

Swelling of *Phaseolus* Mitochondria Induced by the Action of Phospholipase A¹

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ABSTRACT

Phaseolus vulgaris mitochondria incubated in sucrose swell rapidly upon the addition of phospholipase A. Bovine serum albumin inhibits the swelling. The release of free fatty acids as a result of phospholipase A action on the mitochondria is detected only in the presence of bovine serum albumin, which promotes the hydrolysis of both mitochondrial phospholipids and purified lecithin. Either free fatty acid or lysolecithin is able to initiate an extensive mitochondrial swelling in sucrose. It is suggested that phospholipase A-induced swelling results from the release of lysophosphatides plus free fatty acids and their subsequent detergent action on the membranes rather than phospholipid loss *per se*.

lipase A to provide a clear separation of the inner and outer membrane systems of beef heart mitochondria. It has also been reported that the addition of venom to rat liver mitochondria produced a marked swelling response (26).

As part of a detailed study of volume changes exhibited by *Phaseolus* mitochondria (11, 12), it was of interest to determine whether phospholipase A was able to induce swelling in plant mitochondria as well as in animal. *Phaseolus* mitochondria show rapid spontaneous swelling in tris-buffered KCl in a process strongly inhibited by bovine serum albumin but do not swell appreciably in tris-buffered sucrose (12). We wished to determine whether phospholipase A would induce swelling in sucrose and whether BSA⁴ affected the system.

MATERIALS AND METHODS

Isolation of Mitochondria. Seed of dwarf French bean, *Phaseolus vulgaris* (var. Canadian wonder) was sown and germinated as described by Opik and Simon (21). Mitochondria were isolated from the etiolated hypocotyls on the 6th day following planting by a method similar to the procedure of Kenefick and Hanson (17) as described previously (11). Mitochondrial nitrogen content was determined by a method involving digestion and nesslerization.

Volume Changes. Changes in light scattering were followed by changes in absorbance at 520 m μ as described previously (11). The routine procedure for measuring volume changes was to add, at zero time, 0.1 ml of mitochondrial suspension (0.07-0.10 mg of N) to 2.9 ml of 200 mM sucrose, 20 mM tris-HCl (pH 7.5) \pm 1 mg/ml BSA maintained throughout at 30 C. The initial reading of each cuvette was recorded at 1 min and subsequently for 10 min. The following additions were then made in a variety of experiments: 100 μ l (2 enzyme units) of a phospholipase A suspension in sucrose-tris; 100 μ l of lysolecithin dispersed in sucrose-tris at a range of concentrations using an ultrasonic disintegrator (Measuring and Scientific Equipment Ltd., Crawley, Sussex); 10 μ l of oleate, linoleate, or linolenate in absolute ethanol at a range of concentrations. Readings were then continued for a further 20 to 30 min. Corrections have been made to the results to allow for the small dilution occasioned by additions to the reaction system.

Isolation and Determination of Mitochondrial Free Fatty Acids. At zero time, 0.3 ml of mitochondrial suspension was added to 8.7 ml of 200 mM sucrose, 20 mM tris-HCl (pH 7.5) \pm 1 mg/ml BSA and incubated at 30 C for 10 min. Six enzyme units of a phospholipase A suspension (300 μ l) in sucrose-tris were then added and the incubation was continued for a further 20 min. Fatty acids were then isolated by the method of Dole and Meinertz (9) and estimated according to the procedure of Duncombe (10) as described previously (12). The results are expressed in

Phospholipase A (phosphatide acyl-hydrolase, EC 3.1.1.4) of snake venom catalyzes the hydrolysis of diacylated nitrogen-containing phosphoglycerides with the liberation of the fatty acid esterified at the β -position and formation of the lysophosphoglyceride. The metabolic results of adding snake venom or a phospholipase A preparation to animal mitochondria are well documented. Petrushka *et al.* (22) observed, following the addition of heated snake venom to rat mitochondria, an initial stimulation of the respiratory rate followed by a rapid inhibition. Phospholipase A has been shown to inhibit NADH oxidase, succinate- and NADH-cytochrome *c* reductase (28) and succinoxidase activities (13). Several workers have shown a decrease in the P/O ratio of isolated mitochondria as a result of phospholipase A action *in vitro* (23, 25) and also *in vivo* (2).

In addition to the effects of phospholipase A on metabolism, there are several reports indicating that structural disorganization also occurs. The treatment of an NADH oxidase preparation resulted in extensive solubilization of the NADH dehydrogenase activity (24), and cytochrome *c* release occurred upon incubation of electron transport particles with phospholipase A (1). Petrushka *et al.* (23) showed that incubation with venom resulted in release of soluble proteins including ATPase and noted a simultaneous destruction of mitochondrial organization as seen with the phase contrast microscope. Bachman *et al.* (3) used phospho-

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⁴ Abbreviations: BSA: bovine serum albumin; FFA: free fatty acid.

terms of calibration for oleic acid. The BSA used in fatty acid determinations was routinely rendered fatty acid-free according to the method of Chen (6).

Lecithin Hydrolysis. One ml (20 enzyme units) of a phospholipase A suspension in sucrose-tris was added, at zero time, to 35.0 ml of 2 mM egg lecithin dispersed in 200 mM sucrose, 20 mM tris-HCl (pH 7.5) \pm 1 mg/ml BSA and incubated at 30 C for 30 min. Samples were removed at regular time intervals, and fatty acids were extracted and determined as above. Duncombe (10) estimations were made against a zero time blank.

Chemicals. Phospholipase A was obtained from Boehringer Corporation Ltd., lysolecithin from Koch-Light Laboratories Ltd., egg lecithin from British Drug Houses, Ltd. fatty acids from L. Light and Co. Ltd., and BSA from Armour Pharmaceutical Company Ltd.

RESULTS

Figure 1 shows that *Phaseolus* mitochondria exhibit only a slight swelling when incubated in tris-buffered sucrose. This is in agreement with Lyons *et al.* (19), who found that mitochondria isolated from a variety of plant tissues failed to swell appreciably in sucrose. The addition of phospholipase A to the mitochondria at the end of a 10-min incubation period produces a rapid swelling response. The rate of swelling is reasonably linear for about 10 min and thereafter rapidly declines, and swelling is essentially complete about 15 min after addition of the enzyme. The incorporation of 1 mg/ml BSA in the system results in a slower rate of swelling which increases for about 10 min and thereafter declines, and swelling continues at a slow rate. In this particular experiment there was a decrease in the initial absorbance of 43% between 10 and 30 min in the incubation lacking BSA and 23% in the incubation with BSA. Petrushka *et al.* (23) have reported that serum albumin protects against the uncoupling action of heated venom. Various workers have found that calcium is necessary for the effective action of phospholipase A (8, 13). The incorporation of 0.5 mM CaCl_2 in the reaction medium produced

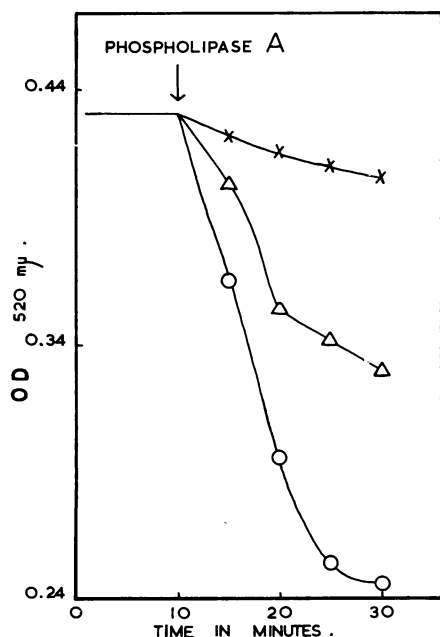


FIG. 1. Swelling of *Phaseolus* mitochondria induced by the action of phospholipase A. At zero time, 0.1 ml of mitochondrial suspension was added to 2.9 ml of 200 mM sucrose, 20 mM tris-HCl (pH 7.5) \pm BSA. Phospholipase A (2 enzyme units) was added at 10 min. X: No additives; O: phospholipase A - BSA; Δ : phospholipase A + BSA.

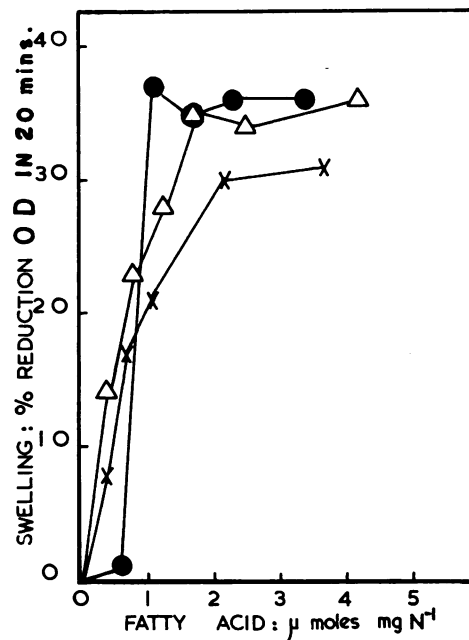


FIG. 2. The effect of free fatty acid concentration on swelling. At zero time, 0.1 ml of mitochondrial suspension was added to 2.9 ml of 200 mM sucrose, 20 mM tris-HCl (pH 7.5). A known concentration of FFA was added at 10 min. and swelling was allowed to proceed for a further 20 min. The results are expressed as the percentage reduction of the 10-min absorbance at the end of the 20-min reaction with FFA and have been adjusted for the small amount of swelling of the control treatment minus FFA. ●: Oleate; Δ : linoleate; X: linolenate.

no increase in the rate of swelling of *Phaseolus* mitochondria or in the final level attained. Calcium was, therefore, omitted from the system.

Several workers have suggested that the action of phospholipase A on mitochondria is partly a result of free fatty acid produced from hydrolysis of the membrane phospholipids (13, 31). In addition, BSA is well known for its ability to bind FFA, thus removing them from any potential sphere of action (4). This suggested to us the possibility that the phospholipase A-induced swelling of *Phaseolus* mitochondria in sucrose (Fig. 1) may be due to FFA produced, which in the presence of BSA are bound, resulting in less swelling. The swelling of *Phaseolus* mitochondria in sucrose induced by the addition of oleic acid has been described previously (12). The process is rapid and is almost completely inhibited by BSA. The effect of varying concentrations of oleate, linoleate, and linolenate on the degree of swelling of *Phaseolus* mitochondria in sucrose is shown in Figure 2. All three fatty acids are able to induce swelling and show a similar relationship between their concentration and degree of swelling produced. It is necessary to add 1 to 2 μ moles of fatty acid per mg of N to attain approximately the same degree of swelling as with an addition of phospholipase A (Fig. 1).

It seemed valuable at this stage to assay the amount of FFA produced as a direct result of phospholipase A action on the mitochondria. It can be seen from Table I that freshly isolated *Phaseolus* mitochondria already contain a high endogenous FFA level, the metabolic significance of which has been discussed elsewhere (12). A 20-min incubation of the mitochondria in sucrose-tris with or without phospholipase A produces no measurable increase over and above the high "background" level. However, the presence of both phospholipase A and BSA results in the production of approximately 1 μ mole of additional FFA per mg of N during the 20-min incubation. From these data, BSA would appear to stimulate mitochondrial phospholipid hydrolysis by

phospholipase A. To test this hypothesis, we examined the effect of BSA on the rate of lecithin hydrolysis by phospholipase A. Figure 3 shows that BSA has no effect on the rate of hydrolysis of lecithin (suspended in sucrose-tris) for approximately the first 15 min of the reaction. Thereafter, lecithin hydrolysis in the system lacking BSA ceases completely but still proceeds, albeit at a slower rate, in the presence of BSA. Although BSA has a less marked effect in this system than in the mitochondrial incubation of Table I, it seems reasonable to suppose that the mechanism involved is the same in both cases.

As well as producing 1 mole of FFA, the hydrolysis of 1 mole of phospholipid by phospholipase A produces 1 mole of lysophosphatide. Lysophosphatides are known to promote swelling and inhibit the contraction of animal mitochondria (29). Thus, it

Table I. Determination of Free Fatty Acids after Incubation of Mitochondria with Phospholipase A

At zero time, 0.3 ml of mitochondrial suspension was added to 8.7 ml of 200 mM sucrose, 20 mM tris-HCl (pH 7.5) \pm 1 mg/ml BSA. Phospholipase A (6 enzyme units) was added where indicated at 10 min. FFA were extracted at zero time from the freshly isolated mitochondria and from the incubated mitochondria at 30 min (see Fig. 1). Values are the means of three experiments.

Treatment	FFA $\mu\text{moles/mg N}$
Freshly isolated	1.34
Incubated in sucrose-tris:	
- phospholipase A, - BSA	1.30
+ phospholipase A, - BSA	1.39
+ phospholipase A, + BSA	2.30

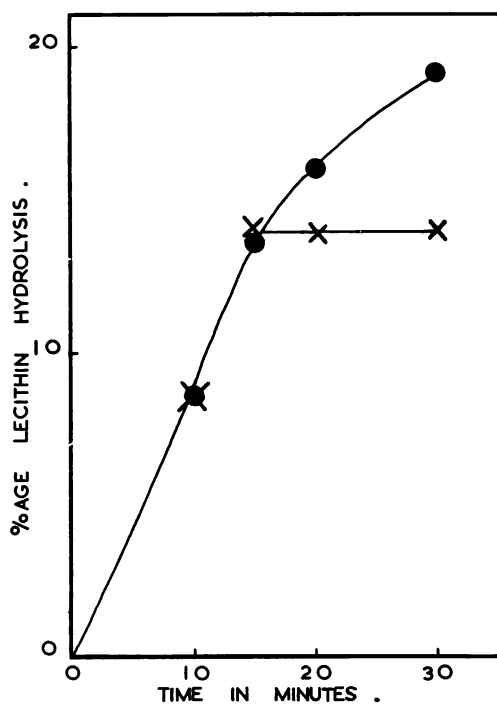


FIG. 3. The effect of bovine serum albumin on the hydrolysis of lecithin by phospholipase A. At zero time, phospholipase A (20 enzyme units) was added to 35.0 ml of 200 mM sucrose, 20 mM tris-HCl (pH 7.5), 2 mM lecithin \pm 1 mg/ml BSA. Samples were withdrawn at the times indicated and analyzed for FFA as described in "Materials and Methods." \times : Without BSA; \bullet : with BSA.

seemed possible that lysophosphatides may be involved in the phospholipase A-induced swelling of *Phaseolus* mitochondria (Fig. 1). Figure 4 shows that the addition of lysolecithin to mitochondria in sucrose causes a rapid swelling which is completely inhibited by BSA. Estrada-O *et al.* (14) have shown that the release of glutamic-aspartic transaminase from rat liver mitochondria as a result of lysolecithin action is strongly inhibited by BSA. This result was interpreted in terms of the formation of a

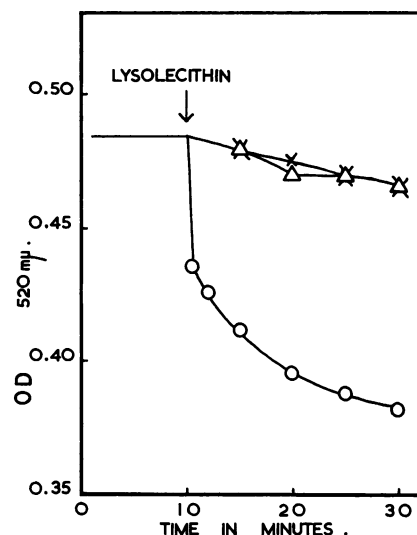


FIG. 4. Swelling of *Phaseolus* mitochondria induced by the action of lysolecithin. The initial experimental conditions were as in Figure 1. At 10 min, 0.24 μmole of lysolecithin per mg of N was added. \times : No additives; \circ : lysolecithin - BSA; \triangle : lysolecithin + BSA.

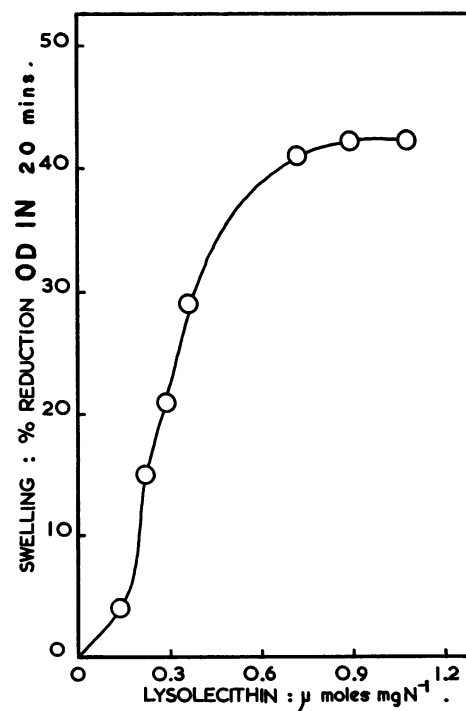


FIG. 5. The effect of lysolecithin concentration on swelling. The initial experimental conditions were as in Figure 2. Following a 10-min incubation period, a known concentration of lysolecithin was added, and swelling was followed for a further 20 min. The results are expressed as in Figure 2.

nonspecific complex of albumin and lysolecithin. In addition, Luzzio (18) found that serum albumin indirectly inhibited the hemolysis of washed red blood cells by binding lysolecithin during or after its formation. The effect of various concentrations of lysolecithin on the degree of swelling of *Phaseolus* mitochondria in sucrose is shown in Figure 5. The addition of about 0.5 μ mole of lysolecithin per mg of N produces approximately the same degree of swelling as a phospholipase A addition (Fig. 1). It should be noted that rather more FFA is needed to produce an equivalent swelling response (Fig. 2).

DISCUSSION

The results show that the addition of phospholipase A or either of the products of phospholipid hydrolysis can induce extensive swelling of *Phaseolus* mitochondria in sucrose. BSA inhibits swelling induced by the action of phospholipase A and lysolecithin. (We have shown elsewhere the inhibition by BSA of oleate-induced swelling [12].) It was not possible to detect the FFA produced as a result of incubating the mitochondria with phospholipase A except in the presence of BSA which stimulated hydrolysis of both mitochondrial phospholipids and purified lecithin.

The increased phospholipase A activity in the presence of BSA (Table I, Fig. 3) is worthy of comment. Wojtczak and Lehninger (30) have shown that BSA does not enhance U factor production in sonically disrupted rat liver mitochondria. Honjo *et al.* (16) found that the protective effect of serum albumin during the aging of pancreas mitochondria was mainly due to inhibition of mitochondrial phospholipid hydrolysis thought to be due to endogenous phospholipase A activity. If the interpretation of these authors is correct, this result would appear to be in direct contrast to our findings (Table I). However, Dawson (8) found that lecithin hydrolysis by phospholipase A was inhibited by FFA. In addition, diethyl ether greatly stimulated the hydrolysis of lecithin, but not phosphatidylethanolamine, and it was concluded that this was due in part to the removal of FFA from the surface of the lecithin particles, enabling fresh substrate molecules to gain access to the enzyme. The data of Figure 3 could indicate that, once approximately 14% of the lecithin has been hydrolyzed, there is sufficient buildup of FFA on the lecithin particles to inhibit the reaction completely. In the presence of BSA some of the fatty acids would be bound, removed from the lecithin, and the inhibition partially released.

There are at least two possible reasons for our inability to detect measurable FFA production following incubation of *Phaseolus* mitochondria with phospholipase A in the absence of BSA (Table I). (a) The endogenous FFA of the freshly isolated mitochondria may inhibit the reaction (8). (b) It has been found that calcium is necessary for the effective hydrolysis of lecithin by phospholipase A (8). Edwards and Ball (13) showed that the inactivation of succinoxidase by venom required calcium and the enzymic production of FFA in intact and sonically disrupted rat liver mitochondria was stimulated by calcium (30). However, Chefurka (5) has suggested that the endogenous phospholipase of aging mouse liver mitochondria is nearly fully activated by endogenous calcium. Thus, although we observed no increase in the phospholipase A-induced swelling of *Phaseolus* mitochondria in the presence of calcium, its incorporation in our system would perhaps have increased FFA release in the absence of BSA. Possibly *Phaseolus* mitochondria retain sufficient calcium to stimulate adequate phospholipase A activity to result in a maximal swelling response.

The mode of action of phospholipase A in disorganizing membrane structure and metabolism has been the subject of some controversy. The importance of mitochondrial phospholipids in the processes of electron transport and energy coupling is now well

established (15). Nygaard and Sumner (20) explained the inactivation of succinoxidase by venom in terms of the hydrolysis of lecithin which they suggested might be a component linking succinic dehydrogenase with cytochrome *c*. The fall in P/O ratio following a venom addition has been explained primarily as due to loss of phospholipid (23, 25). While not excluding the possibility that the phospholipase A-induced swelling of *Phaseolus* mitochondria is a result of hydrolysis of membrane phospholipids *per se* leading to an increase in membrane permeability (14), the data of Table I and Figure 1 suggest that this mechanism is unlikely. Although far more FFA is released and therefore more phospholipid hydrolyzed in the presence of BSA, the degree of swelling is reduced to about one-half of that occurring in the absence of BSA.

In our experiments, both of the hydrolytic products of phospholipase A action are able to produce a swelling response which is inhibited by BSA (Fig. 4, Reference 12). The best recognized effects of FFA on mitochondria are the uncoupling of phosphorylation from oxidation (27) and the promotion of swelling (27, 30). Edwards and Ball (13) found that oleate could stimulate the inactivation of their succinoxidase preparation by venom and suggested that unsaturated FFA may be involved in the inhibition. A similar possibility has been cited by Ziegler *et al.* (31) for the uncoupling of energy transfer and appearance of reverse acceptor control in venom-treated rat liver mitochondria. In addition, a requirement for serum albumin, as well as phospholipid and cytochrome *c*, has been shown in the maximum restoration of oxidase activity in electron transport particles following a prolonged treatment with phospholipase A (1). This requirement was related to inhibition of the oxidase system by released fatty acids. On the other hand, several workers have implicated lysophosphatides as the destructive agents involved in the action of phospholipase A. Lysolecithin has been suggested as a possible causal agent of the fall in P/O ratio of rat mitochondria (23). The hemolysis of red blood cells is considered to be a result of lysolecithin production (18), and Condrea and Rosenberg (7) suggested that the increase in permeability and blocking of axonal contraction in squid giant axons is more likely to be due to lysophosphatide production than to phospholipid splitting *per se*.

In light of the above data, taken from a variety of membrane systems, we would suggest that the swelling of *Phaseolus* mitochondria induced by phospholipase A is a consequence of the action of lysophosphatides and FFA produced rather than phospholipid loss *per se*. However, it should be noted that larger amounts of FFA (or lysolecithin) are required to induce a swelling response (Figs. 2, 5) than can be detected following phospholipase A-induced swelling in the absence of BSA (Table I). It seems reasonable to suggest that, when produced as a direct result of phospholipase A action, the hydrolytic products are close to the site of potential destructive action. But, when added exogenously, possibly only a relatively small proportion is capable of access to this site. Indeed, Edwards and Ball (13) found that inactivation of their succinoxidase preparation by venom could only be simulated by a concentration of oleate 3 times in excess of the FFA concentration produced by the venom action.

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