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MITOCHONDRIAL SWELLING AND UNCOUPLING OF OXIDATIVE PHOSPHORYLATION BY LYSOSOMES

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SUMMARY

Lysosomes catalyze a rapid disruption of mitochondrial structure and function in which oxidative phosphorylation is uncoupled and mitochondrial ATPase is activated. There is a concomitant irreversible high amplitude swelling of mitochondria which is independent of substrate availability. This swelling is not due to lipid peroxidation or digestion by proteolytic enzymes but closely parallels that produced by free fatty acids, in that it is inhibited by low levels of bovine serum albumin. The swelling factor, which is most active in the lysosomal membrane, is associated with the activity of lysosomal lipolytic enzymes. A phospholipid-hydrolyzing enzyme has been found in the lysosome, which liberates free fatty acids from lecithin. Disruption of mitochondrial membranes by these fatty acids, coupled with direct hydrolysis of the membranes by a lysosomal phospholipase, may initiate the digestion of mitochondria by lysosomes.

INTRODUCTION

Lysosomes have been shown to be capable of the lysis of cellular and subcellular membranes as in the hemolysis of erythrocytes[1] and the digestion of mitochondria[2]. This study reports on the capacity of lysosomes to disrupt mitochondria as indicated by the uncoupling of oxidative phosphorylation and by mitochondrial swelling. This investigation of the high amplitude irreversible swelling of mitochondria by lysosomes shows that such mitochondrial damage may be caused by free fatty acids released by the action of lipolytic enzymes of the lysosome.

MATERIALS AND METHODS

Preparation of lysosomes and mitochondria

Male Sprague–Dawley rats (200–250 g) were fasted for 24 h and killed by decapitation. The livers and hearts were quickly removed and transferred to ice-cold sucrose solution. Rat heart mitochondria were isolated in 0.32 M sucrose by the method of CLELAND AND SLATER[3]. Rat livers were homogenized and fractionated, and the mitochondrial and lysosomal fractions were purified, by methods previously described[4,5].

Lysosome fractions. Lysosomes were frozen and thawed ten times to release soluble lysosomal enzymes. The lysosomal membrane was obtained by centrifuging the disrupted lysosomes at 105000 × *g* for 30 min, the lysosomal membrane pellet was washed once with 0.1 M NaCl and finally resuspended in 0.25 M sucrose.

Measurement of oxidative phosphorylation and mitochondrial swelling

Oxidative phosphorylation was measured by the conventional manometric technique[6] or the polarographic method of PACKER[7]. In most experiments swelling of mitochondria was measured at 37° as the change in turbidity at 520 or 540 mμ of a mitochondrial suspension in a Beckman DB spectrophotometer with a thermostatically controlled cell compartment. For measurement of swelling phenomena, mitochondria were suspended in 0.175 M KCl, 0.025 M with respect to tris(hydroxymethyl)aminomethane buffer (pH 7.4). Mitochondrial swelling was also determined as a decrease in light scattering at 540 mμ, measured at a 90° angle using a Phoenix photometer.

Lipid peroxidation catalysis

A Clark oxygen electrode (Yellow Springs Instruments) was used to measure the oxygen consumption of 2.0 ml of a 0.5 % (w/v) suspension of arachidonic acid in 0.1 M phosphate buffer (pH 7.4), after the addition of 1.0 ml of phosphate buffer containing rat liver subcellular fractions at a concentration of about 0.5 mg protein per ml.

Enzyme assays

ATPase activity was measured according to the method of MYERS AND SLATER[8]. Cathepsin and acid phosphatase (EC 3.1.3.2) were estimated by the methods of GIANETTO AND DE DUVE[9]. Non-specific esterase activity was determined by the procedure of RAVIN AND SELIGMAN[10]. Protein was measured by the assay of MILLER[11].

Hydrolysis of ^{14}C-labeled lipids

Glyceryl tri[1-^{14}C]oleate was obtained from Nuclear Chicago Inc. Uniformly labeled [^{14}C]lecithin was obtained from Applied Science Laboratories Inc. and was purified by thin-layer chromatography. The hydrolysis of these ^{14}C-labeled lipids by rat liver fractions was carried out in 0.25 M sucrose, 0.1 M with respect to acetate buffer (pH 5.0). All fractions were treated in a similar manner including freezing and thawing to release enzymes. Sonication at 90 kilocycles in a Sonblaster 200 sonicator was used to disperse the lipids in 0.25 M sucrose solution. The reaction mixture was incubated for 4 h at 37°, under nitrogen, with rapid shaking. Enzyme and substrate controls were run and results were corrected for small blanks. At the end of the incubation neutral lipids were extracted with isooctane by rapid shaking with the solvent for 1 h. The volumes of the isooctane extracts were reduced to 0.25–0.5 ml under nitrogen and free fatty acids were separated from other components by chromatography on thin layers of silica gel. To separate free fatty acids and phospholipids the mobile phase was chloroform–methanol–water (65:25:4, by vol), and to separate the substrate and products of the hydrolysis of triglyceride the mobile phase was light petroleum (b.p. 40–60°)–diethyl ether–glacial acetic acid (80:20:1, by vol.). Lipids were localized on the chromatograms by staining with iodine, appropriate

areas of silicic acid were scraped off, and radioactively labeled lipids were eluted according to the method of ABRAMSON AND BLECHER[12]. After removal of the solvent the radioactivity of each spot was determined by liquid scintillation counting in a Packard Tri-Carb scintillation counter.

RESULTS

Effect of lysosomes on oxidative phosphorylation

Results in Table I show a reduction in the phosphorylation of liver and heart mitochondria and a lowering of the P/O ratio when lysosomes were added to the reaction system. However, addition of heat-denatured lysosomes had no effect on the phosphorylating capacity of mitochondria as indicated by the P/O ratio.

Effect of lysosomes on mitochondrial swelling

A rapid swelling of mitochondria after the addition of lysosomes is demonstrated in Fig. 1. A large decrease in light scattering was observed when lysosomes were

TABLE I

THE EFFECT OF LYSOSOMES ON OXIDATIVE PHOSPHORYLATION

The reaction was carried out in a Warburg apparatus at 37°. Each flask contained 8 mg mitochondrial protein, 80 μmoles P_i, 5 μmoles ADP, 5 μmoles $MgCl_2$, 100 μmoles glucose, 0.5 mg hexokinase and 50 μmoles succinate, in a total volume of 3.0 ml. Lysosomes (1.6 mg protein) were added from the side arm after equilibration. Lysosomes were denatured by heating at 100° for 30 min.

	*Phosphorylation rate** (*P*)	*Oxidation rate*** (*O*)	*P/O*
Liver mitochondria	0.100	0.052	1.96
Liver mitochondria + lysosomes	0.046	0.059	0.78
Liver mitochondria + denatured lysosomes	0.123	0.056	2.20
Heart mitochondria	0.083	0.030	2.77
Heart mitochondria + lysosomes	0.025	0.031	0.82

* μmoles P_i esterified per mg mitochondrial protein per min.
** μatoms oxygen per mg mitochondrial protein per min.

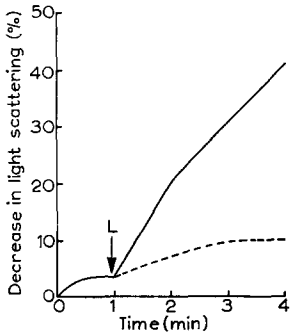

Fig. 1. The swelling of mitochondria by lysosomes (L). Denatured lysosomes (----) and lysosomes (———) were added at the level of 50 μg lysosomal protein per mg mitochondrial protein.

TABLE II

SWELLING OF RAT LIVER MITOCHONDRIA BY LYSOSOMES

Rat liver mitochondria were incubated with rat liver lysosomes at pH 7.4. Lysosomes were denatured in some experiments by heating at 100° for 30 min. In all cases the concentration of added lysosomal protein was 20% of the mitochondrial protein. The mitochondrial protein concentration was chosen so that the initial absorbance of the suspension was 0.60–0.70. Absorbance was measured in a Beckman DB spectrophotometer with a temperature-controlled cell compartment. The suspending medium was 0.175 M KCl, 0.025 M with respect to tris(hydroxymethyl)-aminomethane buffer (pH 7.4). Mitochondria (P), rat liver mitochondria in the oxidative phosphorylation medium described in Table I.

	Temp.	*Reduction in absorbance at 520 mμ in 30 min*
Mitochondria	0°	0.000
Mitochondria + lysosomes	0°	0.000
Mitochondria	37°	0.020
Mitochondria + lysosomes	37°	0.080
Mitochondria + denatured lysosomes	37°	0.010
Mitochondria (P)	37°	0.000
Mitochondria (P) + lysosomes	37°	0.060

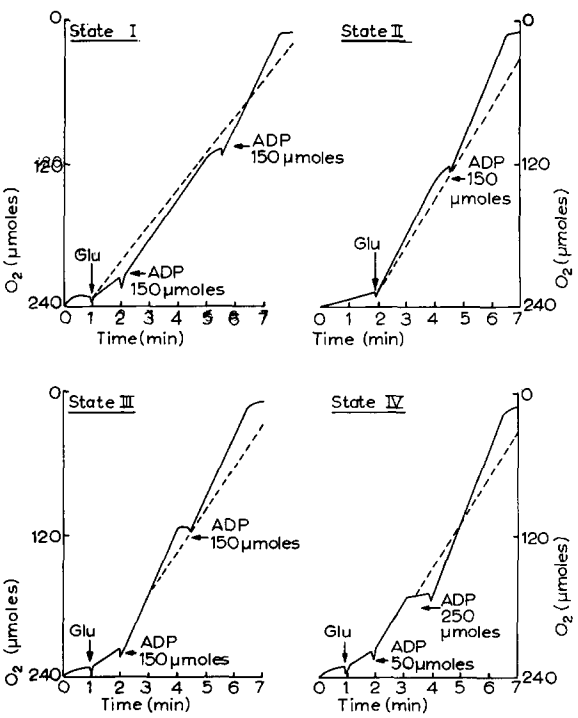

Fig. 2. Uncoupling of phosphorylation of mitochondria in four different metabolic states, by lysosomes. The normal respiration of 10 μg mitochondrial protein (———) is compared with respiration after the addition of 0.5 mg lysosomal protein (– – – –). In State II the mitochondrial suspension contained 150 μmoles ADP.

added to the mitochondrial suspension. Addition of heat-denatured lysosomes, or water, did not cause a decrease in light scattering. Table II shows the temperature-dependence of mitochondrial swelling by lysosomes. Incubation of a mitochondrial suspension with lysosomes at 0° did not cause any decrease in light scattering. However, during incubation at 37°, lysosomes catalyzed a rapid swelling of mitochondria. Heat-denatured lysosomes were again unable to change mitochondrial swelling. Use of phosphorylating medium during the incubation period did not prevent mitochondrial swelling by lysosomes.

Effect of lysosomes on oxidative phosphorylation of mitochondria in various metabolic states

The capacity of lysosomes to uncouple mitochondrial respiration in different metabolic states was determined by polarographic measurement of oxygen consumption as shown in Fig. 2. For mitochondria in State I the rate of glutamate respiration was increased after addition of lysosomes. Under these conditions of increased mitochondrial respiration there was no further response after addition of adenosine diphosphate. When lysosomes were added to mitochondria in the States II, III and IV it was observed that mitochondria did not require the second addition of adenosine diphosphate before reaching the anaerobic state. Addition of EDTA to the medium prevented uncoupling of mitochondria by lysosomes. This effect was observed in all the metabolic states of mitochondria.

The increased utilization of glutamate after the addition of lysosomes to mitochondria in State I may be due to membrane damage, which could increase the availability of glutamate. Such damage would be reflected in mitochondrial swelling and uncoupling of oxidative phosphorylation.

Heat-denatured lysosomes were ineffective in uncoupling oxidative phosphorylation, and were previously found to be inactive in promoting mitochondrial swelling. This suggests that both effects are catalyzed by proteins, a hypothesis supported by separate experiments in which the uncoupling of oxidative phosphorylation was achieved by lysosomes after dialysis.

Effect of lysosomes on mitochondrial ATPase

Results in Table III show the activation of ATPase of mitochondria in the

TABLE III

THE EFFECT OF LYSOSOMES ON MITOCHONDRIAL ATPase

Lysosomes were added at a level of 5% of the mitochondrial protein. The mixture was incubated for 30 min at 37° and then ATPase activity was measured as described in the text. Mitochondria were "aged" *in vitro* by incubation at 37° for 3 h.

	*ATPase activity**	
	Fresh mitochondria	*"Aged" mitochondria*
Mitochondria	0.43	1.10
Mitochondria + lysosomes	0.97	1.13
Mitochondria + lysosomes + EDTA	0.40	1.02

* P_i liberated, mμmoles/mg mitochondrial protein per min.

presence of lysosomes. A similar activation of ATPase can be observed during the spontaneous "aging" of mitochondria *in vitro*. Since lysosomes did not increase the ATPase activity of "aged" mitochondria it appears that both processes, "aging" and uncoupling by added lysosomes, are caused by similar processes of membrane damage. In agreement with the findings on swelling and uncoupling of mitochondria, the presence of EDTA prevented the activation of ATPase of freshly prepared mitochondria by lysosomes. Under the conditions of this experiment no ATPase activity could be detected in the lysosome fraction in the absence of mitochondria.

Properties of the swelling factor

Table IV shows that the swelling factor is localized in the lysosomal membrane, and this fraction of the lysosome was used for further studies on the properties of the swelling factor.

The same table shows the effect of a variety of substances on the capacity of the lysosome membrane to cause swelling of mitochondria. Neither of the antioxidants tested, α-tocopherol and diphenyl-*p*-phenylenediamine, nor the metal-coordinating agents, EDTA and thenoyltrifluoroacetone, inhibited the swelling of mitochondria. This indicates that the membrane damage is not a peroxidative deterioration catalyzed by metal ions. The inability of EDTA to inhibit the swelling factor of the lysosomal membrane contrasts with the findings that EDTA inhibited the uncoupling and swelling of mitochondria, and the activation of mitochondrial ATPase induced by whole lysosomes. An organophosphorus inhibitor of esterases, diisopropyl fluorophosphate, and an inhibitor of catheptic activity, 6-aminocaproic acid, were ineffective in preventing the swelling. Malonate was ineffective in preventing the mito-

TABLE IV

THE EFFECT OF SOME COMPOUNDS ON THE SWELLING OF MITOCHONDRIA BY A LYSOSOME MEMBRANE FRACTION

Conditions are described in the text: To 400 μg mitochondrial protein was added 100 μg lysosomal membrane protein or lysosomal soluble enzymes with other additions. Turbidity changes were measured for 10 min.

Additions	*Percentage of swelling by lysosomal membrane protein* *
Lysosomal membrane protein	100
Lysosomal soluble enzymes	10
Lysosomal membrane protein + 8 mM malonate	85
Lysosomal membrane protein + 1 mM 6-aminocaproic acid	130
Lysosomal membrane protein + 10 μM diisopropylfluorophosphate	104
Lysosomal membrane protein + 10 μM diethyl-*p*-nitrophenylphosphate	103
Lysosomal membrane protein + 100 μM EDTA	143
Lysosomal membrane protein + 100 μM thenoyltrifluoroacetone	145
Lysosomal membrane protein + 1 mM α-tocopherol	118
Lysosomal membrane protein + 100 μM diphenyl-*p*-phenylenediamine	223
Lysosomal membrane protein + 1% bovine serum albumin	0

* $100 \times \frac{\text{Decrease in absorbance at 520 m}\mu}{\text{Decrease in absorbance at 520 m}\mu \text{ with lysosomal membrane protein only}}$. Corrected for the spontaneous swelling of the mitochondria.

chondrial swelling. This indicates that the swelling is not linked to the oxidation of substrate but results from irreversible membrane damage.

Further evidence that the swelling is not caused by peroxidative damage or by enzymatic hydrolysis by cathepsins or non-specific esterases is shown in Table V. These results compare the capacity of various subcellular fractions to cause mitochondrial swelling, with their ability to catalyze lipid peroxidation and their catheptic and non-specific esterase activities. No correlation can be seen between these enzymes and the swelling capacity of the fractions. The ability to catalyze lipid peroxidation is highest in the fraction composed of nuclei and cell debris, which contains a high concentration of heme compounds within erythrocytes, and in the supernatant which contains soluble heme proteins. Heme compounds are potent catalysts of lipid peroxidation.

The only compound which effectively inhibited the swelling was bovine serum

TABLE V

SOME PROPERTIES OF RAT LIVER SUBCELLULAR FRACTIONS COMPARED WITH THEIR CAPACITY TO SWELL MITOCHONDRIA

The individual assays are described in the text. (a) Uptake of oxygen, μmoles/mg protein per min. (b) Decrease in absorbance at 520 mμ in 10 min. (c) Phenylalanine-equivalent liberated, mμmoles per mg protein per min. (d) β-Naphthol released, mμmoles/mg protein per min.

Fraction	*Peroxidation catalysis* (*a*)	*Swelling capacity* (*b*)	*Catheptic activity* (*c*)	*Esterase activity* (*d*)
Nuclear + cell debris	161.1	0.12	6.2	2,380
Mitochondrial	15.4	0.12	9.3	1,230
Microsomal	14.1	0.07	3.1	3,833
Supernatant	48.0	0.06	2.0	2,039
Lysosomal membrane	5.1	0.37	15.0	802

TABLE VI

THE EFFECT OF BOVINE SERUM ALBUMIN ON MITOCHONDRIAL SWELLING INDUCED BY LYSOSOMAL MEMBRANE

The assay conditions are described in the text, and in Table II.

	*Mitochondrial swelling**
Mitochondria	0.047
Mitochondria + 20 μg lysosomal membrane protein	0.070
Mitochondria + 50 μg lysosomal membrane protein	0.350
Mitochondria + 100 μg lysosomal membrane protein	0.427
Mitochondria + 50 μg lysosomal membrane protein + 1 % bovine serum albumin	0.035
Mitochondria + 50 μg lysosomal membrane protein + 0.01 % bovine serum albumin	0.045
Mitochondria + 50 μg lysosomal membrane protein + 0.0001 % bovine serum albumin	0.162
Mitochondria + 0.1 mM sodium oleate	0.658
Mitochondria + 1.0 μM sodium oleate	0.207
Mitochondria + 1.0 μM sodium oleate + 0.01 % bovine serum albumin	0.156

* Decrease in absorbance at 520 mμ in 10 min.

albumin which is a well-known inhibitor of mitochondrial swelling induced by free fatty acids[13]. Table VI shows the swelling of mitochondria catalyzed by the lysosomal membrane, compared with that produced by oleic acid, and the inhibitory effect of bovine serum albumin upon both modes of swelling. From this table it can be seen that the swelling produced by 100 μg of lysosomal membrane is equivalent to that produced by 10 mμmoles of oleic acid.

Hydrolysis of ^{14}C-labeled lipids by lysosomes

To measure the possible production of free fatty acids by lysosomes, lipolytic enzyme activity was investigated using radioactively labeled substrates. Table VII shows the hydrolysis of glyceryl tri[1-^{14}C]oleate and [^{14}C]lecithin by subcellular fractions of rat liver, compared with the distribution of acid phosphatase, a marker enzyme for lysosomes. The results indicate that both lipase and phospholipase activities are present in the lysosome. It is important to note that these hydrolyses were carried out at pH 5.0 where the activities of previously reported lipases and phospholipases of other subcellular fractions would be very low. The phospholipase activity of the lysosome membrane showed a 19-fold increase over the activity of the homogenate, compared with a 14-fold increase for the lysosomal soluble enzyme, acid phosphatase.

TABLE VII

THE HYDROLYSIS OF [^{14}C]LECITHIN AND GLYCERYL TRI[1-^{14}C]OLEATE COMPARED WITH ACID PHOSPHATASE ACTIVITY IN RAT LIVER SUBCELLULAR FRACTIONS

Rat liver fractions	*[^{14}C]Lecithin hydrolysis**	*Glyceryl tri[1-^{14}C]oleate hydrolysis***	*Acid phosphatase activity****
Homogenate	326	29 404	54.4
Nuclear	64	2 589	36.5
Mitochondria	89	3 318	69.7
Microsomes	193	11 505	29.9
Supernatant	110	3 338	22.0
Lysosome membrane	6200	35 478	506.6
Lysosome soluble enzymes	1586	49 986	757.7

* Free fatty acids released, counts/min per mg protein per h.
** Free fatty acids and diglycerides released, counts/min per mg protein per h.
*** P_i released, mμmoles/mg protein per min.

Though the lysosomal fractions appear to contain considerable lipase activity, the increase in purification compared with the activity of the homogenate is not as marked as for the phospholipase activity.

DISCUSSION

The disruption of mitochondria by lysosomes deserves attention because the lysosome is capable of the intracellular digestion of mitochondria, and because all liver mitochondrial preparations contain lysosomes. The results show that the first stage of this digestion is a loss of mitochondrial membrane function as seen in the uncoupling of oxidative phosphorylation and the activation of mitochondrial ATPase.

This disorganization is reflected in the accompanying high amplitude irreversible swelling, which is independent of the availability of substrates for mitochondrial oxidation. Three possible mechanisms for this type of swelling have been investigated: damage by peroxidation of mitochondrial lipids, changes induced by a surface-active lipid, and damage to mitochondrial membrane lipids by enzymic attack. Peroxidative damage is unlikely as there was no inhibition of swelling by antioxidants, and the lysosome did not have a high capacity for the catalysis of lipid peroxidation in model systems. The presence in lysosomes of a non-dialyzable, heat-labile swelling factor which is effectively inhibited by low concentrations of bovine serum albumin indicates that lysosomal enzymes can catalyze the release of free fatty acids or other surface-active lipids from membranes. Thus damage to mitochondria could arise by two possible mechanisms, direct enzymic degradation of the mitochondrial membrane, and destabilization of the membrane by surface-active lipids produced by lipolytic enzymes of the lysosome. Inhibition by bovine serum albumin indicates that the latter mechanism is most effective in promoting swelling by lysosomes. Bovine serum albumin will also inhibit the uncoupling of oxidative phosphorylation by snake venoms[14], suggesting that similar lipolytic enzymes may be present in the lysosome.

EDTA effects should be considered in the perspective of the reaction sequences. The first reactions of hydrolysis of lecithin and trioleate to produce free fatty acids (Table VII) are not blocked by EDTA. The second reaction of mitochondrial swelling is not inhibited (Table IV) by EDTA. Reactions subsequent to these are inhibited EDTA; namely, activation of ATPase (Table III) and uncoupling of phosphorylation (Fig. 2). These latter two reactions could be inhibited through metal coordination, assuming metals available during mitochondrial swelling activate ATPase. If ATPase activity is a cause of the uncoupling, the latter would also be inhibited.

Phospholipase or lipase activity has not been demonstrated previously in the lysosome, although DE DUVE[15] has postulated that such activity should be present. Evidence has been presented that lysosomes contain a sphingomyelin-cleaving enzyme[16] and a phosphatidic acid phosphatase[17]. Several groups have looked for phospholipase activity in the lysosome but have reported it to be absent. A phospholipase A (EC 3.1.1.4), said to be responsible for the spontaneous aging of rat liver mitochondria *in vitro*, was reported to be localized in mitochondria, and to be separable from acid phosphatase activity by sucrose density-gradient separation[18]. The presence of this endogenous phospholipase A in mitochondria has been confirmed by SCHERPHOF AND VAN DEENEN who also showed that the lysophosphatide products could undergo reacylation[19]. This group has reported the presence in rat liver of two phospholipases A (ref. 20), one located in the microsomes which cleaves phosphatides at the C-1 position to yield 2-monacyl-3-glycerophosphatides and one located in the mitochondria which cleaves at the C-2 position to yield 1-monoacyl-3-glycerophosphatides.

BJØRNSTAD[21,22] has confirmed that both the microsomes and the mitochondria of rat liver contain phospholipase A activity, and was unable to show the presence of phospholipase activity in a lysosome-rich fraction. Failure by this worker to observe phospholipase activity in lysosomal fractions may be due to the use of reaction mixtures at neutral pH where the lysosomal enzymes are less active.

The evidence indicates that the active swelling factor is the membrane-lytic lipid produced by lipolytic enzymes of the lysosome. The lysosome membrane fraction is rich in complex lipids, especially phospholipids. Free fatty acids may be released

from membrane lipids by soluble enzymes of the lysosome during fractionation, storage, or during the freezing and thawing procedure used to disrupt lysosomes.

The properties of the phospholipid-hydrolyzing enzyme of lysosomes have been studied in detail and the results will be reported elsewhere. The enzyme cleaves both fatty acids from lecithin and the optimum pH for the hydrolysis is pH 4.5. EDTA does not inhibit the enzyme significantly. It is probable that inhibition of uncoupling of oxidative phosphorylation by EDTA in this study was caused by the inhibition of mitochondrial ATPase. The swelling phenomenon was not inhibited by EDTA (Table IV).

This lysosomal phospholipase could be the initiator of the digestion of mitochondria and the hemolysis of erythrocytes by lysosomes. Lysis of protective phospholipid membranes by a phospholipase would expose a wide variety of substrates that the lysosome is known to hydrolyze. Studies[23,24] of the hydrolysis of mitochondrial phospholipids by snake venon enzymes have shown that phospholipases are powerful agents for the release of membrane sub-units and the disorganization of the mitochondrion. The reported release of β-hydroxybutyrate dehydrogenase from mitochondria by freezing and thawing[25] might be mediated by a lysosomal phospholipase, by a process analogous to the release of β-hydroxybutyrate dehydrogenase from mitochondria by treatment with snake venom phospholipases[26]. This lysosomal phospholipase may be the cationic protein in polymorphonuclear leucocyte granules that has been reported to be the inflammatory agent in phagocytosis[27]. Such a mechanism would be consistent with the well-known properties of snake and bee venoms as inflammatory agents. Thus in tissue damage and repair, and in the turnover of subcellular organelles, the lysosomal phospholipase could be an important initiator of catabolism.

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