Studies on the Phospholipases of Rat Intestinal Mucosa

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1. Subcellular distribution and characteristics of different phospholipases of rat intestinal mucosa were studied. 2. The presence of free fatty acid was necessary for the maximal hydrolysis of lecithin (phosphatidylcholine), but there was no accumulation of lysolecithin (1 or 2-acylglycerophosphorylcholine); lysolecithin accumulated when the reaction was carried out in the presence of sodium deoxycholate and at or above pH 8.0. 3. The fatty acid-activated phospholipase B as well as lysolecithinase showed optimum activity at pH 6.5, whereas for the phospholipase A it was about pH 8.6. 4. The bulk of the phospholipase A was present in the microsomal fraction, whereas the phospholipase B and lysolecithinase activities were distributed between the microsomal and soluble fractions of the mucosal homogenate. 5. Phospholipase A was equally distributed between the brush border and brush-border-free particulate fraction, with the brush border having highest specific activity, whereas the other two activities were distributed between the brush-border-free particulate and soluble fractions. 6. Various treatments showed marked differences between the phospholipase A and phospholipase B activities, but not between phospholipase B and lysolecithinase activities. 7. By using (β [1- 14 C]-oleoyl) lecithin it was shown that the mucosal phospholipase A was specific for the β -ester linkage of the lecithin molecule.

Work from several laboratories has shown that the intestinal mucosa is rich in several hydrolytic enzymes and that most of them are localized in the microvillus membrane of the mucosal cell. Thus disaccharidases (Miller & Crane, 1961), leucine aminopeptidase (Holt & Miller, 1962), alkaline phosphatase (Eicholz & Crane, 1965), adenosine triphosphatase (Taylor, 1962) and sphingomyelinase (Nilsson, 1969) were localized in the isolated brush border of intestinal mucosa of several species of animals. It has been shown that all of the cholesterol ester hydrolase and the bulk of the retinyl ester hydrolase are concentrated in the brush border of rat intestine; the possible role of these enzymes in the absorption of cholesterol and retinyl esters was also discussed (David, Malathi & Ganguly, 1966; David & Ganguly, 1967). As an extension of our work on these two hydrolases, we have now investigated the subcellular distribution and properties of various phospholipases in rat intestinal mucosa. [In accordance with the nomenclature followed by Ansell & Hawthorne (1964) the term phospholipase A denotes the enzyme that hydrolyses one of the acyl ester bonds of the phospholipid molecule (EC 3.1.1.4), and phospholipase B denotes the enzyme or enzyme system that hydrolyses both the acyl ester bonds of the phospholipid molecule. The term lysolecithinase denotes the enzyme that

hydrolyses the fatty acid from lysolecithin (EC 3.1.1.5).]

The presence of phospholipases has been demonstrated in several tissues of various species of animals. Although the enzymes from the pancreas of many species have been studied extensively, information on the intestinal phospholipases is rather insufficient to assign physiological functions to these enzymes in the digestion and absorption of phospholipids. Epstein & Shapiro (1959) reported that a particulate preparation from rat intestinal mucosa contains a 'lecithinase', that hydrolyses lecithin (phosphatidylcholine, 1,2-diacyl-*sn*-glycero-3-phosphorylcholine) to glycerylphosphorylcholine, without showing any accumulation of lysolecithin (1- or 2-acyl-*sn*-glycero-3-phosphorylcholine). This enzyme required the presence of free fatty acids for activity. Ottolenghi (1964) confirmed these observations and suggested that the 'lecithinase' is probably bound to the ribosomes. By incubating acetone-butanol-dried powders of rat intestine with lecithin and in the presence of sodium deoxycholate, Gallai-Hatchard & Thompson (1965), on the other hand, were able to demonstrate an accumulation of lysolecithin. All these investigations were carried out with different types of intestinal preparations and therefore the results obtained are not strictly comparable. It is

also not clear as to whether the fatty acid-activated phospholipase is a mixture of phospholipase A and lysolecithinase or whether it is similar to the phospholipase B from *Penicillium notatum* (Dawson, 1958), which hydrolyses both the acyl ester bonds of the lecithin molecule simultaneously.

Results presented below show that the brush border of rat intestine is rich in phospholipase A, whereas phospholipase B and lysolecithinase are more concentrated inside the mucosal cell. Such differential intracellular distribution and other evidence presented here suggest that the rat intestinal phospholipase A is different from the fatty acid-activated phospholipase B. In the light of these results the possible role of the mucosal phospholipases in the absorption and metabolism of phospholipids in the intestinal mucosa is discussed.

MATERIALS AND METHODS

Male albino rats of this Institute strain weighing 150–200 g were used without prior starvation. They were killed by cardiac puncture while under light ether anaesthesia, after which the intestinal mucosa was prepared (Ganguly, Krishnamurthy & Mahadevan, 1959). The mucosa was immediately homogenized in 10 vol. of cold 0.3 M-sucrose and the whole homogenate was used as the source of enzymes. Nuclear-plus-mitochondrial, microsomal and soluble fractions were obtained as described by Murthy, David & Ganguly (1963). The method of Miller & Crane (1961) for the isolation of the brush border from hamster intestine has been adopted in this laboratory for the rat intestine (David *et al.* 1966). The purity of the brush border thus isolated has been ascertained by Subbaiah, Raghavan & Ganguly (1968), who found about 70% of the invertase activity of the whole mucosa in the brush-border fraction, and that mitochondrial contamination (as judged by succinate dehydrogenase activity) was about 25%. The brush-border-free particles were obtained by centrifuging the brush-border-free 'supernatant' at 100 000 g in a Spinco model L ultracentrifuge for 1 h.

Lecithin and cephalin (1,2-diacyl-*sn*-glycero-3-phosphorylethanolamine) were prepared from egg yolk by the method of Rhodes & Lea (1957). Lysolecithin was

obtained from lecithin by using Russell's-viper venom by the procedure of Long & Penny (1957). The method described by Kates (1955) was used for the preparation of phosphatidic acid (1,2-diacyl-*sn*-glycero-3-phosphoric acid). The phospholipids gave single spots on t.l.c. plates. Sodium deoxycholate was obtained from Difco Laboratories, Detroit, Mich., U.S.A., and oleic acid was purchased from E. Merck A.-G., Darmstadt, Germany. Crude phosphatidylinositol and phosphatidylserine were obtained from Sigma Chemical Co., St Louis, Mo., U.S.A.

The reaction mixtures and the conditions of assay are described in the legends of the respective tables. All reactions were stopped by the addition of 4 vol. of chloroform-methanol (2:1, v/v), followed by vigorous shaking for 30 s. The mixture was left for 2 h, after which portions of the chloroform layer were taken for subsequent analysis.

Phospholipase B and lysolecithinase activities were based on the determinations of the decrease in acyl ester content, which was measured by the procedure of Skidmore & Entenmann (1962). The phospholipase A activity was calculated on the basis of the amount of lysolecithin formed, which in turn was measured by resolving the lipid extracts on t.l.c. plates of silica gel (Subbaiah *et al.* 1968) with the solvent system chloroform-methanol-aq. 14% (w/v) ammonia (65:25:4, by vol.). Lipid phosphorus was determined in the presence of silica gel by the method of Bartlett (1959), as modified by Marinetti (1962); the silica gel was centrifuged off before the colour was measured. Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951), with bovine serum albumin as standard.

RESULTS

Hydrolysis of lecithin, lysolecithin and cephalin by the whole mucosal homogenates. Table 1 shows that the whole homogenate was active against all the three substrates used. Addition of equimolar amounts of oleic acid considerably activated the hydrolysis of lecithin, which is in agreement with the observations of Epstein & Shapiro (1959). The fatty acid was slightly inhibitory to the hydrolysis of cephalin, but it had no effect on the lysolecithinase activity. Sodium deoxycholate, on the other hand, inhibited the hydrolysis of lysolecithin and cephalin and had no effect on lecithin hydrolysis in the

Table 1. *Hydrolysis of phospholipids by the whole homogenates of rat intestinal mucosa*

Chloroform solutions of the substrates (6 μ mol of lecithin or cephalin or 10 μ mol of lysolecithin) were taken, the chloroform was evaporated off under vacuum and the residue was dispersed in 200 μ mol of NaH_2PO_4 - Na_2HPO_4 buffer, pH 6.5. After the addition of 3.0 mg of enzyme protein incubations were carried out at 37°C for 30 min in a final volume of 2.0 ml.

Addition	Enzyme activity (μ equiv./h per mg of protein)		
	Lecithin	Lysolecithin	Cephalin
None	0.40	3.50	2.10
6 μ mol of potassium oleate	1.90	3.40	1.40
6 μ mol of sodium deoxycholate	0.50	2.0	0.75
25 μ mol of sodium deoxycholate	0.60	0.20	—
6 μ mol of potassium oleate + 6 μ mol of sodium deoxycholate	1.00	1.20	1.50

absence of added fatty acid. At higher concentrations deoxycholate slightly stimulated the hydrolysis of lecithin, and lysolecithin hydrolysis was further inhibited. However, sodium deoxycholate showed considerable inhibition of the fatty acid-activated hydrolysis of lecithin. Several other anionic amphipathic substances like sodium dodecyl sulphate (0.04–0.20mM), phosphatidic acid (0.2–0.5mM), phosphatidylinositol (0.5mM) or phosphatidylserine (0.5mM) could not replace the fatty acid in the activation of hydrolysis of lecithin, either at pH 6.5 or at pH 4.0.

Accumulation of lysolecithin during the hydrolysis of lecithin. The results in Table 2 demonstrate that the amounts of lysolecithin formed during the hydrolysis of lecithin were rather low. The addition of oleic acid, alone or with deoxycholate or fluoride at pH 6.5, did not significantly increase the amounts of lysolecithin formed, although there was considerable disappearance of total ester content, indicating hydrolysis of the lecithin molecule to the water-soluble compounds. Heating the enzyme preparation at 60°C for 10 min, a procedure known to inactivate lysolecithinase activity, resulted in a complete loss of the fatty acid-activated hydrolysis of lecithin. In contrast, reactions with the mucosal homogenates at higher pH values, and in the presence of sodium deoxycholate, led to an accumulation of larger amounts of lysolecithin. These results thus provide evidence for the occurrence of phospholipase A activity in intestinal mucosal homogenates.

When the effect of pH on the three enzyme activities was studied it was found that the pH

optimum for the phospholipase B and lysolecithinase activities was at pH 6.5 in phosphate buffer, whereas that for phospholipase A was about pH 8.6 in tris-maleate buffer. The pH optimum found for the phospholipase B and lysolecithinase activities is in close agreement with the value reported by Epstein & Shapiro (1959), and that for the phospholipase A activity agrees with the value found for the human pancreatic enzyme (Magee, Gallai-Hatchard, Sanders & Thompson, 1962).

Subcellular distribution. Most of the phospholipase A activity of the mucosa was recovered in the microsomal fraction, whereas the phospholipase B and lysolecithinase activities were distributed between the microsomal and soluble fractions (Table 3). The specific activities of all the three enzymes were highest in the microsomal fraction. Such intracellular distribution of phospholipase B broadly agrees with the findings of Ottolenghi (1964), which showed it to be associated with the ribosomal structures of the cell.

When the mucosa was fractionated into the brush-border, brush-border-free particulate and soluble fractions, most of the phospholipase A activity was distributed between the brush-border and brush-border-free particulate fractions, whereas the phospholipase B and lysolecithinase activities were distributed between the brush-border-free particulate and soluble fractions, with very little of the last two activities being present in the brush-border fraction (Table 4). The specific activity of phospholipase A was highest in the brush border, whereas those of phospholipase B and lysolecithinase were highest in the brush-border-free particles. We have

Table 2. *Accumulation of lysolecithin during the hydrolysis of lecithin by whole homogenates of rat intestinal mucosa*

The lecithin dispersion (6 μ mol/reaction mixture) was prepared as described in Table 1. For pH 6.5 and 7.6 0.1M-sodium phosphate buffers were used, and for pH 8.0 0.05M-tris-maleate buffer was used. The reactions were carried out for 60 min at 37°C with 3.0 mg of enzyme protein in a final volume of 2.0 ml.

Addition	pH	Lysolecithin formed (μ mol)	Lecithin recovered (μ mol)	Disappearance of total ester bonds* (μ mol)
Control	6.5	0.10	5.40	Nil
None	6.5	0.25	5.22	0.21
6 μ mol of potassium oleate	6.5	0.23	3.25	4.17
6 μ mol of potassium oleate + 25 μ mol of sodium deoxycholate	6.5	0.15	4.67	1.41
6 μ mol of potassium oleate + 150 μ mol of NaF	6.5	0.10	3.32	4.16
6 μ mol of potassium oleate added to enzyme, previously heated at 60°C for 10 min	6.5	0.10	5.20	0.40
6 μ mol of potassium oleate	7.6	0.18	4.93	0.86
25 μ mol of sodium deoxycholate	6.5	0.45	4.71	1.03
25 μ mol of sodium deoxycholate	8.0	0.90	4.48	1.04

* Calculated on the basis of the difference between the ester values of control and experimental tubes.

Table 3. *Subcellular distribution of phospholipase A, phospholipase B and lysolecithinase activities in rat intestinal mucosa*

For the assay of phospholipase A lecithin (6 μ mol) was dispersed with 25 μ mol of sodium deoxycholate in 100 μ mol of tris-maleate buffer, pH 8.0. The amounts of enzyme proteins added were 3.0 mg for the whole homogenate, 3.0 mg for the nuclear-plus-mitochondrial fraction, 1.0 mg for the microsomal fraction and 2.5 mg for the soluble fraction. Incubations were carried out for 60 min at 37°C in a final volume of 2.0 ml. For the assay of phospholipase B 6 μ mol of lecithin and 6 μ mol of potassium oleate were dispersed in 200 μ mol of NaH_2PO_4 - Na_2HPO_4 buffer, pH 6.5. The enzyme proteins were: 3.0 mg for the whole homogenate and for the nuclear-plus-mitochondrial fraction, 1.0 mg for the microsomal fraction and 2.0 mg for the soluble fraction. Incubations were carried out at 37°C for 30 min in a final volume of 2.0 ml. The reaction mixtures for the assay of lysolecithinase activity contained 10 μ mol of lysolecithin in 200 μ mol of the phosphate buffer, pH 6.5, and 1.0 mg of enzyme protein of whole homogenate or of nuclear-plus-mitochondrial fraction or 0.8 mg of protein of microsomal or of soluble fraction. Incubations were carried out for 30 min at 37°C in a final volume of 2.0 ml. The specific activity of phospholipase A is expressed as μ mol of lysolecithin formed/h per mg of protein, and those of phospholipase B and lysolecithinase are expressed as μ equiv. of ester bonds hydrolysed/h per mg of protein.

Fraction	Phospholipase A		Phospholipase B		Lysolecithinase	
	Specific activity	% of total activity	Specific activity	% of total activity	Specific activity	% of total activity
Homogenate	0.33	100	2.15	100	4.4	100
Nuclear + mitochondrial	0.23	22.5	1.21	13	2.6	10
Microsomal	0.76	62.3	3.20	26	7.5	34
Soluble	0.13	18.7	2.60	47	3.4	45

Table 4. *Distribution of the phospholipase activities in the brush-border, brush-border-free particulate and soluble fractions of rat intestinal mucosa*

The enzyme activities were assayed as described in Table 3, except that the amounts of enzyme protein used for phospholipase A were 1.0 mg for the whole homogenate, 0.8 mg for the brush border, 1.0 mg for the brush-border-free particles, and 0.7 mg for the soluble fraction; for phospholipase B the amounts of enzyme protein used were 1.0 mg for the whole homogenate, 2.0 mg for the brush border, 1.5 mg for the brush-border-free particles and 0.7 mg for the soluble fraction, and those for the lysolecithinase were 0.6 mg for the whole homogenate, 1.0 mg for the brush border, 1.0 mg for the brush-border-free particles and 0.5 mg for the soluble fraction. The specific activities are expressed as described in Table 3.

Fraction	Phospholipase A		Phospholipase B		Lysolecithinase	
	Specific activity	% of total activity	Specific activity	% of total activity	Specific activity	% of total activity
Homogenate	0.35	100	1.10	100	1.60	100
Brush border	0.90	43.2	0.40	19	1.50	17
Brush-border-free particulate	0.51	42.0	1.80	25	3.20	40
Soluble	0.20	23.0	0.80	37	2.00	45

also noted that the activities of the latter two enzymes were low after exposure to the EDTA medium used for the isolation of brush border, when compared with their activities after treatment with the sucrose medium (cf. Tables 3 and 4).

Effects of various treatments on the phospholipase activities. The effects of various treatments on the three types of phospholipase activities, as presented in Table 5, show that the free fatty acid, which is necessary for the maximal activity of phospholipase B, was inhibitory to the phospholipase A. As

shown above (Table 1) sodium deoxycholate inhibited the phospholipase B as well as the lysolecithinase activities. The thiol-group inhibitor *p*-hydroxymercuribenzoate had a marked effect on the phospholipase B and lysolecithinase activities, but at the same time had no such effect on phospholipase A. Heating at 60°C abolished the lysolecithinase activity as it did the lecithinase activity (Table 2), but with only partial loss of the phospholipase A activity. Ca^{2+} had a slightly stimulatory effect on lysolecithinase and phos-

Table 5. *Effect of various treatments on the activities of phospholipase A, phospholipase B and lysolecithinase of the whole homogenate of rat intestinal mucosa*

Acetone–butanol treatment of the whole mucosa and extraction of the powder thus obtained were as described by Gallai-Hatchard & Thompson (1965). The procedures for the assays were as described in Table 3. The values given in parentheses are the specific activities of the three enzymes of the homogenate without any treatment.

Treatment	Enzyme activity (% of control)		
	Phospholipase A	Phospholipase B	Lysolecithinase
None	100.00 (0.32)	100.0 (2.62)	100.0 (4.70)
25 μ mol of deoxycholate	—	37.5	0.0
6 μ mol of potassium oleate	40.6	—	102.0
20 mM- Ca^{2+}	119.0	25.5	119.0
5 mM-EDTA	75.0	102.0	117.0
1 mM- <i>p</i> -Hydroxymercuribenzoate	107.0	15.2	0.0
60°C for 10 min	62.5	1.5	2.10
Acetone–butanol drying	125.0	3.5	21.30

Table 6. *Positional specificity of phospholipase A of rat intestinal mucosa*

Mucosal homogenate containing 2mg of protein was incubated with 3 μ mol of (β [1- ^{14}C]-oleoyl) lecithin, 12 μ mol of sodium deoxycholate and 48 μ mol of tris–maleate buffer, pH 8.3, in a final volume of 0.8 ml. After the incubation was carried out for 1 h the lipids were extracted and separated on silica gel t.l.c. plates into lysolecithin, lecithin and free fatty acids with chloroform–methanol–water (65:25:4, by vol.) as the solvent system. Treatment of the labelled lecithin with *Crotalus adamanteus* venom was by the method of Long & Penny (1957). The reactions in the control samples were stopped immediately after the addition of the enzyme.

Treatment	Radioactivity (% of total)		
	Lysolecithin	Lecithin	Free fatty acids
Control	4.3	94.0	1.7
Mucosal homogenate	5.0	77.0	18.0
<i>C. adamanteus</i> venom	3.0	2.5	94.5

pholipase A, but was inhibitory to the phospholipase B activity, probably by forming an insoluble soap of the free fatty acid. Acetone–butanol treatment resulted in a marked loss of the phospholipase B and lysolecithinase activities, but the phospholipase A was unaffected. In most cases, therefore, the effects of various treatments run parallel with the phospholipase B and lysolecithinase activities, although differing from that of phospholipase A.

Positional specificity of the phospholipase A. Lecithin, labelled with [1- ^{14}C]oleic acid at the β -position, synthesized by the procedure of Robertson & Lands (1962), was used as a substrate for the phospholipase A. After reaction, the lipids were separated on silica-gel t.l.c. plates, eluted (Abramson & Blecher, 1964) and their radioactivities measured (Murthy *et al.* 1963). Almost all the radioactivity that disappeared from the lecithin was found in the fatty acids released, which demonstrated that this enzyme specifically hydrolyses the β -ester linkage of the lecithin molecule (Table 6).

DISCUSSION

The results reported above show the presence of two types of phospholipase activity in homogenates of rat intestinal mucosa; one of them hydrolyses lecithin to lysolecithin in the presence of sodium deoxycholate (phospholipase A), and the other hydrolyses lecithin to glycerylphosphorylcholine in the presence of free fatty acids (phospholipase B). The present work has further shown that the deoxycholate-activated phospholipase A is different from the fatty acid-activated phospholipase B and the lysolecithinase because of the differences in their pH optima, in their behaviour towards activators and inhibitors and in their heat-stability. More compelling evidence was obtained from the results on the subcellular distribution of these enzymes and by acetone–butanol-drying of intestinal mucosa. Thus the investigations on the subcellular distribution showed that, whereas the phospholipase A is distributed between the brush-border and the brush-border-free particulate fractions of the mucosa,

the phospholipase B and lysolecithinase activities are distributed between the brush-border-free particulate and soluble fractions. Acetone-butanol treatment led to a loss of the phospholipase B and lysolecithinase activities, but not of the phospholipase A activity. These observations are in general agreement with those of Epstein & Shapiro (1959), who showed that the phospholipase B activity of rat mucosa is lost on acetone-drying, and also with those of Gallai-Hatchard & Thompson (1965), who demonstrated the presence of phospholipase A activity in the acetone-butanol-dried powder of rat intestine.

It has, however, not been possible to effect a separation of the phospholipase B from lysolecithinase activity, and this agrees with the earlier results of Robertson (1966) and of Ottolenghi (1967). Of the various agents tested, Ca^{2+} exhibited some significant differences between the two activities. Ca^{2+} readily removes free fatty acids as insoluble soap; phospholipase B activity, and not lysolecithinase activity, requires free fatty acids, and therefore this effect of Ca^{2+} can readily be explained. In their experiments with phospholipase B from *P. notatum* Dawson (1958) and Bangham & Dawson (1959) had similarly observed that lecithinase and lysolecithinase activities cannot be separated and that the former required the presence of anionic amphipathic compounds. Here also the addition of Ca^{2+} led to a significant inhibition of the lecithinase activity, and these authors explained their observations on the basis of the ability of Ca^{2+} to bind with the activator.

Earlier work from several laboratories has shown that the intestinal microvillus membrane is rich in several hydrolytic enzymes, which may be important in the terminal digestion of various dietary constituents. The present work has demonstrated that it is rich in yet another hydrolytic enzyme, namely phospholipase A, and that it is similar in positional specificity to the enzyme from snake venom (Tattrie, 1959) and from human pancreas (van Deenen, De Haas & Heemskerck, 1963).

We have also shown here that in rat mucosa lysolecithinase activity is more pronounced than the phospholipase A activity, and that it is mostly found in the brush-border-free particles and non-particulate supernatant fraction. It is possible that the mucosal phospholipase A, which is apparently concentrated in the brush border, plays a role secondary to that of the pancreatic phospholipase A in the absorption of phospholipids, because it has been reported that lecithin is hydrolysed to lysolecithin (1-acyl-*sn*-glycero-3-phosphorylcholine) before absorption (Scow, Stein & Stein, 1967; Nilsson, 1968).

Once absorbed into the mucosal cell the lysole-

cithin can be metabolized in several ways. First, it can be reacylated to lecithin; we have demonstrated the presence of an active acyl-CoA-acylglycerophosphorylcholine acyltransferase in the same tissue (Subbaiah, Sastry & Ganguly, 1969, 1970). Secondly, the present work has shown that the mucosa contains a rather powerful lysolecithinase, which would rapidly hydrolyse the lysolecithin to glycerylphosphorylcholine. Finally, our unpublished observations have indicated the presence in the mucosa of an enzyme that can convert two molecules of lysolecithin into lecithin and glycerylphosphorylcholine by a mechanism of transesterification. The mucosal cell thus seems to be well equipped for the rapid removal of excess of lysolecithin that might gain entry into the cell, and probably this is physiologically significant, because lysolecithin is a powerful cytolytic agent.

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