The Action of Phospholipase A on Purified Phospholipids, Plasma and Tissue Preparations

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Earlier work in this Laboratory on the action of phospholipase A in aqueous systems indicated that the phospholipase A (phosphatide acyl-hydrolase, EC 3.1.1.4) in human pancreas, unlike the enzyme in snake venom from Cottonmouth moccasin (*Agkistrodon piscivorus piscivorus*), does not hydrolyse purified ovolecithin readily in the system 2,4,6-collidine–diethyl ether described by Magee & Thompson (1960). Nor does it readily attack purified ovolecithin in a glycylglycine buffer system unless sodium deoxycholate is present (Magee, Gallai-Hatchard, Sanders & Thompson, 1962). Vogel & Zieve (1960), working with the phospholipase A of human duodenal contents, also obtained evidence of activation of the hydrolysis of lecithin by deoxycholate.

In unpublished work, however, H. Sanders & R. H. S. Thompson found that the pancreatic enzyme is readily capable of hydrolysing the lecithin present in rat-brain homogenates without the need for activation by deoxycholate, and van Deenen, de Haas & Heemskerk (1963) showed that the pancreatic phospholipase A also is able to hydrolyse purified phosphatidylethanolamine at a rapid rate without deoxycholate activation. Using the 2,4,6-collidine–diethyl ether system mentioned above, Condrea, de Vries & Mager (1962) found that snake-venom phospholipase A splits the phospholipids present in crude egg yolk or in human serum at a very much faster rate than it does purified lecithin, and concluded that the susceptibility of the unpurified substrates to hydrolysis by the enzyme was due to the specific nature of the combination of their contained lecithins with protein in the form of lipoprotein complexes. Condrea, Klibansky, Keret & de Vries (1963) have reported that human and bovine pancreatic phospholipase A also split plasma lipoprotein-bound phospholipids more rapidly than purified ovolecithin.

In view of these various observations, and of the rapid effects produced by certain snake venoms on red blood cells, nerve cells and muscle cells *in vivo*, which have frequently been ascribed to the action of the phospholipase A contained in the venom, it seemed desirable to study in more detail the action of both venom and mammalian phospholipase A on purified phospholipids, on plasma lipoproteins and on the phospholipids in the cell membranes of both homogenized and whole-cell preparations of different tissues. In addition, the requirement of these different substrates for activation by deoxycholate has been studied.

EXPERIMENTAL

Materials

Ovolecithin. This was prepared from fresh egg yolks and was purified by chromatography on an alumina column according to the method of Rhodes & Lea (1957). It was stored at $-10°$ as a 5% solution in chloroform. In some experiments it was further purified by chromatography on silicic acid columns.

Phosphatidylethanolamine. This was prepared by Dr G. R. Webster from egg-yolk lipids by chromatography on a silicic acid column. Paper chromatography of the separated sample showed one ninhydrin-staining spot.

Phosphatidylserine. This was prepared by Dr L. Rathbone, in this Department. It was obtained from a modified phosphatidylserine-rich fraction III of human brain (Folch, 1942) by chromatography on treated silicic acid–Hyflo Super-Cel columns (Rathbone & Maroney, 1963).

Pancreatic phospholipase A. This was a heat-treated preparation (fraction C) obtained from human pancreas by the method of Magee *et al.* (1962), freshly dissolved in water or in 0·9% NaCl to give a solution containing 50 μg./0·1 ml.

Cobra-venom phospholipase A. Freeze-dried cobra (*Naja naja*) venom was obtained from Ross Allen's Reptile Institute, Silver Springs, Florida, U.S.A. and 100 mg. was dissolved in 10 ml. of water, and brought to pH 4·5 with 2N-HCl. The solution was heated at 75° for 5 min., cooled to room temperature and centrifuged, the supernatant collected and cooled to 0°, and 3 vol. of ice-cold acetone added. A white precipitate formed and the mixture was kept in the cold overnight. After centrifuging, the precipitate was washed twice with 3 vol. of ice-cold acetone and dried *in vacuo*. For use it was freshly dissolved in water or in 0·9% NaCl to give a solution containing 20 μg./0·1 ml.

Sodium deoxycholate. This compound (Hopkin and Williams Ltd.) was used without further purification in most experiments; in some cases it was purified as described by Magee *et al.* (1962).

Silicic acid. This compound (Mallinckrodt A.R., 100 mesh) was washed with redistilled chloroform, kept dry in a desiccator and used directly.

Human plasma. This was obtained from freshly drawn heparinized blood.

Methods

Assay system for phospholipase A. The purified phospholipids were emulsified in glycylglycine buffer alone at a concentration of 2 mg./ml. or with sodium deoxycholate added as a freshly prepared solution in water (5 mg./ml.).

In a typical experiment 10 mg. of the phospholipid was emulsified in 5 ml. of 0·1 M-glycylglycine buffer of the required pH; 4 ml. of water or 4 ml. of deoxycholate solution was then added. Samples (0·9 ml.) of the mixture were then pipetted into glass-stoppered tubes, each tube therefore containing 1 mg. of phospholipid alone or with 2 mg. of sodium deoxycholate. The tubes were placed in a water bath at 38°; after temperature equilibration 0·1 ml. of the enzyme solution, also at 38°, was added at half-minute intervals to the different tubes. The reaction was allowed to proceed for 0, 15, 30 and 60 min., the tubes being shaken constantly throughout. In each experiment a '60 min. control' was included, in which the substrate–buffer mixture was incubated without the enzyme for 60 min. All tubes were set up in duplicate.

Free fatty acid estimations. With lecithin as substrate the enzymic reaction was stopped by the addition of 5 ml. of propan-2-ol–*n*-heptane–N-H_2SO_4 (40:10:1, by vol.) mixture. In the zero-time tubes and the 60 min. controls the solvent mixture was added before the addition of the enzyme. Free fatty acids were then estimated, essentially by the method of Dole (1956) except that the titrations were carried out with methanolic 5 mN-NaOH.

When either phosphatidylethanolamine or phosphatidylserine was used as substrate, difficulties were encountered with the above method since these compounds, like lecithin, were extracted in varying degrees into the heptane phase and, unlike lecithin, were titratable under the conditions used. None of the three lysophosphatides was extracted. To exclude diacylphosphatides from the titration phase enzymic hydrolysis was stopped by the addition of 4 ml. of chloroform–methanol (2:1, v/v). After centrifugation and removal of the upper phase and interfacial protein layer, 2 ml. of the lower phase was evaporated to dryness under N_2, 0·5 ml. of chloroform added and evaporation to dryness repeated. Silicic acid (300 mg.) was then added, followed by 3 ml. of redistilled chloroform. After shaking and centrifugation, 2 ml. of the chloroform layer was carefully withdrawn, added to 1 ml. of indicator (ethanolic Cresol Red) and titrated with methanolic 5 mN-NaOH.

Protein. This was estimated by the method of Lowry, Rosebrough, Farr & Randall (1951).

Glutamic–oxaloacetic transaminase (L-*aspartate-2-oxoglutarate aminotransferase, EC* 2.6.1.1). This was estimated spectrophotometrically by the method of Karmen (1955).

Chromatography of phospholipids. Silicic acid-impregnated paper chromatograms were developed in di-isobutyl ketone–di-*n*-butyl ether–acetic acid–water (20:20:20:3, by vol.) according to the method of Reed, Swisher, Marinetti & Eden (1960). Phosphorus was estimated in cut-out spots by the method of Strickland, Thompson & Webster (1956).

RESULTS

Action on purified phospholipids. In an aqueous system at pH 7·3 the hydrolysis of lecithin by either the pancreatic or the cobra-venom enzyme required activation by deoxycholate to proceed rapidly; the pancreatic enzyme in particular appeared to be highly dependent on deoxycholate, lecithin alone being hydrolysed by this enzyme at only 3–4 % of the rate in the presence of the activator (Table 1).

Activation of hydrolysis of phosphatidylserine was much less marked, and with phosphatidylethanolamine deoxycholate strongly inhibited hydrolysis by both the enzymes. Of the three substrates phosphatidylethanolamine was hydrolysed most rapidly, particularly by the pancreatic enzyme; phosphatidylserine was a poor substrate, and was attacked only very slowly by the cobra-venom enzyme.

Enzymic hydrolysis of these substrates was also measured at pH 6·0 and at pH 9·0. In agreement with the results of earlier workers it was found (Fig. 1) that lecithin was split more rapidly at pH 9·0 than at the physiological pH. At pH 6·0 the pancreatic phospholipase had no action on it, even in the presence of deoxycholate. Phosphatidylserine was also split more rapidly by the pancreatic enzyme at pH 9·0; the cobra venom was almost completely inert against this substrate, except at pH 9·0 and in the presence of deoxycholate. Phosphatidylethanolamine showed maximal hydrolysis at pH 7·3.

Action on phospholipids in whole plasma. Plasma (4 ml.) obtained from freshly drawn heparinized blood was added to 5 ml. of glycylglycine buffer, pH 7·3, containing where necessary sodium deoxycholate (4 mg./ml.). The reaction system consisted of 0·9 ml. of this mixture, to which was added 0·1 ml.

Table 1. *Estimated initial reaction velocities of hydrolysis of purified phospholipids by phospholipase A*

Values are the means of two experiments with each enzyme. Reaction system: 1 mg. of phospholipid/ml.; 0·05 M-glycylglycine buffer, pH 7·3, with (+) and without (−) 2 mg. of sodium deoxycholate.

	Free fatty acids released (μm-equiv./hr.)					
	Lecithin		Phosphatidylethanolamine		Phosphatidylserine	
Deoxycholate	+	−	+	−	+	−
Pancreas enzyme (50 μg.)	1600	56	2020	6920	860	624
Cobra venom (20 μg.)	1760	260	320	1860	64	59

of the enzyme solution; each tube therefore contained 0·4 ml. of plasma (approx. 0·7–0·8 mg. of 'hydrolysable' phospholipids) with or without 2 mg. of sodium deoxycholate.

The free fatty acid released over four successive 15 min. intervals at 38° is shown in Fig. 2; with plasma as substrate cobra-venom phospholipase A does not require activation by deoxycholate. Paper chromatography confirmed that almost complete conversion of the lecithin and phosphatidylethanolamine spots into the corresponding lyso derivatives had occurred after incubation for 60 min. Marinetti (1961) reported the breakdown of both lecithin and phosphatidylethanolamine in rat serum by *Crotalus adamanteus* venom.

The addition of deoxycholate did not inhibit the hydrolysis of the plasma phosphatidylethanolamine, as was the case with the purified substrate. The pancreatic enzyme, on the other hand, required the presence of deoxycholate for optimum activity on the plasma phospholipids.

Action on brain homogenates and on brain slices. Homogenates (1:20) of rat cerebrum were prepared in glycylglycine buffer, pH 7·3. Each reaction tube contained 0·4 ml. of homogenate (0·8 mg. of hydrolysable phospholipids) and 0·5 ml. of glycylglycine buffer, containing where necessary 2 mg. of sodium deoxycholate.

Both pancreatic and cobra-venom phospholipase A caused a rapid breakdown of the phospholipids in these homogenates and neither required activation by deoxycholate (Table 2). In 1 hr. at 38° approx. 70–80 % of the hydrolysable phospholipids underwent hydrolysis, as judged by fatty acid production; chromatography confirmed the extensive breakdown of lecithin, phosphatidylethanolamine and phosphatidylserine to the corresponding lyso derivatives.

Similar experiments were made with rat-brain slices, to provide as substrate a preparation containing intact brain cells.

Brain slices weighing 35–45 mg. were incubated in 0·9 ml. of iso-osmotic buffer with 0·1 ml. of enzyme at 38° and with mechanical shaking. Tubes were set up in duplicate, together with controls in which the enzyme was added at the end of the incubation period. Table 3 shows that a considerable release of free fatty acids by either of the two

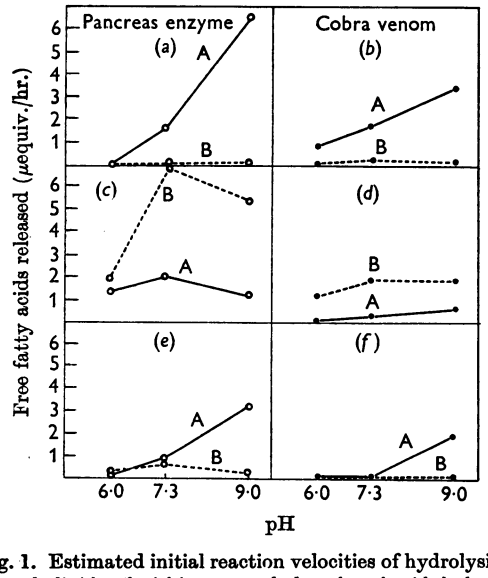

Fig. 1. Estimated initial reaction velocities of hydrolysis of phospholipids (lecithin, *a* and *b*; phosphatidylethanolamine, *c* and *d*; phosphatidylserine, *e* and *f*) by phospholipase A at pH 6·0, 7·3 and 9·0, in the presence (curves A) or absence (curves B) of added sodium deoxycholate.

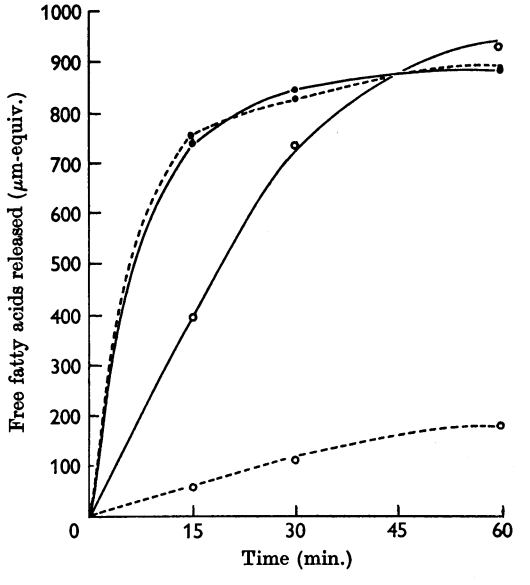

Fig. 2. Hydrolysis of human plasma phospholipids by phospholipase A: ○—○, pancreas enzyme with, and ○---○ without, sodium deoxycholate; ●—●, cobra venom with, and ●---● without, sodium deoxycholate. Values are the means of three experiments with each enzyme.

Table 2. *Hydrolysis of rat-brain homogenates by phospholipase A of pancreas or cobra venom*

Values are the means of two experiments with each enzyme.

	Sodium deoxycholate	Free fatty acids released (μm-equiv./hr.)
Pancreas enzyme (50 μg.)	Present	670
	Absent	723
Cobra venom (20 μg.)	Present	785
	Absent	788

Table 3. *Release of free fatty acid from rat-brain slices by phospholipase A of pancreas and cobra venom*

Each tube contained 35–45 mg. of brain slice.

Time of reaction	No. of expts.	Free fatty acids released (μm-equiv.) 15 min.	30 min.	60 min.	2 hr.	3 hr.	4 hr.
Pancreas enzyme (50 μg.)	5	56	136	216	—	—	475
(160 μg.)	2	131	231	349	—	—	—
Cobra venom (20 μg.)	6	120	203	409	584	765	1014
(160 μg.)	3	257	442	660	—	—	—

enzymes occurred, although the rate was appreciably less than that found with homogenates of brain.

Because of trauma to cells on the cut surfaces of the slices it was important to determine to what extent the enzyme action was exerted on these damaged cells, and to what extent, if any, on the intact cells in the slice. It is known that the phospholipase A in cottonmouth-moccasin venom can damage intact cells in brain slices sufficiently to allow the outflow of significant amounts of the intracellular glutamic–oxaloacetic transaminase from the slice into the fluid in which it is suspended (McArdle, Thompson & Webster, 1960). To discover whether the action of these enzymes under these conditions also resulted in the escape of intracellular constituents, and if so to attempt to relate the extent of their outflow with the extent of enzymic formation of free fatty acid, duplicate tubes were set up; in one free fatty acid release was estimated in the usual way; the other was centrifuged at the end of the incubation period at 1800*g* for 2 min. The supernatant was then pipetted off, and samples were taken for estimation of total protein and of glutamic–oxaloacetic transaminase. The outflow of protein and transaminase was also measured in the supernatants obtained from control tubes in which the slices had been incubated without the addition of phospholipase A. By this means a measure was obtained of the extra outflow from the slices of these intracellular components induced by the presence of the enzyme. It was found that the greater the enzyme action (as determined by free fatty acid formation) the greater was the amount of extra protein and transaminase released over and above the small amount released from the control slices. From these relationships (Figs. 3 and 4) it was concluded that the greater part of the free fatty acids formed by both the pancreatic and cobra phospholipases represented an action of these enzymes on intact cells, resulting in a degree of damage to the cell membrane sufficiently great to allow the escape in measurable amounts of intracellular protein.

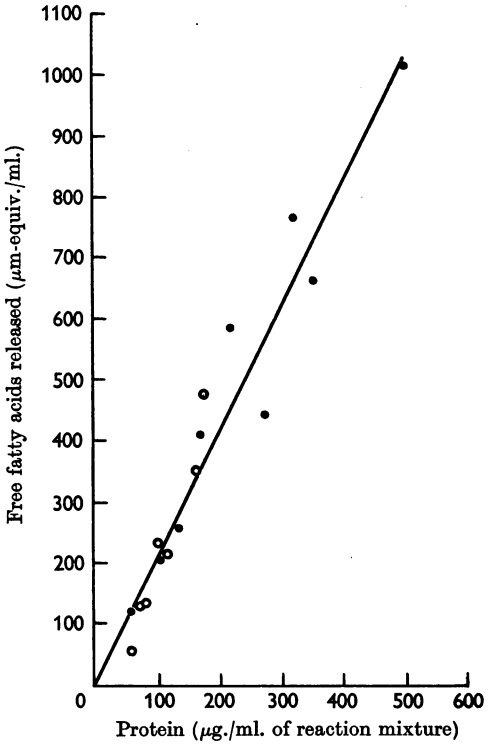

Fig. 3. Relation between release of free fatty acids and outflow of protein from brain slices in the presence of ○, pancreas enzyme and ●, cobra venom. Slope of the curve is given by $y = 4{\cdot}9 + 2{\cdot}05x$.

Action on rat-diaphragm homogenates and 'intact' quarter-diaphragms. The phospholipids in muscle were next used as substrates. Diaphragms were carefully dissected from freshly killed rats and, after removal of adherent blood together with the central tendonous portion and any fat or connective tissue, were either homogenized in water or cut with sharp scissors into quarter-diaphragms. Three diaphragms, weighing about 1·5 g., were finely

minced with scissors; 0·5 ml. of 0·9 % sodium chloride was added and the mincing continued. The material was then homogenized in an all-glass homogenizer with 8 ml. of water; 0·4 ml. of homogenate was added to 0·5 ml. of buffer in each reaction tube (0·4 ml. of homogenate contained about 0·6 mg. of hydrolysable phospholipid; B. McArdle, personal communication).

Both enzymes caused a rapid and almost complete hydrolysis of the phospholipids in rat-diaphragm homogenates in 1 hr., and were not activated by deoxycholate, evidence of a slight but consistent inhibition by deoxycholate being in fact found (Table 4).

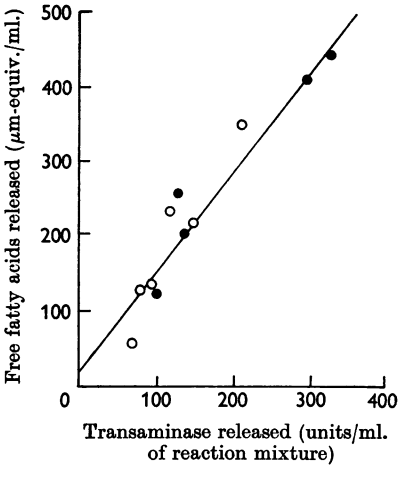

Fig. 4. Relation between release of free fatty acids and outflow of glutamic–oxaloacetic transaminase from brain slices in the presence of ○, pancreas enzyme and ●, cobra venom. Slope of the curve is given by $y = 18{\cdot}5 + 1{\cdot}36x$.

Quarter-diaphragms were used to provide a preparation of intact muscle cells. In each reaction tube one quarter-diaphragm (weighing 90–100 mg. and containing about 1 mg. of hydrolysable phospholipids) was added to 0·9 ml. of iso-osmotic glycylglycine buffer. Fatty acid, protein outflow and release of glutamic–oxaloacetic transaminase were measured as for the brain slices. Table 4 shows that the cobra venom caused a very considerable release of free fatty acids, accompanied by the outflow from the diaphragm of large amounts of protein and glutamic–oxaloacetic transaminase, the two latter showing a rough proportionality to the fatty acid produced. On the other hand, 50 μg. of pancreatic enzyme showed only a slow, inconsistent and very slight rate of free fatty acid release, accompanied by only a slight outflow of protein and glutamic–oxaloacetic transaminase. It seemed likely that the low activity of the pancreatic enzyme on the quarter-diaphragm implied an action restricted to cells already damaged, rather than an effect on intact cells.

DISCUSSION

The results described above with purified substrates confirm the finding of van Deenen *et al.* (1963) that, unlike lecithin, the hydrolysis of phosphatidylethanolamine is not accelerated by deoxycholate. Indeed, its hydrolysis by either the pancreatic or cobra-venom enzyme is strongly inhibited by deoxycholate. It would seem there-

Table 4. *Action of phospholipase A of pancreas and cobra venom on rat-diaphragm homogenates and 'intact' quarter-diaphragms*

Values are the means of two experiments with each enzyme.

		Free fatty acids released (μm-equiv.)		
(A) Diaphragm homogenates	Time of reaction (min.)	15	30	60
	Sodium deoxycholate			
Pancreas enzyme (50 μg.)	Present	360	493	561
	Absent	498	599	692
Cobra venom (20 μg.)	Present	468	580	690
	Absent	592	645	733
(B) 'Intact' quarter-diaphragms				
	Time of reaction (min.)	15	30	60
Pancreas enzyme (50 μg.)	Fatty acids (μm-equiv.)	14	36	104
	Protein outflow (μg./ml.)	58	103	145
	Enzyme* outflow (units/ml.)	60	135	150
Cobra venom (20 μg.)	Fatty acids (μm-equiv.)	147	313	574
	Protein outflow (μg./ml.)	488	650	1220
	Enzyme* outflow (units/ml.)	810	1275	3273

* Glutamic–oxaloacetic transaminase.

fore that for many purposes, particularly when working with the pancreatic enzyme and possibly with extracts of other mammalian tissues, phosphatidylethanolamine would provide a more suitable substrate than lecithin since, not only does it not require the presence of deoxycholate in the reaction system, but it is hydrolysed more rapidly.

Bangham & Dawson (1959) have shown that the phospholipase in *Penicillium notatum* splits lecithin only when the substrate micelles have a net negative ζ potential. Consideration of our results with the different substrates at different pH values, and in the presence and absence of deoxycholate, did not appear to support this relationship for the enzymes which we have studied. However, Dawson (1963) has recently concluded that, unlike the enzyme in *P. notatum*, the action of cobra-venom phospholipase A is not dependent on the net ζ potential of the substrate, and our results would support his view.

In the experiments with plasma or tissue preparations as substrates, i.e. where the phospholipids were present in a more physiological state than when isolated, conditions were chosen so as to give roughly comparable concentrations of 'hydrolysable' phospholipids in all cases. The experiments with cobra venom confirm the findings of Condrea *et al.* (1962) that the phospholipids in human serum are split more rapidly than purified ovolecithin, although in the presence of deoxycholate the increase in rate is only about fourfold, as opposed to the 10- to 20-fold increase found by them for the venom of *Vipera palestinae* in the collidine–diethyl ether system. Moreover, in the presence of deoxycholate the pancreatic enzyme hydrolyses purified lecithin at about the same rate as the plasma phospholipids (Table 1 and Fig. 2). The cobra-venom and the pancreatic enzymes are strikingly different in their actions on plasma phospholipids as regards activation by deoxycholate (Fig. 2).

Although the release by phospholipase A of free fatty acids from brain slices proceeds at a slower rate than from brain homogenates, the estimation of protein and outflow of glutamic–oxaloacetic transaminase from the slices would seem to indicate that both the pancreatic and cobra-venom enzymes are capable of hydrolysing phospholipids in the intact cells of the central nervous system. The results obtained with rat quarter-diaphragms indicate that in this tissue also the cobra-venom enzyme can apparently attack the membranes of intact muscle cells. The pancreatic phospholipase A gave results with quarter-diaphragms which are not easy to assess. When acting on muscle homogenates the free fatty acid release by 50 μg. of pancreatic enzyme is of the same order as that obtained with 20 μg. of cobra venom, and yet when acting on quarter-diaphragms its action is very much less, amounting in the first 15 min. to only about 10 % of the rate obtained with cobra venom. Further, the outflow of either protein or glutamic–oxalo-acetic transaminase/μm-equiv. of free fatty acid released is much less with the pancreatic enzyme than with the venom; thus a liberation of 104 μm-equiv. of fatty acid by the pancreatic enzyme in 60 min. is accompanied by the outflow of only 150 units of glutamic–oxaloacetic transaminase/ml., whereas the liberation of 147 μm-equiv. of fatty acids by the venom is accompanied by the release of 810 units of enzyme/ml. in 15 min. Because of these facts we think it likely that the pancreatic enzyme is acting largely on damaged cells rather than on cells still endowed with the normal permeability properties of their cell membranes.

Phospholipase A, of either mammalian or snake-venom origin, can therefore attack the phospholipids in brain without the need for any activation by deoxycholate, although with muscle, when the cells are intact, their susceptibility to attack by pancreatic phospholipase A appears to be greatly modified.

SUMMARY

1. The actions of phospholipase A on purified lecithin, phosphatidylethanolamine and phosphatidylserine on the phospholipids in plasma lipoproteins and on the phospholipids present in brain and muscle have been studied in an aqueous reaction system.

2. A partially purified heat-treated human pancreatic phospholipase A and the phospholipase A present in heated freeze-dried cobra venom have been used.

3. The need for activation by deoxycholate, when isolated phospholipids were used as substrates, depended on the type of phospholipid chosen.

4. Phospholipids in plasma were rapidly attacked by the cobra-venom enzyme without activation. The pancreatic enzyme showed only slight activity against plasma unless deoxycholate was added.

5. Both enzymes rapidly hydrolysed the diacylphospholipids present in brain and muscle homogenates without the need for activation. They also both attacked the phospholipids in intact brain cells. Cobra-venom enzyme split phospholipids in intact muscle cells but the pancreatic enzyme attacked these only slowly, if at all.

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The Estimation of Renin in Human Plasma

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A method was previously developed in this Laboratory for estimating the enzyme renin in rabbit plasma (Lever, Robertson & Tree, 1963*a*, *b*,*c*, 1964). The present paper describes the adaptation of the method to the measurement of renin in the plasma of man. Preliminary accounts of this work have been published (Brown, Davies, Lever & Robertson, 1963*a*, *b*, 1964*a*).

METHODS

The technique consists of an estimation of renin concentration by determining the initial velocity of angiotensin formation under standard conditions of incubation with substrate. Angiotensinase-free ox-serum substrate, as prepared for the estimation of rabbit renin (Lever *et al.* 1964), was used throughout the present studies. The technique of incubation and assay, and the tests for contamination with angiotensinase and endogenous substrate, were also as described by Lever *et al.* (1964). The methods of preparation of standard human renin, and of extracting renin from human plasma, however, differed from those used with the rabbit.

Preparation of standard human renin. Human kidneys (4·5 kg.) were obtained *post mortem*, and excess of fat and fibrous tissue was removed. The kidneys were then minced and allowed to stand in 10 l. of water at 8° for 36 hr. The mince was filtered through muslin, and 10% (w/v) trichloroacetic acid was added to the filtrate at 8°, with constant stirring, to give pH 2·9. Then NaCl (52 g. to each litre) was added slowly with stirring; the pH was checked after 15 min., and if necessary readjusted to pH 2·9 by adding 10% trichloroacetic acid or N-NaOH. The solution was then filtered at 8° overnight through Whatman no. 50 paper and the filtrate adjusted to pH 5·0 with N-NaOH. Then 2 l. batches of this solution were dialysed in Visking cellophan sacs (28/32 in.) against three 15 l. changes of water at 8° over 48 hr.

The method of renin preparation used to this stage was basically that described by Dexter, Haynes & Bridges (1945). It was found, however, that this renin solution had considerable angiotensinase activity, and therefore further purification was carried out as follows. These later stages were based on results obtained by W. S. Peart, N. N. Mendelsson & N. E. Stone (see Peart, 1959) and W. S. Peart, A. F. Lever, M. W. Lloyd, N. N. Payne, N. E. Stone & A. Taylor (unpublished work) during the purification of pig renin.

The 2 l. batches of renin solution were dialysed in Visking cellophan sacs (28/32 in.) against three 15 l. changes of 5 mM-sodium phosphate buffer, pH 7·0 (0·0240% NaH_2PO_4; 0·1074% $Na_2HPO_4,2H_2O$), over 48 hr. at 8°. This solution was then applied at room temperature to a column (65 cm. × 2·5 cm.; dry wt. 100 g.) of lightly-packed DEAE-cellulose equilibrated with the 5 mM-sodium phosphate buffer, pH 7·0. Renin was adsorbed during this application. The column was washed with 3 l. of 0·03M-sodium phosphate buffer, pH 7·0 (0·140% NaH_2PO_4; 0·653% $Na_2HPO_4,12H_2O$), and the eluted protein discarded. Renin was then eluted with 600 ml. of 0·35M-phosphate–saline buffer, pH 6·0 (0·05M-Na_2HPO_4; 0·3M-NaCl, adjusted to pH 6·0 with 6N-HCl), containing neomycin sulphate (0·01%).

The eluate was dialysed against three 10 l. changes of glycine–HCl–saline buffer, pH 3·0 (0·1M-glycine; 0·9M-NaCl; 0·018N-HCl), at room temperature over 24 hr. The precipitate that then formed was removed by filtration