Relation of mitochondrial phospholipase A activity to mitochondrial swelling

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ABSTRACT Assays of mitochondrial phospholipase A activity and mitochondrial swelling demonstrated that the phospholipase A activity is related to the swelling under the experimental conditions used. Both were stimulated by added free fatty acid and $CaCl_2$, not affected greatly by the addition of monoacyl phosphoglycerides, and inhibited by EDTA. The amount of fatty acid hydrolyzed from endogenous phosphatidyl ethanolamine and phosphatidyl choline during swelling was calculated to be 20–30 times less than the amount of added free fatty acid that gave comparable swelling. Under the experimental conditions about 4% of the phospholipid was hydrolyzed.

Mitochondrial swelling was studied by electron microscopy and turbidity measurements. The results found were in agreement, whether oleic acid was present or not, except for those values obtained after very brief incubation (1 min) and after incubation for longer than 35 min.

The lack of direct proportion between swelling and the concentration of lysosomes present indicated that the swelling is related mainly to mitochondrial phospholipase A, although swelling due to contaminating lysosomes cannot be excluded entirely.

The temperature dependence of spontaneous, fatty acid-induced, or $CaCl_2$-induced swelling suggested that enzymatic activities are responsible for swelling.

SUPPLEMENTARY KEY WORDS correlation · phospholipase A · swelling · oleic acid · $CaCl_2$ · EDTA · electron microscopy · temperature dependence

THE PHENOMENON of mitochondrial swelling has received a great deal of attention in the past decade, much of which is summarized by Lehninger in "The Mitochondrion" (1). In 1959 Lehninger and Remmert (2) first described the enzymatic formation of "U-factor" which could cause swelling. They found that "U-factor" had many properties identical with those of fatty acids and that oleic acid could duplicate many activities of the "U-factor"; this suggested that the appearance of "U-factor" was due to lipolytic activity. Wojtczak and Lehninger (3) found that ATP could cause contraction of the mitochondria concomitant with the esterification of labeled oleic acid. Aged mitochondria required the addition of either phosphatidyl inositol, palmitoyl carnitine, or palmitoyl CoA as well as ATP to contract (4). To further support the contention that the "U-factor" was fatty acid, Wojtczak and Lehninger demonstrated protection against "U-factor"-induced swelling by the addition of serum albumin, which binds fatty acids. Calcium, which was known to stimulate phospholipase activity, caused rapid swelling. Several other compounds were shown to stimulate swelling, although not all were thought to be activators of the phospholipase activity.

The concept of involvement of lipids in swelling received support from the work of Johnson (5), who found that mitochondria isolated from the livers of rats deficient in essential fatty acids underwent swelling much more rapidly than those from normal rats. Waite and van Golde (6) demonstrated that feeding a corn oil diet to essential fatty acid-deficient rats caused a rapid decrease in the rate of swelling and phospholipase A activity in the isolated liver mitochondria. This change in the swelling characteristics was accompanied by the replacement of oleic and eicosatrienoic acids by linoleic and arachidonic acids on the phospholipids. Condrea,

Abbreviations: PE, phosphatidyl ethanolamine; PC, phosphatidyl choline; GP, *sn*-glycerol 3-phosphate; GPE, glycerophosphoryl ethanolamine; GPC, glycerophosphoryl choline.

Avi-Dor, and Mager (7) and Allmann, Bachmann, Orme-Johnson, Tan, and Green (8) found that the swelling of rat liver mitochondria caused by snake venom was accompanied by a rapid breakdown of the membrane phospholipids. Their observations confirmed the conclusions made by Aravindakshan and Braganca (9) from studies both in vivo and in vitro.

The finding that the mitochondrial phospholipase A requires $CaCl_2$ for activity and is stimulated by fatty acids (10) suggested a closer study of the relationship between phospholipase A activity and mitochondrial swelling. This paper presents evidence that the two phenomena are related and that the fatty acid-induced swelling can be attributed to one of two mechanisms. One mechanism, manifested at all concentrations of fatty acid used, is related to the stimulation of the phospholipase A. The second, manifested at high concentrations of fatty acid, is the result of the detergent action of fatty acids on the membranes.

METHODS

Rat liver mitochondria and lysosomes (the latter being designated F_4) were prepared as described in a recent communication (10). Phospholipase A activity and swelling were assayed as follows: mitochondria (either labeled or nonlabeled) were incubated with the indicated additions at 36°C in 5.0 ml of 0.125 M KCl buffered with 0.01 M Tris, pH 7.4. The suspensions of oleic acids or monoacyl GPC, prepared daily by sonication of 8.0 μmoles of lipid in 2.0 ml of 0.125 M KCl buffered with 0.01 M Tris, pH 7.4, were stable for at least several hours. Suspensions of 2-(linoleoyl-1′-^{14}C)-PE were prepared as before (11).

At the indicated times 1.0 ml of the incubation mixture was pipetted into 2.0 ml of methanol at 0°C for determination of the hydrolysis of phospholipid by thin-layer chromatography (10, 11). Labeled free fatty acid and PE were counted when 2-(linoleoyl-1′-^{14}C)-PE was the substrate [mitochondrial phospholipase acts primarily on the 2-fatty acid (10, 11)] and labeled monoacyl GPE and PE were counted when endogenous ethanolamine-^{14}C-PE was the substrate. 0.03–0.06 ml of the incubation mixture (depending on protein concentration) was pipetted into 3.0 ml of cold 0.125 M KCl, buffered with 0.01 M Tris at pH 7.4, for measurement of the optical density at 520 nm in a Unicam SP 500 spectrophotometer. Usually the initial optical density was 0.5 unit, although in some experiments in which the amount of protein was limiting, the initial optical density was as low as 0.3 unit. Koch (12) demonstrated that the light absorbancy of a suspension of particles, like mitochondria, that change in size by solute transfer is inversely proportional to the area of an equatorial section. Swelling is therefore expressed as decrease in optical density. Except where otherwise stated, experiments were done with the mitochondrial fraction M-I (see Ref. 10), which contained some lysosomes.

Electron microscopic studies were done as follows: aliquots of the mitochondrial suspensions were taken out of the incubation tubes at various times and centrifuged. The resulting mitochondrial pellets were fixed for 1 hr with 1% OsO_4 solution buffered with 0.10 M Tris, pH 6.0. With this fixation medium it was possible to obtain electron micrographs showing nearly circular cross-sections of the mitochondria. After the pellets had been dehydrated with ethanol and embedded in an epoxy resin, sections were cut on a Porter–Blum MT-1 microtome, and collected on copper grids. For staining, these grids were floated on a solution of 1.8 g of ammonium acetate in 10 ml of a saturated lead acetate solution for 45 min (13). Electron micrographs were taken at an initial magnification of 1940 with a Philips EM 200 at 60 kv.

Mitochondrial sections that showed sharply delineated membranes were considered as equatorial and thus indicative of the size of the mitochondria. As the mitochondrial areas on the micrographs are not completely circular, partly because of compression during sectioning, they were measured by means of an Ott planimeter. In the plotting of Fig. 6, the mean of 50 measurements of mitochondrial area from each sample was used. In this study only the size of the mitochondria was taken into account; structural differences that developed during swelling were not analyzed.

MATERIALS

The bovine serum albumin used was Fraction V of the Sigma Co., St. Louis, Mo. Other materials were prepared or purchased as described before (10).

RESULTS

Correlation of Swelling and Phospholipase A Activity

Fig. 1 shows the results of an experiment in which the effect of various concentrations of oleic acid was determined on (*a*) mitochondrial phospholipase activity on exogenous PE, and (*b*) swelling. In the absence of oleic acid there was almost no phospholipase activity or swelling in 20-min incubations. The lowest concentration of oleic acid used, 15 μM, considerably stimulated the hydrolysis of added PE after a slower initial rate and caused some stimulation of swelling. With 75 μM oleic acid the phospholipase activity was nearly at the maximal observed rate and much greater stimulation of

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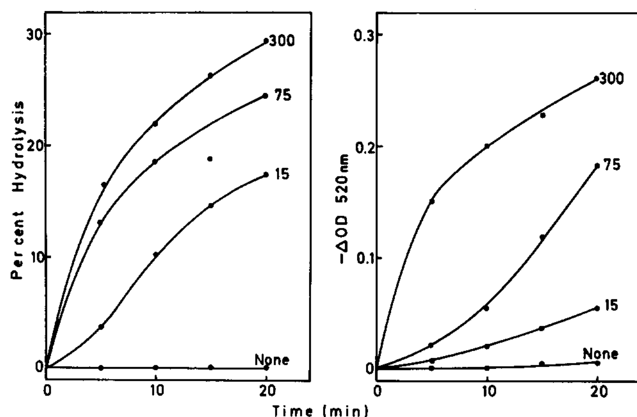

FIG. 1. Effect of different concentrations of oleic acid on mitochondrial phospholipase A activity (assessed by means of exogenous 2-linoleoyl-1′-^{14}C PE) and mitochondrial swelling. Mitochondria (preparation M-I, 50 mg of protein) were suspended in 5.0 ml of 0.125 M KCl buffered with 0.10 M Tris pH 7.4 containing 10 μM PE-^{14}C and the indicated concentrations (μM) of oleic acid, and incubated at 36 °C for the times designated.

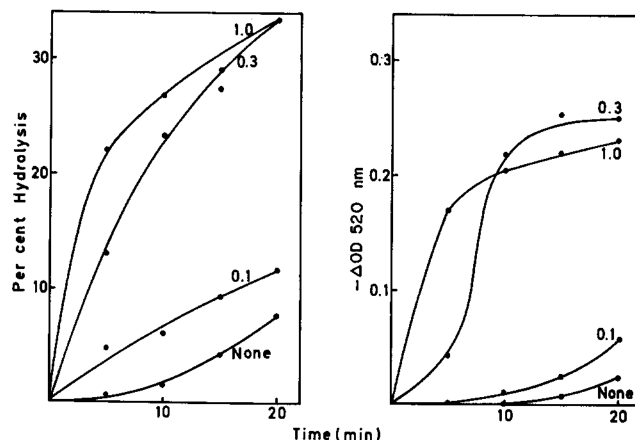

FIG. 2. Stimulation of mitochondrial phospholipase A activity and mitochondrial swelling by $CaCl_2$. Conditions were as described in Fig. 1 except for the indicated concentrations (mM) of $CaCl_2$ and the omission of oleic acid.

swelling could be seen, even though the slower initial rate was still evident. With 300 μM oleic acid both swelling and phospholipase A activity proceeded rapidly. Comparable effects were found with mitochondrial fraction M-II.

A similar study was done with $CaCl_2$ instead of oleic acid as the stimulant (Fig. 2). The lowest concentration of $CaCl_2$ employed, 0.1 mM, caused slight stimulation of both swelling and phospholipase activity on added substrate. An increase to 0.3 mM gave roughly a threefold increase in the hydrolytic activity and caused the mitochondria to be almost completely swollen in 10 min, even though there was little activity at 5 min. In 1.0 mM $CaCl_2$ the mitochondria were almost completely swollen in 5 min. The slight lowering of the total extent of optical density change at the highest $CaCl_2$ concentration has been found in several experiments. The results presented in Figs. 1 and 2 demonstrate that phospholipase A and swelling are stimulated by the same concentrations of $CaCl_2$ or oleic acid, which suggests a relationship between the two activities.

The effect of monoacyl phosphoglycerides, the other product of phospholipase A activity, was compared with that of fatty acid. The results of such an experiment, presented in Fig. 3, indicate that 300 μM 1-oleoyl-GPC was less effective as a stimulator of swelling and much less effective in stimulating phospholipase A activity than 300 μM oleic acid. In the presence of EDTA, which inhibits phospholipase A by chelating the required cofactor, Ca^{++} (10), oleic acid-induced swelling was

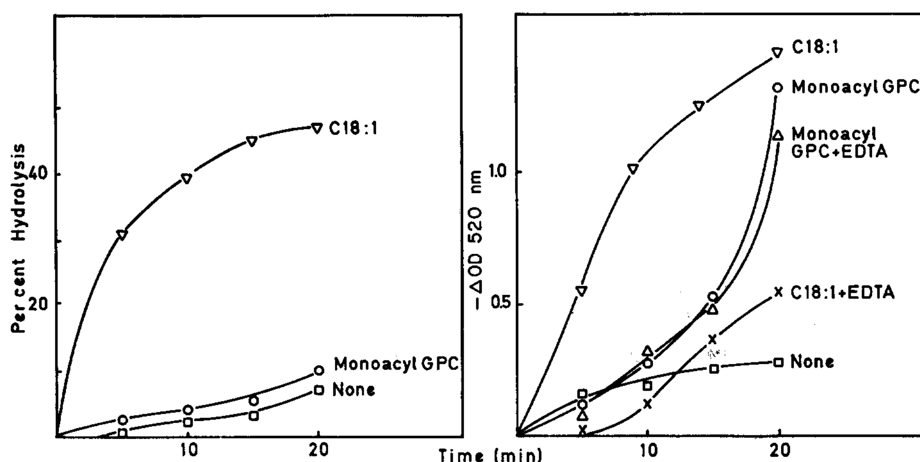

FIG. 3. Comparison of stimulating effect of equimolar quantities of oleic acid (C18:1) and monoacyl GPC on mitochondrial phospholipase A and swelling. Conditions were as described in Fig. 1 except for the indicated presence of 300 μM synthetically prepared 1-oleoyl GPC (kindly supplied by Dr. H. van den Bosch) or 300 μM oleic acid, with or without 5.0 mM EDTA.

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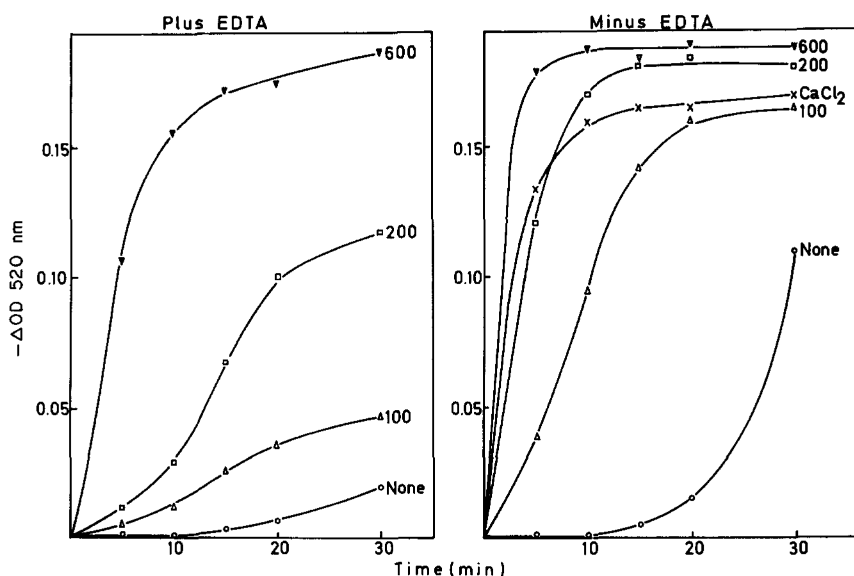

FIG. 4. EDTA inhibition of oleic acid-induced swelling of mitochondria. Ethanolamine-^{14}C-labeled mitochondria, 3.7 mg protein/ml, were incubated under the same conditions as for Fig. 1 except for the omission of PE-^{14}C. EDTA and $CaCl_2$ were added at a concentration of 5.0 mM where indicated. The indicated concentrations of oleic acid are μM.

greatly reduced, whereas there was almost no effect on the swelling induced by 1-oleoyl-GPC. It was concluded that swelling induced by monoacyl phospholipids was due to a direct lytic effect rather than to stimulation of phospholipase A activity. This conclusion is supported by the observation that doubling the 1-oleoyl-GPC concentration caused almost instantaneous swelling of the mitochondrial suspension at 0°C. The swelling effect at 0°C was not found with oleic acid. Addition of monoacyl GPC to oleic acid in the system did not cause further stimulation of swelling.

Extent of Hydrolysis During Swelling

To measure the extent of phosphoglyceride hydrolysis during swelling, we studied this hydrolysis and the swelling in mitochondria from the livers of rats that had been injected with ethanolamine-^{14}C or choline-^{14}C and which therefore contained ethanolamine- or choline-labeled phospholipids. Also, the amount of monoacyl GPE-^{14}C and monoacyl GPC-^{14}C formed during the enzymatic hydrolysis was determined and compared with the (molar) amount of added fatty acid required to cause swelling in the presence of EDTA.

In this experiment (see Fig. 4) 100 μM oleic acid caused rapid swelling in the absence of EDTA but only one-fifth that level of swelling (comparing the 20-min values) in the presence of EDTA. 600 μM oleic acid was required to overcome the protective effect of EDTA, although a 30 min incubation was required to obtain the full extent of swelling. In the absence of EDTA, maximal swelling occurred in about 5 min when the oleic acid concentration was 600 μM, 15 min at 200 μM, and 20 min at 100 μM.

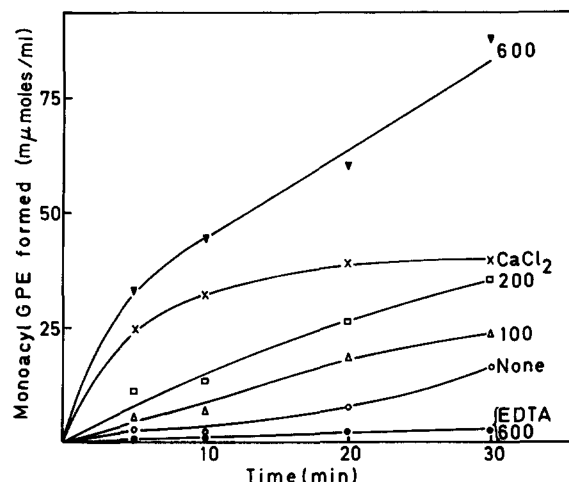

FIG. 5. Effect of EDTA and oleic acid on hydrolysis of ethanolamine-^{14}C-labeled mitochondrial PE. These assays were performed simultaneously with those of Fig. 4. The PE-^{14}C content was about 9000 cpm/ml of reaction mixture. Monoacyl GPE formed is expressed as mμmoles formed per ml of reaction mixture, determined from the specific radioactivity of the PE-^{14}C (10). EDTA suppressed phospholipase activity completely.

Fig. 5 presents data on the hydrolysis of endogenously labeled PE with or without $CaCl_2$, or with oleic acid plus or minus EDTA. Here again, it can be seen that the addition of oleic acid or 5 mM $CaCl_2$ causes stimulation of the endogenous PE hydrolysis. 5 nM EDTA, on the other hand, inhibits the phospholipase activity, even in the presence of fatty acid.

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TABLE 1 CONCENTRATIONS OF FATTY ACID LIBERATED AT 100% SWELLING

	Time of Maximal Swelling (without EDTA)	Oleic Acid Added	Per Cent of Maximal Swelling in Presence of EDTA*	Calculated Concn. of Liberated Oleic Acid at 100% Swelling† (without EDTA)	Calculated Concn. of Added Oleic Acid Causing 100% Swelling‡ (with EDTA)
	min	*mμmoles/mg protein*		*mμmoles/mg protein*	*mμmoles/mg protein*
PE	20	27	15	5.0	299
	15	54	40	5.4	135
	5	163	65	8.1	180
Average (A)	—	—	—	6.2	188
PC	20	20	15	2.9	186
	15	40	40	1.9	100
	5	121	65	2.1	133
Average (B)	—	—	—	1.6	140
Total fatty acid liberated (sum of A and B)	—	—	—	7.8	—

* Values were determined from Fig. 4 at time of maximal swelling in the absence of EDTA.

† Calculated from the amount of monoacyl GPE (Fig. 5) or monoacyl GPC formed at the time of maximal swelling in the absence of EDTA.

‡ Calculated as the amount of oleic acid added divided by per cent of maximal swelling in the presence of EDTA.

The calculations based on the data from Figs. 4 and 5 and from a similar experiment using mitochondria from rats previously injected with choline-^{14}C are compiled in Table 1. These calculations quantitate differences between the swelling related to the phospholipase A activity (without EDTA) and that caused by high levels of the fatty acid (with EDTA, which prevents phospholipase A activity). At the time 100% swelling had occurred without EDTA, an average of 6.2 mμmoles of fatty acid (per mg protein) had been hydrolyzed from PE and 1.6 mμmoles of fatty acid had been released from PC, a total of 7.8 mμmoles. However, in the presence of EDTA, maximum swelling required an average of 160 mμmoles of oleic acid per mg protein. An example of this calculation is: 27 mμmoles of oleic acid per mg protein (100 μM) gave 15% maximal swelling in 20 min; 27/15% = 180. This is roughly 20–30 times the amount of fatty acid and of monoacyl phosphoglyceride released from endogenous phospholipid during swelling in the absence of EDTA.

The amount of PE and PC undergoing hydrolysis during maximal swelling corresponded to about 4% of the total PE and PC present. Similar data were obtained from $CaCl_2$-induced or noninduced swelling experiments. These results show that the extent of membranous phosphoglyceride hydrolysis related to swelling is low.

Electron Micrographic Study of Swelling

The mitochondrial swelling was studied simultaneously by electron microscopy and spectrophotometry. Micrographs of the stock mitochondrial suspension (Fig. 6A) showed mitochondria with a dense matrix and intact cristae, much like those found in intact liver cells. Samples of the mitochondrial preparations were incubated without addition or in the presence of 1.0 mM EDTA (Fig. 6B), 1.0 mM EDTA and 0.1 mM oleic acid (Fig. 6C), or 0.1 mM oleic acid alone (Fig. 6D, E, and F). Micrographs of samples incubated without additions or with $CaCl_2$ are not shown. In these experiments about 5 min were required for preparation of the mitochondria for fixation.

After 1 min incubation, either without addition or with addition of oleic acid (6D), the equatorial cross-section actually decreased. It might be expected that such a contraction would be observed spectrophotometrically as an increase in absorbancy. This was not the case, however (Fig. 7B), possibly because of other, offsetting structural changes. Micrographs of the sample with $CaCl_2$ showed two distinct mitochondrial forms after 1 min incubation, one form similar to stock suspension and one form that was swollen. The two forms were seen in the micrographs of mitochondria incubated 17 min with oleic acid (Fig. 6E) and 25–30 min without addition. In the presence of $CaCl_2$ most of the mitochondria were swollen in 17 min, whereas 35 min was required to swell all mitochondria in the presence of oleic acid (Fig. 6F). The mitochondria incubated without addition were nearly all swollen at 57 min, the longest incubation time.

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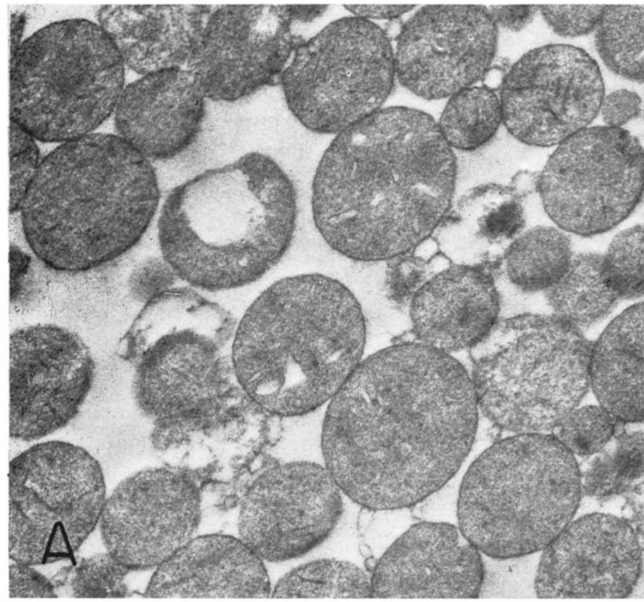

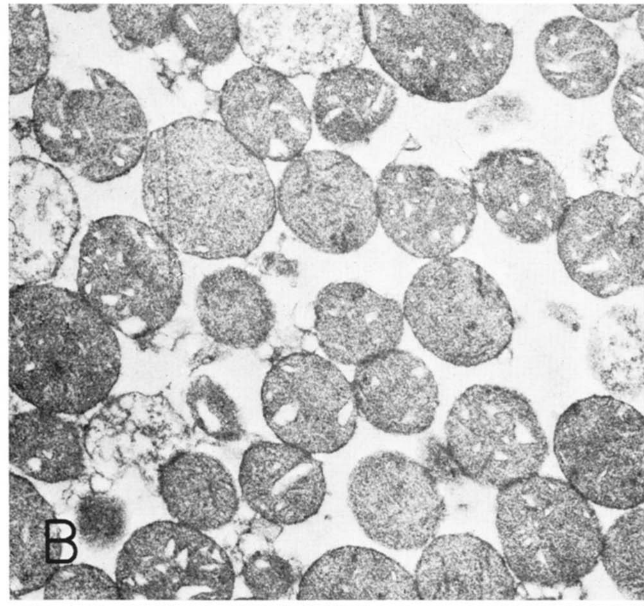

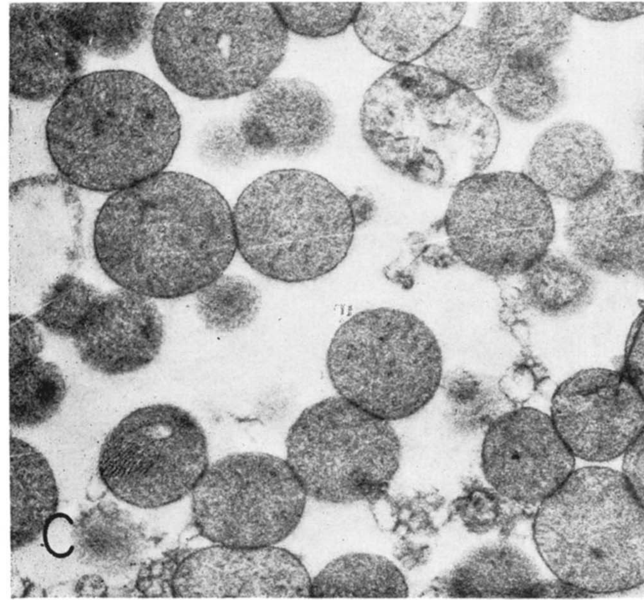

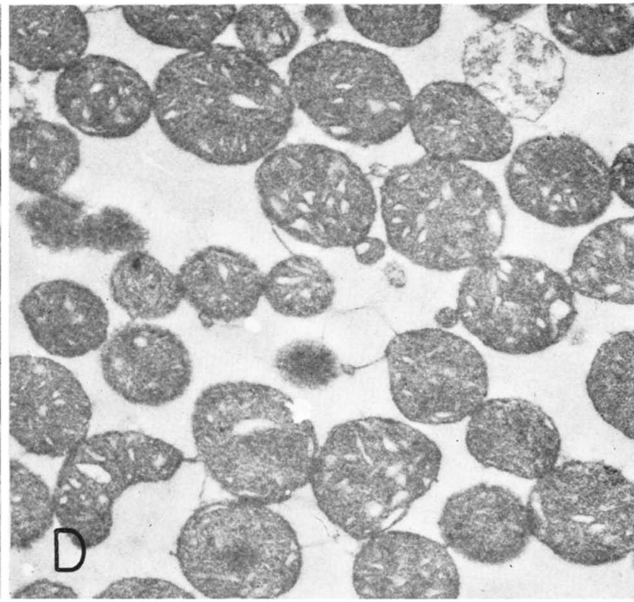

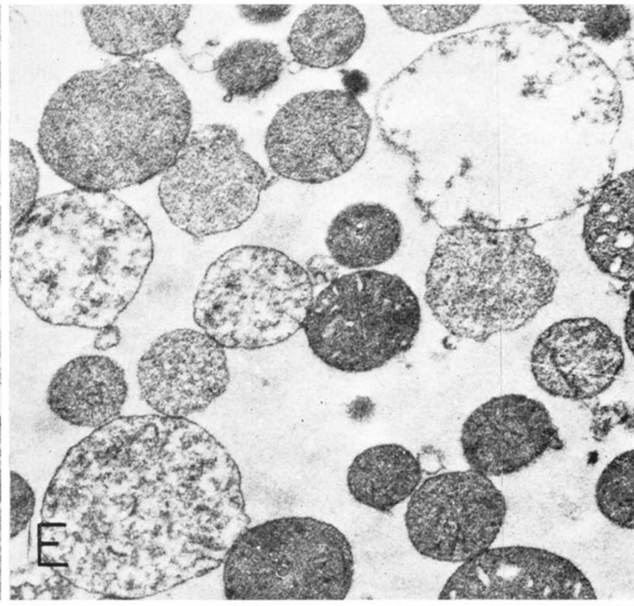

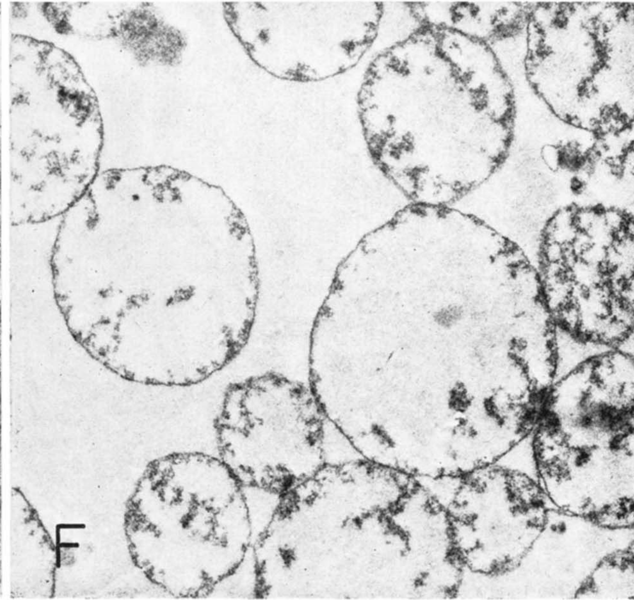

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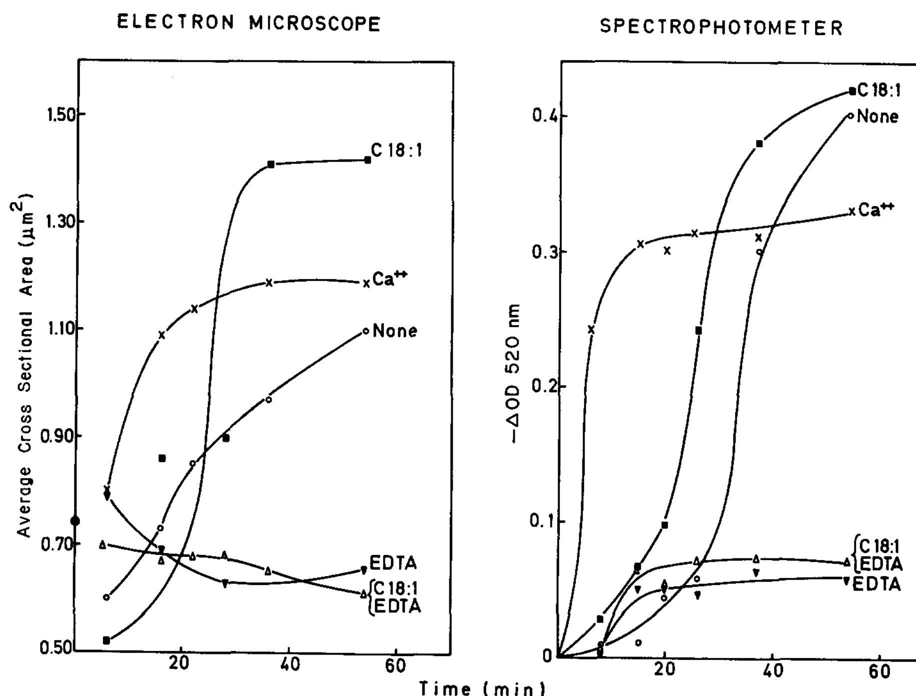

FIG. 7. Comparison of spectrophotometric and electron microscopic swelling studies. Values of surface area of cross sections were determined from the electron micrographs. The closed circle designates the average cross-sectional area of mitochondria in the stock specimen at zero time.

The rate of swelling measured from the electron micrographs and by the optical density were in good agreement between 5 and 35 min (Fig. 7). Mitochondria treated with $CaCl_2$ had electron-dense material within the membrane (cf. Ref. 14), whereas those incubated without addition or in the presence of oleic acid were nearly empty. This difference would change the light absorbancy properties of the mitochondria and prevent the correlation of these two methods at incubation periods longer than 35 min.

The majority of the mitochondria in the presence of EDTA, either without (Fig. 6B) or with oleic acid (Fig. 6C), were unchanged in size although the population seemed to be less homogeneous than that in the stock suspension. The studies with the electron microscope confirmed the observation that EDTA greatly reduces the swelling effect of oleic acid.

Possible Role of Lysosomes in Mitochondrial Swelling

The recent studies by Mellors, Tappel, Sawant, and Desai (15) demonstrated that lysosomes can stimulate mitochondrial swelling. The extent of lysosomal contribution to swelling in our preparation was studied in two ways: first, by comparing the swelling of mitochondrial preparations M-I (less pure) and M-II (more purified) and second, by comparison of the swelling of M-II with and without added F_4, a lysosomal preparation. Comparison of curves 4 (M-II) and 5 (M-I) of Fig. 8 shows that mitochondria underwent a slower spontaneous swelling when they had undergone further purification. This nearly 100% difference could not be attributed directly to lysosomal contamination, however, since there was a five-fold decrease in the lysosomal contamination of the mitochondria (according to the acid phosphatase, assayed using β-glycerol phosphate, and ribonuclease specific activities) after the density gradient centrifugation that converted M-I to M-II (10). A five-fold increase in the lysosomal concentration, caused by the addition of F_4 (which had been frozen and thawed three times) to M-II, caused only a twofold increase (curve 6) in the rate of swelling. This was inhibited by EDTA (curve 2). Raising the pH of the swelling medium from 7.0 to 8.8, which leads to higher phospholipase A activity (10), caused an increase in the rate of swelling (curve 7), although at this pH EDTA inhibition was not as great as at pH 7.0 (curves 1 and 3).

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FIG. 6. Electron micrographs of mitochondria. The reaction mixture from which samples were taken for the electron micrographs contained 9.1 mg of mitochondrial protein per ml of 0.125 M KCl with 0.01 M Tris, pH 7.4. *A*, the stock suspension before incubation. *B–F*, incubations at 36°C as follows: with 1.0 mM EDTA for 49 min (*B*), with 1.0 mM EDTA and 0.10 mM oleic acid for 49 min (*C*), and with 0.10 mM oleic acid (*D*), 1 min; *E*, 17 min; *F*, 49 min. The samples were prepared as described under Methods. All are at a final magnification of 24,000×.

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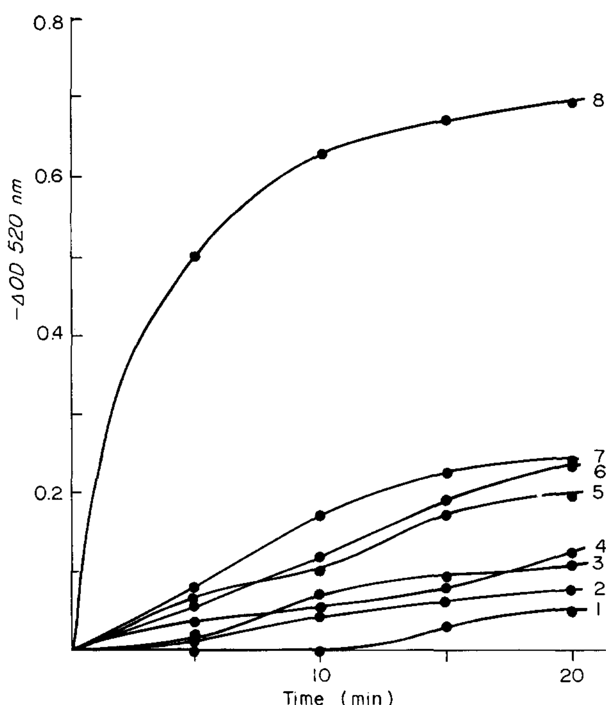

FIG. 8. Influence of lysosomes and pH on mitochondrial swelling. Mitochondrial preparation M-I or M-II (4.3 mg of protein) was incubated in 1.0 ml of 0.125 M KCl with 0.02 mM glycylglycine at the specified pH's for the indicated periods of time. A 0.10 ml aliquot was then pipetted into 2.9 ml of cold 0.125 M KCl buffered with glycylglycine buffer at the pH of the incubation and the OD at 520 nm was determined. The initial OD was 0.85. Incubation mixtures were as follows: No. 1, pH 7.0, 5.0 mM EDTA and M-II; No. 2, pH 7.0, 5.0 mM EDTA, M-II, and 1.08 mg of F_4; No. 3, pH 8.8, 5.0 M EDTA and M-II; No. 4, pH 7.0 and M-II; No. 5, pH 7.0 and M-I; No. 6, pH 7.0, M-II and 1.08 mg of F_4; No. 7, pH 8.8 and M-II; and No. 8, pH 7.0, M-II and 1.0 mM $CaCl_2$.

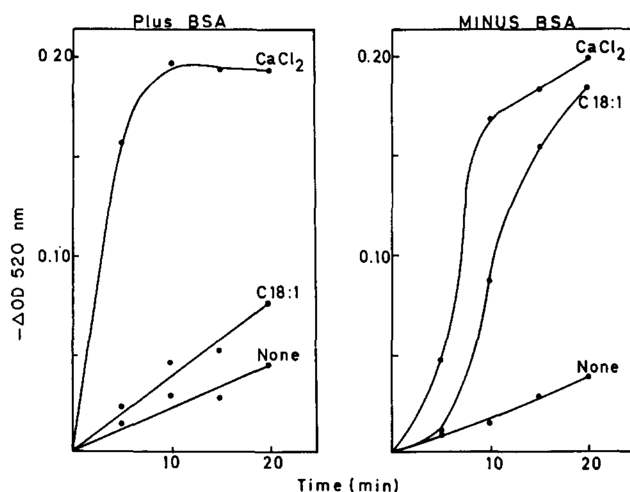

FIG. 9. Albumin reduction of oleic acid-induced swelling. Conditions were as described in Fig. 1 except for the indicated additions of 7.5 mg of serum albumin in 0.125 M KCl with 0.010 M Tris, pH 7.4, 160 μM oleic acid, or 1.0 mM $CaCl_2$ where indicated, and the omission of PE-^{14}C. The mitochondrial (M-I) protein concentration was 6.8 mg/ml.

Since mitochondrial proteins precipitated at pH 4.5, it was not possible to study swelling at this pH. $CaCl_2$ caused a very rapid swelling of M-II (curve 8). It appears, therefore, that the swelling of the mitochondria in these preparations is primarily the result of mitochondrial rather than lysosomal activity.

Effect of Albumin and Temperature on Swelling

The reports of Wojtczak and Lehninger (3) and of Condrea et al. (7) indicate that serum albumin protection against swelling is more complete when fatty acid is added as the swelling agent than when the fatty acid is liberated from the mitochondrial phospholipid as a result of incubation with $CaCl_2$ or of the action of snake venom. Some of these experiments were repeated using the experimental conditions reported here. Buffered serum albumin (Sigma, Type V) greatly decreased the oleic acid-induced swelling, as previously found (see Fig. 9). On the other hand, the addition of serum albumin to the mitochondria in the presence of $CaCl_2$ caused an increase in the rate of swelling, a result that was not expected. Albumin did not stimulate phospholipase A activity. Lowering the level of albumin diminished the stimulation of the $CaCl_2$-induced swelling, but allowed more swelling in the presence of added oleic acid. Prior incubation of the serum albumin with oleic acid (at the same level used for swelling) eliminated the stimulation of swelling due to the albumin. This finding suggests that the albumin was interacting with the mitochondrial lipids rather than altering conditions such as pH or salt concentration in the incubation mixture.

The results of an experiment on the effect of temperature on swelling are presented in Fig. 10. $CaCl_2$- and oleic acid-induced and noninduced swelling are temperature-dependent (compare rates at 22 and 36°C). Also, when swelling occurs at 22°C, the stimulation by the combination of $CaCl_2$ and oleic acid (curves 5 and 6) was greater than the sum of the stimulation by the compounds alone (curve 2 plus 3 and curve 2 plus 4). This was demonstrated both at moderate (160 μM) and high (400 μM) levels of oleic acid. Further, the swelling caused by 400 μM oleic acid at 22°C is somewhat more rapid than that caused by 160 μM at 36°C, but does not proceed to anywhere near the same extent. These data are consistent with the concept that swelling is related to reactions catalyzed by an enzyme or enzymes, one of which has been shown to be phospholipase A.

DISCUSSION

Direct measurement of phospholipid hydrolysis with concomitant swelling studies provided evidence that the mitochondrial phospholipase A is one of the factors related

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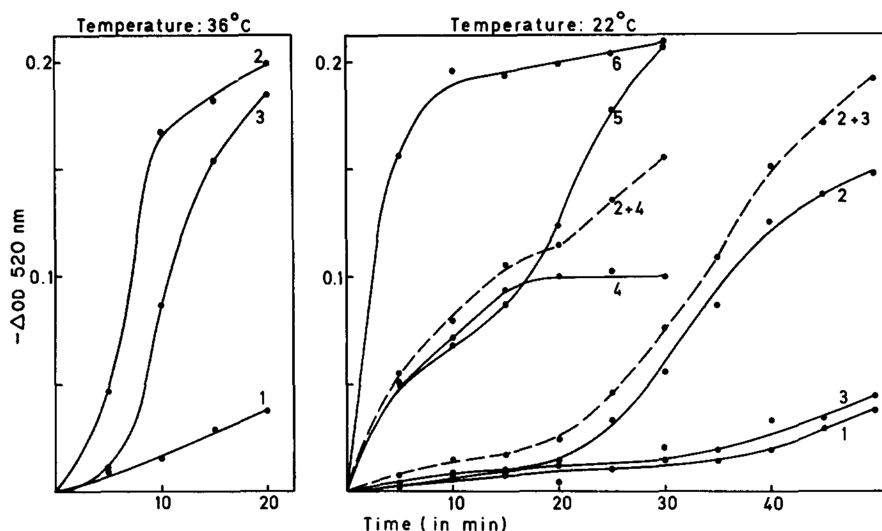

Fig. 10. Effect of temperature on swelling. Conditions were the same as for Fig. 9 except for the indicated change in temperature. The various incubations contained the following added substances: No. 1, none; No. 2, 1.0 mM $CaCl_2$; No. 3, 160 μM oleic acid; No. 4, 400 μM oleic acid; No. 5, 1.0 mM $CaCl_2$ and 160 μM oleic acid; and No. 6, 1.0 mM $CaCl_2$ and 400 μM oleic acid.

to swelling of mitochondria. Although this was postulated previously by Lehninger and Remmert (2), their experiments did not show directly the presence of a phospholipase in the mitochondria. We conclude from the data presented here that the swelling effect of fatty acid involves two mechanisms. The first mechanism, manifested at the lower concentrations of added fatty acid, is related to the stimulation of phospholipase A, which splits membrane phospholipid into fatty acid and monoacyl phosphoglyceride with consequent change of membrane permeability. The second mechanism, manifested at higher concentrations, is direct lysis of the membrane resulting from the detergent action of the soaps, in addition to the stimulation of the phospholipase activity.

These conclusions are based on the following observations: (*a*) EDTA, which chelates Ca^{++}, inhibited the phospholipase A activity (Fig. 5) and oleic acid-induced swelling (Fig. 4) at lower concentrations (i.e., 100–200 μM) of fatty acid. (*b*) Added monoacyl phosphoglycerides had less effect on swelling and phospholipase A activity than equimolar amounts of added oleic acid (Fig. 3), even though monoacyl phosphoglycerides had pronounced lytic properties. The swelling caused by monoacyl phosphoglycerides was not inhibited by EDTA, which suggested that they lysed the membrane but did not stimulate the phospholipase A activity. (*c*) The amount of PE and PC hydrolyzed during swelling (Table 1) was equivalent to about 4% of the PC and PE in the preparation. (*d*) Albumin (fatty acid-binding agent) protected against oleic acid stimulation but not against $CaCl_2$ induction of swelling, which indicated that the swelling effect is primarily brought about by the phospholipase A activity and secondarily by the added oleic acid. (*e*) The temperature dependence of the swelling stimulated by oleic acid or $CaCl_2$ (Fig. 10) indicated an enzyme-catalyzed reaction rather than a purely physical interaction.

The possibility that the oleic acid-induced swelling was due to lipid peroxidation does not seem likely. We have shown (10) that the saturated acids lauric and myristic stimulated phospholipase A activity to the same degree as oleic and linoleic acid. This pattern of stimulation was similar to that found in studies on fatty acid stimulation of swelling (16). Also, Hunter et al. (17) demonstrated that fatty acid-induced swelling was independent of ascorbate-induced swelling, which was the result of lipid peroxidation. We found that antioxidants such as α-tocopherol or hydroquinone had little effect on linoleic acid-induced swelling at concentrations of the antioxidant which protected against ascorbate-induced swelling. Similar conclusions were reached by Mellors et al. (15).

Mellors et al. (15, 18) demonstrated that rat liver lysosomes stimulated mitochondrial swelling and contained a phospholipase with maximal activity at pH 4.5 and almost no activity at pH 7.4. It was shown that swelling induced by the addition of lysosomes at pH 7.4 parallels the release of free fatty acid. They ascribed the lysosomal stimulation of mitochondrial swelling to action of the hydrolysis products from the lysosomal membrane rather than to the direct hydrolysis of mitochondrial membranes by the lysosomal phospholipase A. We were able to repeat their experiments but found only limited

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induction of swelling by added lysosomes. It is possible that the release of fatty acid shown by them was catalyzed not only by the phospholipase A with an acid pH optimum, but was catalyzed also by mitochondrial phospholipase A and by the lysosomal phospholipase A with a pH optimum between 6.0 and 7.0 (10, 19). Since lysosomes have been shown to be heterogeneous in some tissues (20), it is difficult to rule out the possibility that a species of lysosomes with low acid phosphatase and ribonuclease activity and rich in phospholipase A activity at neutral and alkaline pH's was contaminating the mitochondrial preparations and was responsible for the activity we attribute to the mitochondria. This seems unlikely, however, since lysosomes prepared by different methods from various tissues all have phospholipase activity at acid pH's (10, 18, 19, 21). It was not surprising that the lysosomal phospholipase A added to mitochondria can cause some swelling since it has been shown that the phospholipase in snake venom can do the same (7–9).

Electron microscopic analysis of size changes demonstrated mitochondrial shrinking in incubation periods less than 6 min. This was not observed by optical density measurements, and these are evidently some limitations of the theoretical correlation described by Koch (13). Electron micrographs indicated that some mitochondria have a greater tendency to swell (on longer incubation) than others, and therefore that more than one type of mitochondria is present. This difference in the rate of swelling of the two species could be the result of damage to a portion of the mitochondria during isolation or could be due to the manifestation of physiological differences such as age, discussed by Baudhuin and Berthet (22).

The work reported here suggests that the role of the phospholipase A in swelling is to catalyze the hydrolysis of phospholipid at a key locale. Such a preferential site of hydrolysis might explain the observation (10) that not all mitochondrial phospholipid is hydrolyzed at the same rate. Recently Nachbaur and Vignais have shown (23) that mitochondrial phospholipase A is located mainly in the outer membrane. Similar observations in this laboratory confirm the findings of Nachbaur and Vignais but also show some phospholipase A to be associated with the inner membrane (24). It is possible that the critical sites of hydrolysis are in the outer membrane. Breakdown of the outer membrane could allow the inner membrane to unfold and swell. On the other hand, these results suggest that it is possible that hydrolysis of both membranes is required for swelling under these conditions. Schnaitman and Greenawalt (25) have been able to isolate morphologically and biochemically intact inner mitochondrial membranes, which indicates that the outer membrane is not required for the maintenance of the inner membrane structure.

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