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Purification and Characterization of an A Type Phospholipase from Potato and Its Effect on Potato Mitochondria[1, 2]

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ABSTRACT

A potato (*Solanum tuberosum*) phospholipid acyl-hydrolase, which—in the pH range 7.5 to 8.5—is at least 10,000 times more effective with phospholipids than with galactolipids, has been purified and characterized. It is a soluble enzyme readily distinguished from a neutral lipid lipase and a third lipid acyl-hydrolase which, while acting on phospholipid, shows a decided preference for glyceryl monoolein. The phospholipase in question has a pH optimum of 8.5, is stimulated by Ca^{2+} at pH above 7.5 and inhibited by Ca^{2+} at lower pH, is not dependent on detergents although stimulated by Triton X-100 to a moderate extent, and remains very active at temperatures close to zero. The phospholipids of intact potato mitochondria are highly susceptible to degradation by potato phospholipase, and it is suggested that this enzyme is involved in the extensive lipid breakdown which occurs in fresh potato slices following cutting, and in the deterioration of mitochondria during their preparation and aging.

In the preceding paper (14), we established the presence of three distinct lipid acyl-hydrolases in potato tuber with different specificities. Enzyme I was shown to be an acyl-hydrolase for neutral lipids. Enzyme II showed a preference for phospholipids and galactolipids. Enzyme III, while attacking phospholipids and galactolipids to some extent, displayed a pronounced specificity and activity towards glycerol monoolein. A generic lipolytic acyl-hydrolase from potato described by Galliard (8, 9) and, more recently, shown to comprise several isoenzymes (11) seemingly encompasses our enzymes II and III. Disparities between our enzyme II and Galliard's enzyme with respect to phospholipid acyl-hydrolase activity, in particular, caused us to describe further the phospholipase characteristics of our enzyme II, and to compare it with Galliard's enzyme and with our enzyme III. Recently, a phospho- and galactolipase from potato has been isolated (15) which resembles our enzyme II.

MATERIALS AND METHODS

Lipases were prepared from intact tubers (*Solanum tuberosum* var. Russet Burbank) or from fresh or aged slices, as described in the previous paper (14).

Substrate Preparation and Enzyme Assay. Substrate was prepared according to Gatt and Barenholz (13). 1,2-Dipalmityl-1-^{14}C lecithin was dissolved in chloroform-methanol (2:1) and added either to water or water containing a suitable quantity of Triton X-100. The mixture was gradually brought to 70 C and the organic solvents were evaporated under a stream of nitrogen. An appropriate amount of 0.5 M citrate, phosphate, or tris buffer solution was added and mixed well, and the solution was shaken for several min in a boiling water bath. The solution was subsequently incubated for 15 min at the reaction temperature, at which time 0.1 mg of enzyme protein was added in a final volume of 1.2 ml.

The reaction mixture was gently shaken for 20 min in a water bath. Enzyme activity was terminated with Dole's reagent (5) consisting of isopropyl alcohol-heptane-1 N H_2SO_4 (40:10:1, v/v). Palmitate-1-^{14}C partitioned into heptane, and its radioactivity was determined by scintillation counting. When the reaction was run in ether when all reaction products were to be identified, 1 ml of ether was added to the lecithin residue resulting from the evaporation of the organic solvents from a solution of labeled lecithin in chloroform-methanol (2:1 v/v). Enzyme was added in a small volume of buffer and the reaction mixture was incubated for 30 min with very rapid shaking in a water bath. Enzyme action was terminated with 1 ml of hot alcohol, and the entire mixture then dried under a stream of nitrogen. The residue was taken up in 1:1 chloroform-methanol and spotted and developed on thin layer plates (21).

Estimation of Potato Phospholipase Action on Potato Mitochondria. Mitochondria were prepared as previously described (16). To a suspension containing approximately 15 mg of mitochondrial protein in a total volume of 0.6 ml was added 0.1 mg of potato phospholipase (enzyme II). The mixture was incubated for 15 min at 10 C after which the reaction was terminated with hot alcohol, and lipids were extracted under acid conditions, cleaned by the methods of Bligh and Dyer (2) and Folch *et al.* (7), and finally suspended in 0.5 ml of benzene-ether (4:1, v/v). The latter solution was banded on a TLC plate of Silica Gel G and developed by the method of Randerath (21). Standards chromatographed at one side of the plate were visualized with iodine vapor to establish the positions of fractions of interest. The latter were scraped from the plate and methylated as per Metcalf *et al.* (18). Methylated fatty acids were subsequently separated by gas chromatography and estimated quantitatively.

RESULTS

The three lipolytic acyl-hydrolases isolable from potato tuber tissue are all soluble enzymes found in the supernatant solution following centrifugation of the homogenate at 104,000*g* for 1 hr. Of the three enzymes, enzyme II, which is precipitated by $(NH_4)_2SO_4$ in the range 40 to 60%, and which precedes enzyme III in the eluant of a Sephadex G-200 column, contains the bulk of the phospholipase activity (Table I).

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[2] In honor of Leon Bernstein—warm friend, dedicated plant physiologist, and devoted editor.

Table I. *Purification and Activity of Potato Phospholipase*

The enzyme was assayed at pH 8.5 in the presence of 1 mM Ca^{2+}. The substrate was lecithin 1,2-dipalmityl-1-^{14}C.

Fraction	Relative Protein	Relative Activity	Activity	Purification
	%		*μmoles free fatty acid/mg protein·hr*	*relative units*
Supernatant	100	100	10.4	1
40–60% $(NH_4)SO_4$ fraction	12	32	36.5	3.4
Sephadex G-200				
Enzyme II	3.8	53	145.6	14
Enzyme III	1.5	5.4	37.4	3.5
DEAE-cellulose				
Enzyme II	2	50	260.5	25
Enzyme III	0.2	0.6	62.4	3

Effect of pH and Ca^{2+} on Potato Phospholipase Activity. The effect of pH and Ca^{2+} on potato phospholipase is depicted in Figure 1. In the absence of Ca^{2+} the pH profile of enzyme II is very broad from pH 4 to pH 10 with the hint of an optimum at pH 8.5. In the presence of 1 mM Ca^{2+} there is a sharp stimulation above pH 7.5, with an optimum at pH 8.5, and increasing inhibition from pH 7.5 to pH 4 (Fig. 1). Enzyme III is maximally effective in the range of pH 9, with or without Ca^{2+}, and is virtually inactive at pH 7. The Ca^{2+} response curves are shown in Figure 2, and the divalent cation specificity is indicated in Table II. The Ca^{2+} effect is maximal at 1 mM. Mg^{2+} is less effective than Ca^{2+}, and Mn^{2+} even less so. While EDTA has no effect on the untreated enzyme, it prevents Ca^{2+} stimulation at high pH and Ca^{2+} inhibition at low pH.

Effect of Temperature on Potato Phospholipase. In temperature studies enzyme and substrate were brought to temperature for 15 min before mixing. Enzyme II is markedly active at 2 C and responds to increasing temperature with a Q_{10} of roughly 2 between 2 and 15 C. There is little effect of temperature between 25 and 50 C (Fig. 3). The temperature response is independent of the presence of Ca^{2+} and indifferent to pH. Enzyme III, by contrast, shows no activity at 15 C, while responding sharply to temperatures between 15 and 25 C.

Effect of Triton X-100 on Potato Phospholipase. Potato phospholipase as prepared herein does not require detergent for activity, albeit 0.2% Triton X-100 (2 moles/mole lecithin) increases activity some 60% (Fig. 4). By contrast, the lipolytic acyl-hydrolase described by Galliard (9) is essentially inactive in the absence of Triton X-100, its activity being increased 30- to 40-fold on addition of detergent. Triton X-100 at levels above the optimum caused marked inhibition (Fig. 4), as did dodecyl sulfate at low concentrations, and Na deoxycholate at concentrations as low as 0.02% (90% inhibition).

Phospholipase activity was determined by measuring the rate of release of labeled palmitic acid from 1,2-dipalmityl-1-^{14}C lecithin. This gross measurement of enzyme activity, wherein released free fatty acids are taken into heptane, and the radioactivity of the heptane solution is subsequently measured, says nothing about the specificity of the enzyme with respect to positions 1 and 2. Hence, an alternative procedure was used where reactants and products were separated on TLC plates. The only radioactive components were found to be free palmitic acid and lecithin—there being no evidence of lysolecithin—indicating an attack at both positions 1 and 2 (15). By contrast, the endogenous degradation of mitochondrial phospholipid in cauliflower yields stoichiometric quantities of FFA[3] and lysolecithin (6). It is noteworthy in this connection that the fatty acids esterified at position 2 in plant mitochondrial phospholipids comprise predominantly the highly unsaturated linoleic and linolenic acids (6).

Influence of Potato Phospholipase on Potato Mitochondria. It has been pointed out that a pronounced degradation of phospholipid occurs rapidly in freshly prepared potato slices (14), and that organelle ultrastructure deteriorates as well (23). The implication that potato phospholipase might be involved in the disorganization of potato mitochondria *in situ* was tested by submitting isolated mitochondria from both fresh and aged potato slices to enzyme II. Table III indicates that as much as 35% of the mitochondrial polar lipid fatty acids are hydrolyzed in 15 min at 10 C by a small quantity of enzyme II. It is to be noted that the FFA which are isolated chromatographically fall far short of accounting for the fatty acids which are lost from the polar lipids. The disparity is seemingly due to the high rate of fatty acid peroxidation which accompanies hydrolysis. Lipoxygenase is plentiful in potato extracts (8) and has been observed in potato mitochondrial fractions as well (B. Axelrod and G. G. Laties, unpublished). Peroxidatic products range from the hydroperoxides of linoleic and linolenic acids primarily, through

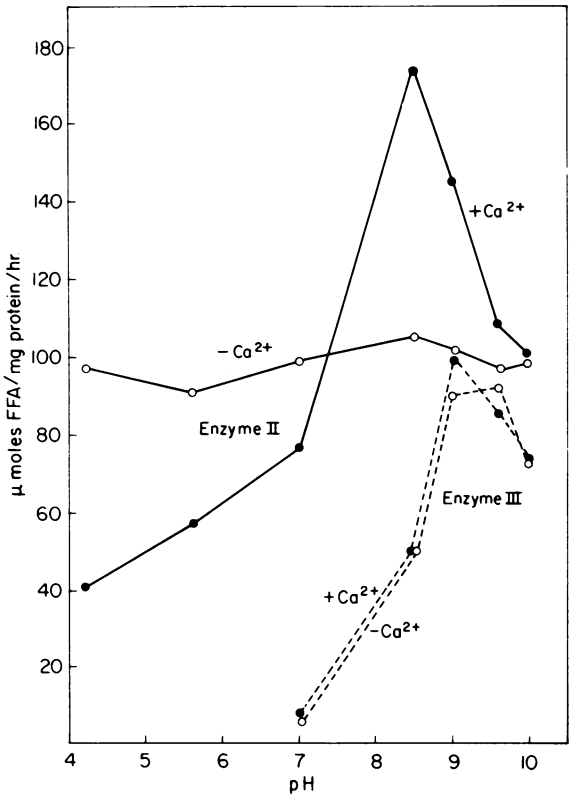

FIG. 1. Effect of pH and Ca^{2+} on phospholipase activity of enzyme II and enzyme III. The substrate was 1,2-dipalmityl-1-^{14}C-lecithin and Ca^{2+} was 1 mM. The temperature was 25 C. The buffers were pH 4.5–5.5, 0.05 M citrate buffer; pH 6–8, 0.05 M K phosphate buffer; pH 8.5–10, 0.05 M tris·HCl buffer.

[3] Abbreviation: FFA: free fatty acids.

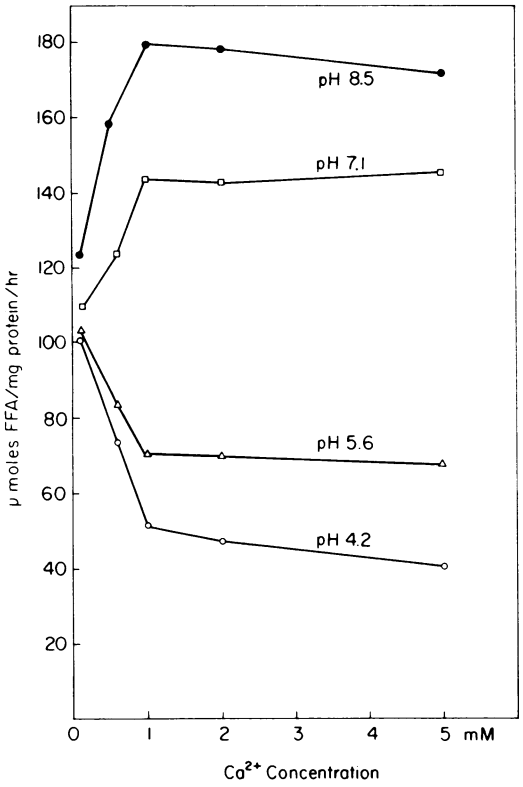

FIG. 2. Effect of Ca^{2+} concentration on potato phospholipase (enzyme II).

Table II. *Comparison of Ca^{2+}, Mg^{2+}, and Mn^{2+} Effects on Potato Phospholipase (Enzyme II) and Counteracting Effect of EDTA*

Ca^{2+}, Mg^{2+}, and Mn^{2+} (1 mM) were added as the choride. The substrate was lecithin 1,2-dipalmityl-1-^{14}C.

Treatment	Phospholipase Activity			pH 8.5
	pH 4.2	pH 5.6	pH 7.0	
	μmoles free fatty acid/mg protein·hr			
Control	101	103	115	129
EDTA (1 mM)	95	99	109	114
EDTA (2 mM)	108	97	115	117
Ca^{2+}	63	86	169	246
Ca^{2+} + 2 mM EDTA	103	91	104	148
Mg^{2+}	67	85	146	178
Mg^{2+} + 2 mM EDTA	88	80	95	113
Mn^{2+}	82	88	114	135
Mn^{2+} + 2 mM EDTA	100	96	92	119

colneleic and colnelenic acids respectively derived therefrom (10, 12) and low molecular breakdown products of these divinyl ether fatty acids. The various degradation products move more slowly than the FFA on TLC plates, and seemingly account for the noted disparity. Acetone precipitation is required to free potato phospholipase from lipoxygenase (8) (see below). Potato phospholipase (enzyme II) not only degrades membrane-bound phospholipid, but may bring about the solubilization of membrane-bound enzymes in the process (as described under "Discussion").

DISCUSSION

The most notable difference between our phospholipase purification procedure and Galliard's is that the latter involves an initial acetone precipitation (which frees the acyl-hydrolase

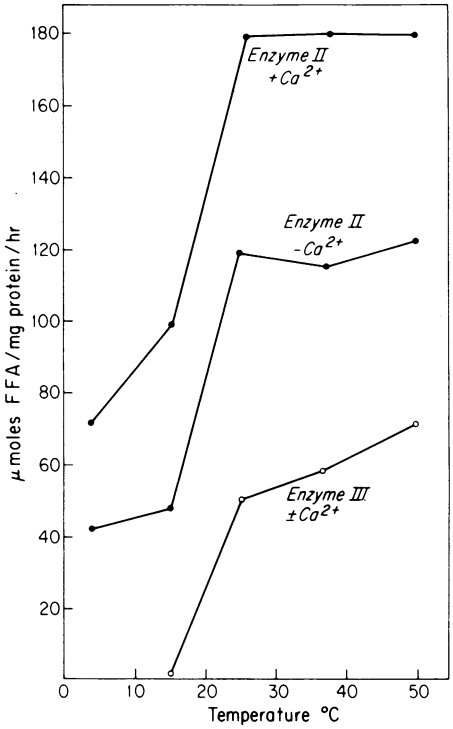

FIG. 3. Effect of temperature on the phospholipase activity of enzymes II and III. Ca^{2+} was 1 mM and tris·HCl buffer was 0.05 M at pH 8.5.

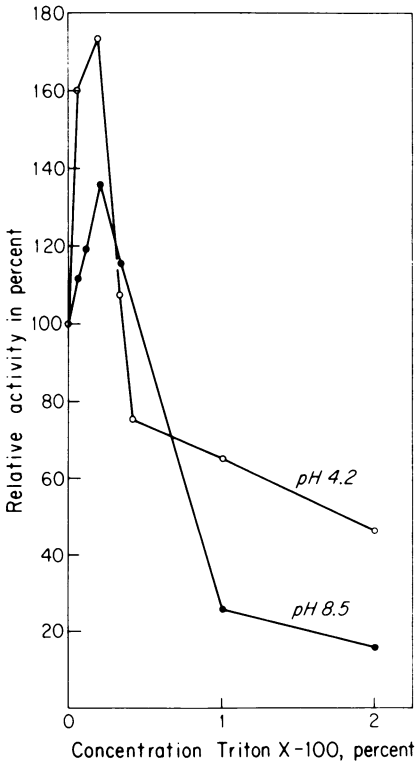

FIG. 4. Effect of Triton X-100 concentration on potato phospholipase (enzyme II).

from lipoxygenase activity (8, 9)). The ways in which phospholipase II differs from Galliard's original lipid acyl-hydrolase suggest the latter may be associated with a large protein or lipoprotein component extraneous to the enzyme itself. The latter prospect is reinforced by the observation that the generic lipid

Table III. *Effect of Potato Phospholipase (Enzyme II) on Potato Mitochondria*

The reaction mixture contained about 15 mg of mitochondrial protein and 0.1 mg of enzyme II. Incubation period was 15 min at 10 C, in 0.05 M K phosphate, pH 7.5. Control incubated in buffer alone. Reaction was terminated with hot alcohol.

Mitochondrial Source	Treatment	Polar Lipid	Free Fatty Acid
		nmoles fatty acid/mg mitochondrial protein	*nmoles/mg mitochondrial protein*
Fresh slices	Control	395	7
	Enzyme II	252	18
Aged slices	Control	450	28
	Enzyme II	292	102

acyl-hydrolase displays three bands on gel electrophoresis (9, 11). It is an open question whether acetone precipitation leads to contamination. Whereas Hirayama *et al.* (15) also use acetone in the extraction of potato lipid acyl-hydrolase, their enzymic assays, which yield high specific activities, are routinely carried out in the presence of 0.1 mM sodium deoxycholate. In any event, the generic lipid acyl-hydrolase (8, 9) and enzyme II differ as follows. (*a*) The pH optimum of enzyme II is approximately 8.5 in the presence or absence of detergent, whereas Galliard's lipid acyl-hydrolase has a pH optimum of about 5.2 without detergent and 8.5 with Triton X-100. (*b*) Enzyme II is enriched 30- to 50-fold during purification while the specific activity of the generic lipid acyl-hydrolase is increased but 4.4 times (*c*) Triton X-100 causes roughly a 60% increase in activity of enzyme II while stimulating Galliard's enzyme 30- to 40-fold. (*d*) Enzyme II is sharply stimulated by Ca^{2+} above 7.5 and inhibited by Ca^{2+} at lower pH. The generic lipid acyl-hydrolase is indifferent to Ca^{2+}.

Both our enzyme II and Galliard's lipolytic acyl-hydrolase actively deacylate mono- and digalactolipids. The deacylation of both phospholipids and galactolipids involves esters in both the 1 and 2 positions. We failed to find lysolecithin on presentation of dipalmityl-1-^{14}C labeled lecithin. Hydrolysis may nevertheless occur sequentially, since the deacylation of monoacyl lipids is rapid, and phospholipase A_2 activity may be rate-limiting (8, 9). In this connection, the nature of the fatty acid esterified at position 2 may determine the kinetics of phospholipase A_2 action. In cauliflower mitochondria, the fatty acids at position 2 of phosphatidyl choline and phosphatidyl ethanolamine are highly unsaturated, being primarily linolenic and linoleic acids (6). When cauliflower mitochondria are kept at 0 C, the aforementioned phospholipids drop markedly with time, while their respective lyso derivatives appear in essentially stoichiometric proportions (4).

Phospholipase II is remarkably active at 0 C. It may be recalled that 40% of *slice* phospholipid and galactolipid is lost in minutes at 0 C following cutting (14). By contrast, enzyme III shows no activity below 15 C. Since enzyme III with its predilection for glyceryl monoolein, and for the N-methyl indoxyl ester of butyrate rather than myristate, has the characteristics of an esterase rather than a *bona fide* lipase, it may well be that the apparent effect of temperature on the phospholipase activity of enzyme III may relate to the physical state, *i.e.*, to the degree of solubilization, of the substrate.

While Triton X-100 has an effect on lecithin hydrolysis by enzyme II, the effect is modest compared with that shown by the lipid acyl-hydrolase prepared by acetone precipitation (9). The optimal level of detergent was found to be similar to that described by Dennis (4) and by Galliard (8, 9), approximately 2 moles Triton X-100 per mole lecithin. While the stimulating effect of detergent has occasionally been imputed to a direct effect on the enzyme (*viz.* deoxycholate on pancreatic lipase) (26), the more general view is that detergent influences the structure of lipid micelles so that substrate becomes more accessible to lipase attack. In acetone preparations, where phospholipase is seemingly associated with a large and presumably extraneous protein or lipoprotein component, acetone may remove one or more endogenous surfactants with the result that the enzyme coprecipitates with other proteins or lipoproteins in a complex which can be dissociated by detergent with consequent stimulation of phospholipase activity (however, see ref. 15).

Reports with respect to mammalian lipase more often than not have implicated Ca^{2+} as a stimulating agent (25). A positive effect of Ca^{2+} has also been observed with chloroplast lipase of spinach (3). Suggestions regarding the mechanism of Ca^{2+} action have included its involvement in complex formation favoring enzyme-substrate binding (17, 25) and its role as a stabilizing influence on enzyme configuration (3, 20, 24). In potato only enzyme II is responsive to Ca^{2+} and the response depends on substrate and pH. At pH above 7.5 phospholipid acylase is markedly stimulated by Ca^{2+}, while at lower pH it is inhibited. The action of the enzyme on galactolipids is unaffected by Ca^{2+} at any pH. The positive effect of Ca^{2+} at high pH notwithstanding, EDTA does not affect the activity of the enzyme in the absence of Ca^{2+}. In the presence of Ca^{2+} EDTA abolishes the stimulation at high pH and the inhibition at low pH.

The solubilizing effect on plant cell membranes exerted by enzyme II may be somewhat unique. Snake venom phospholipase A_2, phospholipase D, pancreatic lipase, and trypsin have variously been used to solubilize membrane-bound, especially microsomal, enzymes. None of the foregoing was found to release an active kaurene hydroxylase system from microsomal membranes from endosperm cells of wild cucumber (*Echinocystis macrocarpa*), while enzyme II readily solubilized the enzymes (S. Hirano, E. P. Hasson, and C. West, unpublished). Enzyme II may prove an effective tool in the isolation of plant membrane-associated enzymes. It seems certain that mitochondrial membranes are attacked by endogenous lipid acyl-hydrolase during tissue homogenization and subsequent differentiatial centrifugation. To date the means of avoiding disaster have been to separate the soluble lipid acyl-hydrolases in the supernatant solution from the mitochondrial pellet as quickly as possible and to provide BSA to adsorb troublesome products of hydrolytic action such as the free fatty acids and lysophosphatides. More recently, nupercaine has been used as a phospholipase inhibitor during organelle preparation (19, 22).

Potato phospholipase would appear to be a soluble enzyme, found in the supernatant solution following differential centrifugation. Potato mitochondria, whether intact, detergent-treated, or disrupted ultrasonically, showed no phospholipase activity either on exogenous lecithin or on convenient experimental substrates such as fatty acid esters of 4-methyl umbelliferone or N-methyl indoxyl. By apparent contrast, 25% of the phospholipid of isolated cauliflower mitochondria is degraded on standing at 20 C for 6 hr, with the production of stoichiometric quantities of lysophospholipids (6). While the foregoing implies a mitochondrial phospholipase in cauliflower, the extremely low level of activity is uncharacteristic of phospholipases which have been studied. For example, when a mitochondrial suspension from roughly 100 g fresh weight of potato tissue is treated with 0.1 mg of potato enzyme II (Table III), which derives from 5 g fresh weight at most, the amount of phospholipid degraded is about 50 times that which is hydrolyzed endogenously in cauliflower mitochondria in the same time interval at the same temperature, albeit the composition of potato mitochondrial phospholipid resembles that of cauliflower mitochondria, particularly with respect to the prevalence of unsaturated fatty acid (1). It remains an open question whether there is an intrinsic difference between cauliflower and potato with respect to mitochondrial phospholipase. The FFA liberated/mg cauliflower mi-

tochondrial protein in unit time is but $^1/_{1000}$ of the FFA released from exogenous lecithin by 0.1 mg of enzyme II. Our regular assay, which involves a measure of the label released from 1,2-dipalmityl-^{14}C-lecithin in several minutes may have failed to detect activity at the low level reported for endogenous phospholipid hydrolysis in cauliflower mitochondria. On the other hand, a mere trace of contamination by an active cytosol phospholipase may be enough to account for the low levels of phospholipid breakdown in 6 hr, even in mitochondria prepared by density gradient centrifugation.

Finally, the phospholipase activity of homogenates of intact tubers, of fresh slices, and of slices aged for 24 hr is much the same. The basic question of why a spate of *in situ* phospho- and galactolipase activity follows slicing remains unanswered, and it will be a matter of great interest to determine whether the effect of cutting is exerted on the enzyme, or on the accessibility of substrate, which in the case of potato slices comprises the phospho- and galactolipids of cell and organelle membranes.

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