrogenase (EC 1.1.1.37). The pellets were fixed for electron microscopy. Appropriate controls were also run in the absence of digitonin. 'Full activity controls' were made by solubilizing mitochondria with the nonionic detergent Lubrol[7] (I.C.I. Organics, Providence, R.I.). No centrifugation was carried out on these samples.

Digitonin was recrystallized from ethanol and freshly dissolved before use.

Oxidation of ferrocytochrome *c* was measured spectrophotometrically in either 250 mM sucrose or 125 mM KCl containing the following additions: 10 mM Tris–HCl buffer (pH 7.4), 1 mM P_i and 1 μM carbonyl cyanide *m*-chlorophenylhydrazone. The velocity constants were calculated as described by SMITH[9]. Ferrocytochrome *c* was obtained by reducing commercial cytochrome *c* with ascorbate and by removing the reductant by dialysis[10] or gel filtration[11].

The activity of cytochrome oxidase with endogenous cytochrome *c* was determined polarographically using a Clark-type oxygen electrode (Yellow Springs Instruments Co., Yellow Springs, Ohio), and ascorbate *plus* *N,N,N',N'*-tetramethyl-*p*-phenylenediamine as electron donors.

Succinate–cytochrome *c* reductase was measured spectrophotometrically in the same media as used for the oxidation of ferrocytochrome *c* which contained, in addition, 3 mM succinate, 1 mM KCN and 1 μM rotenone (ferricytochrome *c* being substituted for ferrocytochrome *c*).

Malate dehydrogenase was determined with NADH and oxaloacetate as substrates[12].

Adenylate kinase was measured in a system containing ADP, $NADP^+$, hexokinase (EC 2.7.1.1) and glucose-6-phosphate dehydrogenase (EC 1.1.1.49) in the medium described by SILVA LIMA *et al.*[13].

Electron microscopy. Mitochondrial pellets obtained after digitonin treatment were fixed with 1 % OsO_4 (phosphate buffered to pH 7.4) during 1 h at 0°, dehydrated in ethanol, embedded in Epon, sectioned with Reichert OMU 2 ultramicrotome, stained with lead[14] and examined with JEM 7 electron microsope.

RESULTS AND DISCUSSION

As shown in Fig. 1, the release of adenylate kinase and the unmasking of cytochrome oxidase approximately parallel each other as plotted *versus* digitonin concentration, although the release of adenylate kinase usually occurs at a slightly lower digitonin concentration than the unmasking of cytochrome oxidase. Experiments with succinate–cytochrome *c* reductase, as exemplified in Fig. 2, also reveal an unmasking of this enzyme activity which proceeds essentially as that of cytochrome oxidase. If it is assumed that adenylate kinase is associated with the outer mitochondrial membrane or with the intermembrane space[7,13,15–18], it should be concluded that the un-

masking of enzymes reacting with exogenous cytochrome *c*, both in the reduced and the oxidized form, goes parallel with the breaking or detachment of the outer membrane. No such increase has ever been observed when the activity of cytochrome oxidase with endogenous cytochrome *c* was measured. In fact, a slight decrease in the activity in the presence of digitonin was usually observed (Fig. 3). This indicates that digitonin does not activate cytochrome oxidase as such but rather acts on a permeability barrier for external cytochrome *c*. Another interpretation of the effect of digitonin might postulate a removal of endogenous cytochrome *c*, thus giving a better

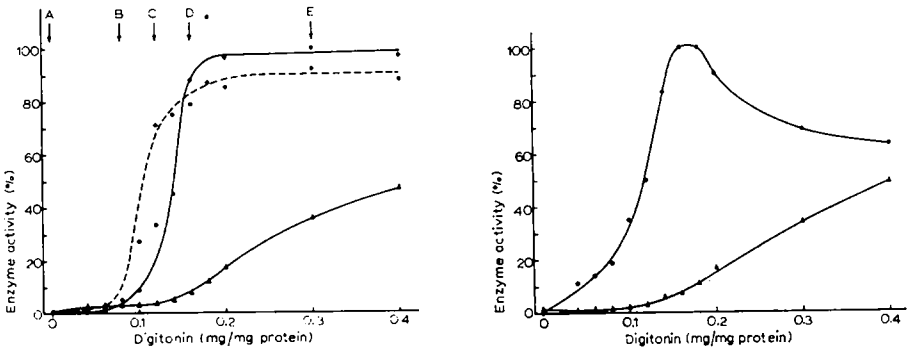


Fig. 1. Effect of digitonin on the release of adenylate kinase and malate dehydrogenase and the unmasking of cytochrome oxidase activity with external cytochrome *c* in rat-liver mitochondria. Sucrose medium. Enzyme activities are expressed as percentage of the activities obtained by solubilization of mitochondria with Lubrol. The following absolute values were obtained (taken as 100%): cytochrome oxidase, velocity constant 1.55 min^{-1}/mg protein; adenylate kinase, 0.20 μmole ATP formed per min per mg protein; malate dehydrogenase, 2.5 μmoles NADH oxidized per min per mg protein. O---O, adenylate kinase; ●——●, cytochrome oxidase; ▲– –▲, malate dehydrogenase. Arrows at the top of the figure indicate samples taken for electron microscopy and shown in Fig. 4. The letters A–E correspond to the lettering of Fig. 4.

Fig. 2. Effect of digitonin on the unmasking of succinate–cytochrome *c* reductase activity with external cytochrome *c* and the release of malate dehydrogenase. Sucrose medium. The activity of succinate–cytochrome *c* reductase is expressed as percentage of the maximum activity obtained after digitonin treatment (when solubilized with Lubrol the activity was 71%). The activity of malate dehydrogenase is expressed in the same way as in Fig. 1. Absolute values (100% activity): succinate–cytochrome *c* reductase, 0.05 μmole cytochrome *c* reduced per min per mg protein; malate dehydrogenase, 2.3 μmoles NADH oxidized per min per mg protein. ●——●, succinate–cytochrome *c* reductase; ▲——▲, malate dehydrogenase.

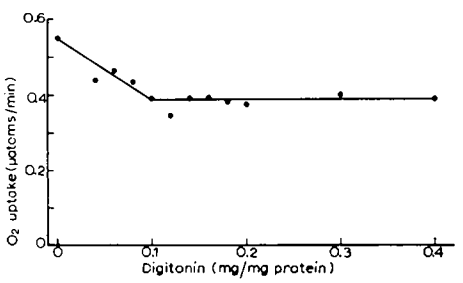


Fig. 3. Effect of digitonin on the activity of cytochrome oxidase with endogenous cytochrome *c*. Incubation medium: 250 mM sucrose, 10 mM Tris–HCl buffer (pH 7.4), 6.7 mM ascorbate (Tris salt), 0.3 mM *N,N,N',N'*-tetramethyl-*p*-phenylenediamine, 1 μM rotenone and mitochondria corresponding to 5.0 mg protein, previously treated with digitonin as described under MATERIALS AND METHODS. Total volume, 3.0 ml; temp., 30°.

access of added cytochrome *c* to sites of cytochrome oxidase and succinate–cytochrome *c* reductase. Such interpretation seems, however, rather unlikely, since there was only a slight diminution of the activity of cytochrome oxidase with endogenous cytochrome *c* (Fig. 3).

The parallellism between the release of adenylate kinase and unmasking of oxidation or reduction of external cytochrome *c* could be observed in both isotonic sucrose and isotonic KCl. Absolute rates of cytochrome *c* oxidation were much higher in 125 mM KCl than in 250 mM sucrose (velocity constants 10–15 min^{-1}/mg protein in KCl *versus* 1.5–3.0 min^{-1}/mg protein in sucrose), due to the marked dependence of cytochrome oxidase on the ionic strength[19]; nevertheless, the degree of stimulation by digitonin was the same in both media.

At 0.16 mg digitonin per mg mitochondrial protein, when about 90 % of adenylate kinase was released from mitochondria, the reactivity of the mitochondria with external cytochrome *c* (both oxidized and reduced) also reached its maximum. At this concentration of digitonin there was, however, only a slight, no more than 10 %, release of malate dehydrogenase (Figs. 1 and 2), an enzyme considered to be located in the compartment enclosed by the inner mitochondrial membrane[7,17,18,20,21]. This suggests that no opening of the inner membrane is necessary to permit the free access of external cytochrome *c* to the active sites of the inner membrane. This is in agreement with the recent postulation of CARAFOLI AND MUSCATELLO[22,23] that cytochrome oxidase and succinate–cytochrome *c* reductase can be accessible to cytochrome *c* at the external surface of the inner membrane.

It is worthwhile mentioning that the minimum concentration of digitonin to produce the release of adenylate kinase and unmasking of cytochrome oxidase and succinate–cytochrome *c* reductase in the present investigation agrees fairly well with that to detach the outer mitochondrial membrane reported by SCHNAITMAN and coworkers[6,7].

Further increase in the concentration of digitonin from 0.16 up to 0.4 mg per mg protein results in no further change in the release of adenylate kinase and of unmasking of cytochrome oxidase but produces a release of malate dehydrogenase, indicating a gradual damage of the inner membrane. The decrease in succinate–cytochrome *c* reductase activity at higher digitonin concentrations (Fig. 2) may also be a reflection of the same phenomenon.

Electron micrographs of digitonin-treated mitochondria support the view that the unmasking of enzyme activities with external cytochrome *c* is associated with the destruction of the outer mitochondrial membrane. Mitochondria preincubated without digitonin mostly retain their typical 'orthodox' structure (Fig. 4A), whereas increasing concentrations of digitonin increase the degree of disorganization, as already observed by SCHNAITMAN *et al.*[6]. Already 0.08 mg digitonin per mg protein gives a rearrangement of the inner structure, although the outer membrane still seems to be mostly intact (Fig. 4B). This is compatible with the fact that there is only a small release of adenylate kinase at that concentration of digitonin as well as only a slight unmasking of cytochrome *c*-connected enzyme activities (Figs. 1 and 2). At 0.12 mg digitonin per mg mitochondrial protein, a considerable number of discontinuities of the outer membrane can be detected, although the spacial relation between the two membranes is still mostly retained (Fig. 4C and 4C'). To this situation corresponds a substantial release and unmasking of the enzymes (Figs. 1 and 2). An apparently

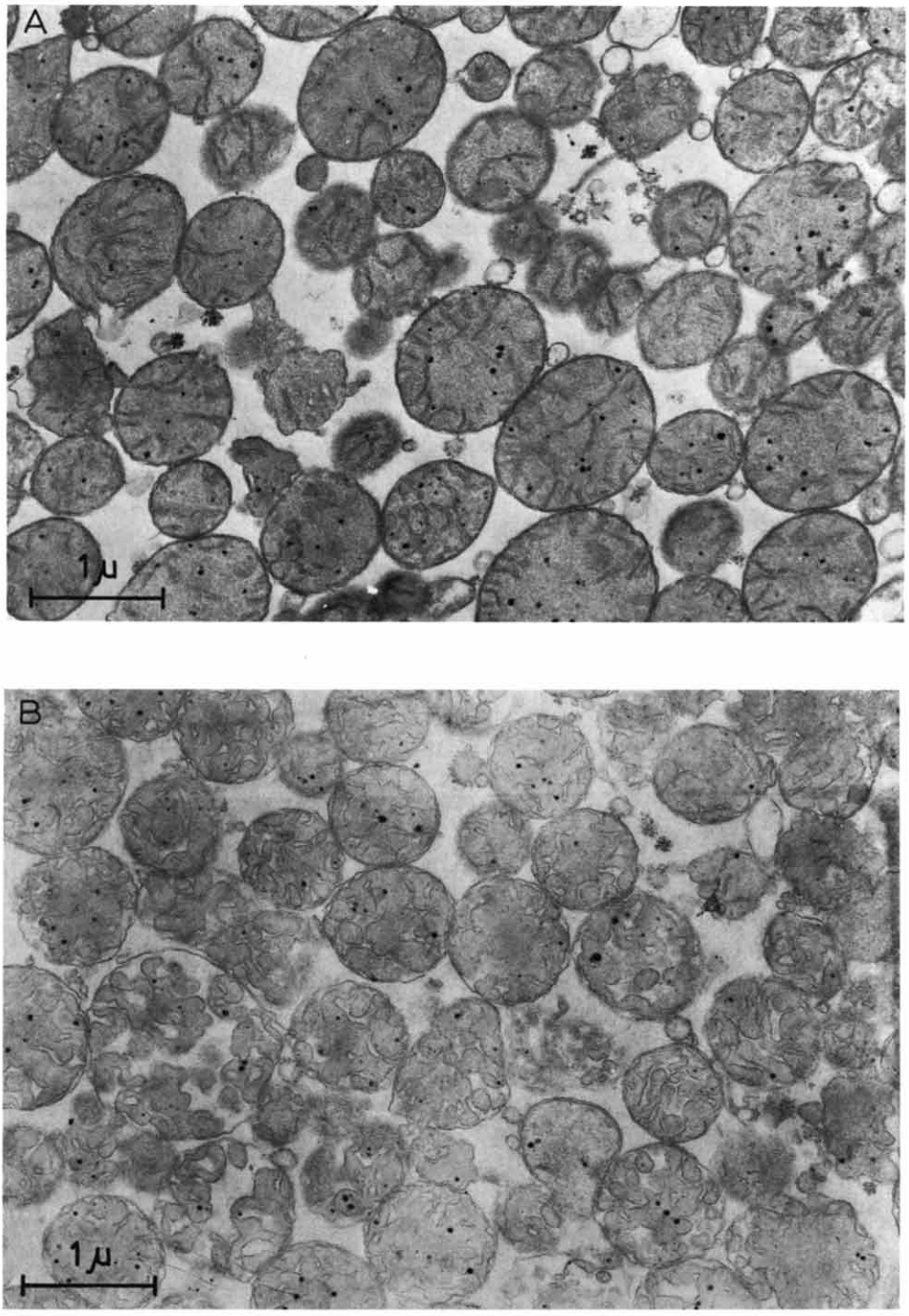
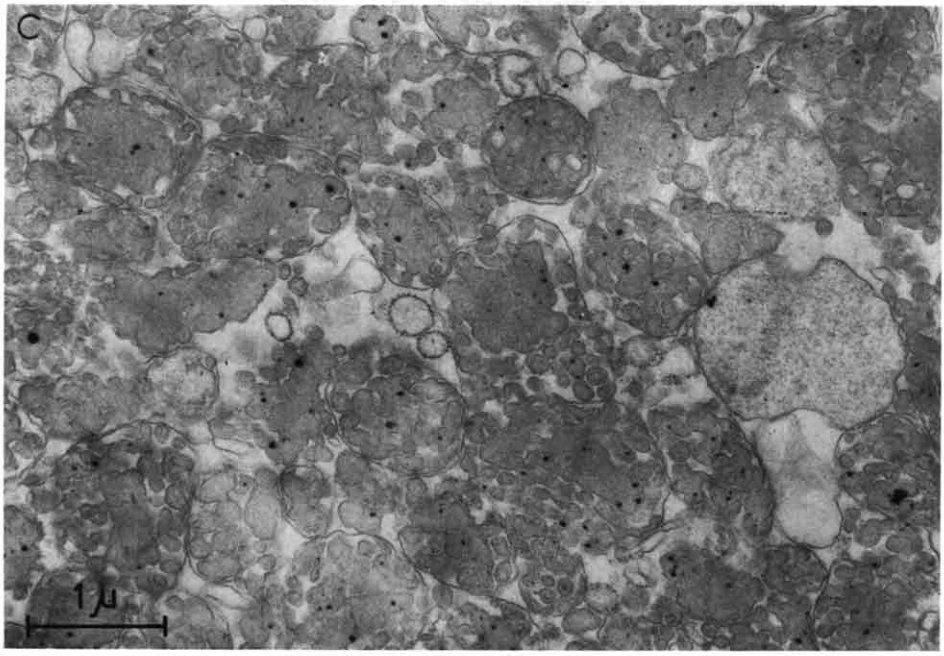


Fig. 4. Electron micrographs of rat-liver mitochondria treated with various amounts of digitonin. Figs. A–E correspond to samples indicated by arrows in Fig. 1. A, no digitonin (control sample); B, 0.08 mg digitonin per mg mitochondrial protein; C, 0.12 mg digitonin per mg protein; C′, same as C, higher magnification; D, 0.16 mg digitonin per mg protein; E, 0.3 mg digitonin per mg protein.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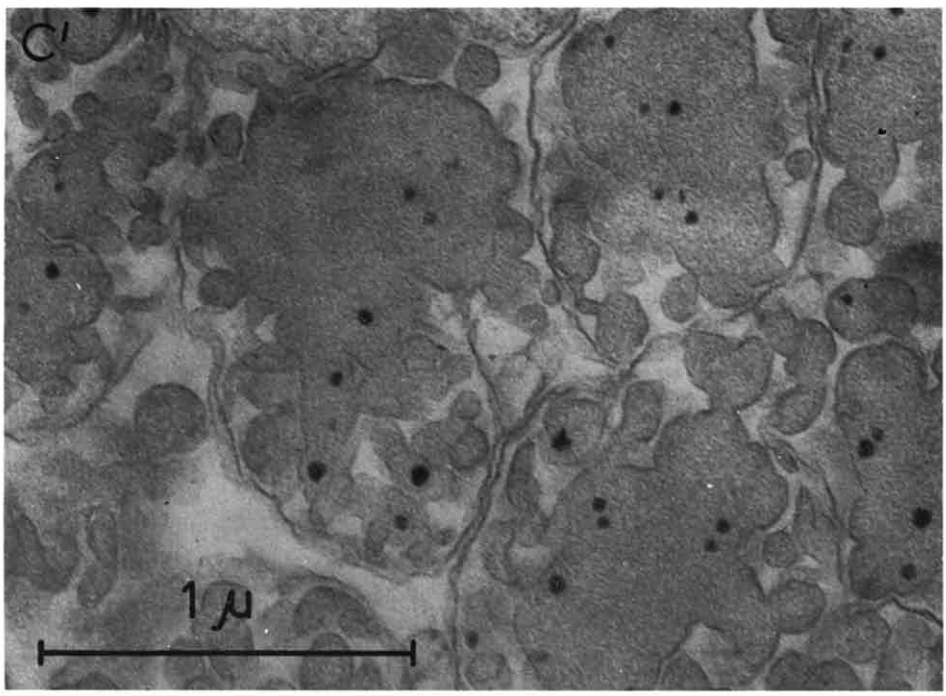
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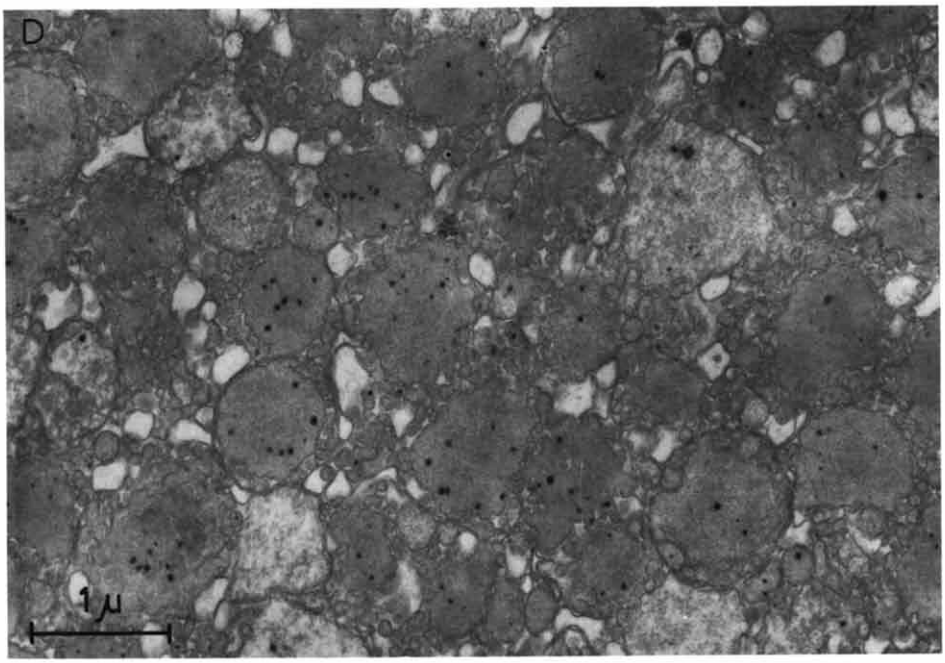
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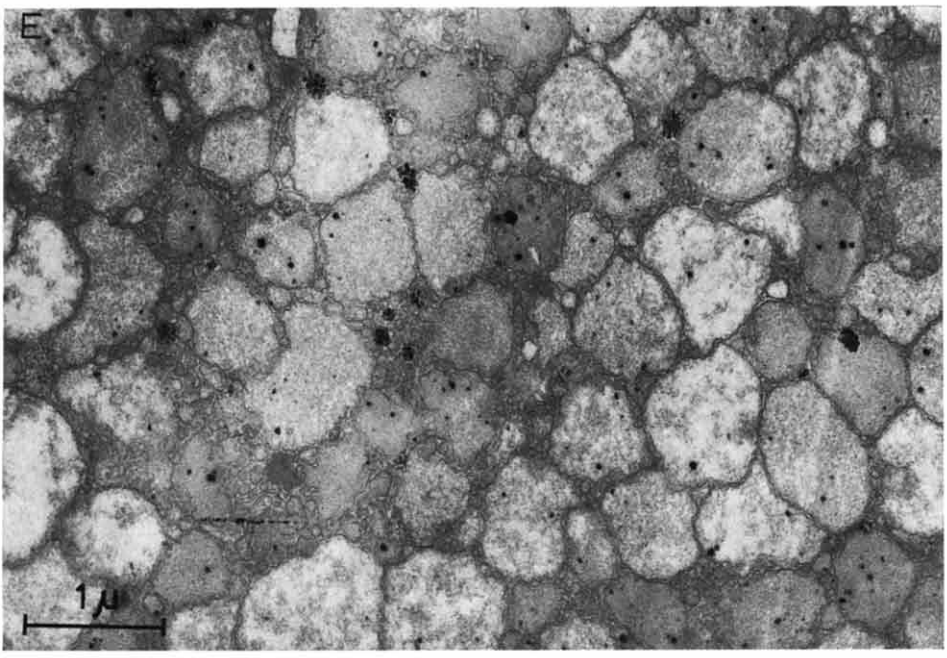
E
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complete opening or removal of outer membranes can be noted at 0.16 mg digitonin per mg protein (Fig. 4D). At this concentration, however, the matrix seems to be still retained inside the inner membrane, as indicated by the electron-opaque appearance of a great part of the particles. Higher concentrations of digitonin result in a leakage of the matrix giving rise to numerous electron-transparent inner membrane ghosts (Fig. 4E). This coincides with a substantial (Figs. 1 and 2) or complete (not shown) release of malate dehydrogenase.

The disruption of mitochondria by digitonin treatment has been recently used[21] to show that enzymes of the citric acid cycle are located in the mitochondrial matrix rather than in the outer membrane as suggested by GREEN and co-workers[24-26]. The present investigation gives an additional support to the view[7,17,18,20] that malate dehydrogenase is located inside the space enclosed by the inner membrane and is not associated with the outer membrane or the intermembrane space.

Morphological changes in mitochondria produced by digitonin as observed in the present study agree fairly well with the pictures shown by SCHNAITMAN *et al.*[6] and by BRDICZKA *et al.*[21] and allow one to follow the effect of varying the concentration of digitonin.

The impermeability of the outer mitochondrial membrane to cytochrome *c* explains the known poor accessibility of intact mitochondria to external cytochrome *c* (*e.g.* refs. 10, 27–29). It also explains why the extraction of cytochrome *c* from mitochondria by salt solutions is effective only when preceded by hypotonic swelling[30], the treatment known to disrupt the outer membrane[31,32].

The next publication of this series[33] will show that the impermeability for cytochrome *c* can also be observed in isolated outer mitochondrial membranes.

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