described. The procedure employs anion- and cation-exchange resins for the major purification steps and produces yields of *N*-phosphoryl-lombricine in excess of 70 % of that present in the original extracts.

Our thanks are due to Dr Joyce Fildes for elementary analyses and to Mr B. Thorpe for skilled technical assistance.

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Biochem. J. (1962) **83**, 17

The Purification and Properties of Phospholipase A from Human Pancreas

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(Received 27 July 1961)

Although phospholipase A (phosphatide acyl-hydrolase, EC 3.1.1.4) has been shown to occur in a number of animal tissues (Zeller, 1951) the pancreas appears to be the richest mammalian source of the enzyme. Its presence was first suggested by the work of Bokay (1877), but more definite proof of its existence in pancreas was not provided until considerably later (Belfanti & Arnaudi, 1932; Gronchi, 1936). Since then pancreatic phospholipase A has been the subject of considerable research, a variety of techniques being used for the assay and isolation of the enzyme, sometimes with conflicting results (Ercoli, 1940).

Gronchi (1936) isolated phospholipase A by extracting commercial pancreatin with aqueous 50 % ethanol at 90°. Hanahan (1952) employed the same starting material to obtain a partially purified preparation that was active in diethyl ether; extracts of fresh rat pancreas also converted lecithin into lysolecithin in the ethereal reaction system. The preparation and purification of phospholipase A from aged ox pancreas was described by Rimón & Shapiro (1959), who used a phosphate-buffered assay system. Very recently, a method has been described for the measurement of phospholipase A in duodenal contents, serum and other

body fluids (Vogel & Zieve, 1960; Zieve & Vogel, 1961).

This paper represents a continuation (Magee & Thompson, 1960) of a project designed to devise a reaction system suitable for the accurate assessment of phospholipase A in animal tissues, and to compare the properties of phospholipase A preparations isolated from various sources. Human pancreas was used as the enzyme source because it contains an active phospholipase A and because human pancreatic tissue had not been investigated previously. A relatively simple method for the purification of the enzyme is described and some of its properties are discussed.

MATERIALS

Ovolecithin. Ovolecithin was prepared, stored and washed free of Ca^{2+} ions as described by Magee & Thompson (1960). For certain experiments, the ovolecithin (1 mg./ml.) was emulsified in mM-EDTA and then mixed with 4 vol. of chloroform-methanol (2:1, v/v). Phase separation was assisted by centrifuging. The lower phase was washed with upper-phase solvents (Folch, Lees & Sloane-Stanley, 1957), taken to dryness and the lipid made up as a stock solution in chloroform.

Lysolecithin. Pure lysolecithin was prepared in this Laboratory by Dr G. R. Webster as described by Marples, Thompson & Webster (1958).

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Tributyryl. Tributyrin (British Drug Houses Ltd.) was made up as a stock solution in ethanol.

Sodium deoxycholate. Sodium deoxycholate (Hopkin and Williams Ltd.) was used directly in some cases, but for certain experiments was purified by running a solution through a column of ion-exchange resin (Zeo-Karb 225, Na form), acidifying the eluate, washing the resulting solid with ice-cold water and recrystallizing from hot ethanol. The deoxycholic acid was then washed with diethyl ether and dried *in vacuo*. Solutions of sodium deoxycholate were made up immediately before use at approximately double the concentration finally added to the reaction system (see below). This technique produced a clear solution, whereas solutions made up directly to the required concentration were often cloudy.

Buffers. Freshly prepared glycylglycine-NaOH and 2,4,6-collidine-HCl buffers were used at a concentration of 0.1M. Final pH adjustments were made with a glass electrode. Glycylglycine (Hopkin and Williams Ltd.) was used without purification. 2,4,6-Collidine (Yorkshire Tar Distillers Ltd.) was redistilled before use.

METHODS

Measurement of enzyme activities

Phospholipase A, phospholipase B and tributyrinase activities were estimated by following the decrease in acyl ester bonds occurring during hydrolysis of lecithin, lysolecithin and tributyrin respectively. The substrates themselves were used as standards for the ester-linkage determinations. All three substrates gave identical colour yields/ μ equiv. of ester linkage, and the method (Stern & Shapiro, 1953) produced coloured solutions which obeyed Beer's law up to 6 μ equiv. of ester linkage/sample. Thus for the conversion of lecithin into lysolecithin, changes of extinction could be converted directly into μ equiv. of ester linkage hydrolysed by means of a factor obtained from an appropriate calibration plot for the particular reaction conditions employed. The procedure can be applied to the determination of phospholipase A only when the lysolecithin produced by the reaction is not itself hydrolysed.

When a quantitative comparison of enzyme activities was required, initial reaction velocities were determined. For this purpose, the amount of enzyme and the reaction times were chosen to give reasonably linear hydrolysis rates. Initial reaction velocities were converted into specific activities (μ m-equiv. of ester linkage hydrolysed/min./mg.).

Phospholipase A-assay system. The 2,4,6-collidine-buffered reaction system described by Magee & Thompson (1960) was used in a few of the experiments reported here. However, in most of the present experiments a glycylglycine-buffered reaction system was used, in which the ovolcithin substrate was solubilized with sodium deoxycholate. In a typical experiment, ovolcithin was emulsified in buffer to a concentration of 2 mg./ml. A solution of sodium deoxycholate in water (5 mg./ml.) was added, so that 0.9 ml. of the resulting clear solution contained 1 mg. of ovolcithin and 2 mg. of sodium deoxycholate. This volume was pipetted into glass-stoppered test tubes (110 mm. $\times$ 17 mm.), which were immersed in a water bath at 38°. After temperature equilibration, 0.1 ml. of enzyme solution (also at 38°) was added at fixed time intervals. After the appropriate

reaction period, during which the samples were shaken, enzymic hydrolysis was stopped by the addition of 4 ml. of chloroform-methanol (2:1, v/v). After centrifuging and removal of the upper phase, together with the interfacial layer of protein, a sample (2 ml.) of the lower phase was taken and evaporated to dryness under reduced pressure. The residue was then dissolved in 3 ml. of ethanol-water-ether (2:1:1), and the acyl ester linkages were determined as described by Magee & Thompson (1960). In the zero-time controls chloroform-methanol was added immediately after addition of the enzyme solution. All samples were prepared in duplicate.

To obtain maximum enzymic activity the samples should not be left standing on the bench or in the bath for long periods before the reaction is initiated. Although sodium deoxycholate at the concentration described above did not materially affect the final colour yield of the ester-linkage assay, it did affect the time-course of colour development. In the biphasic system much of the deoxycholate is removed in the upper-phase solvents and interference avoided thereby.

Phospholipase B-assay system. The system is identical with that described for phospholipase A, except that lysolecithin (2.4 μ moles/sample) replaced ovolcithin as the substrate.

Tributyrylase-assay system. Homogeneous suspensions of tributyrin were difficult to prepare reproducibly in aqueous media. Therefore tributyrin (0.8 μ mole) in ethanol (0.5 ml.) was added to each tube individually. The ethanol was evaporated to dryness under reduced pressure. A mixture of 0.5 ml. of buffer (0.1M, pH 7.3) and 0.4 ml. of 0.5% sodium deoxycholate was added, and the subsequent assay procedure carried out as described with the previous substrates.

The tributyrinase assays were employed only as indicators of non-specific esterase or lipase activity in certain enzyme preparations and cannot be regarded as quantitative. The colour yields of the di- and mono-glycerides produced by the action of the non-specific enzymes on tributyrin were not determined and could vary considerably from that of the parent triglyceride. Also, the distribution of these hydrolysis products in the biphasic system was not investigated. Although ovolcithin, lysolecithin and tributyrin are extracted quantitatively into the lower phase during chloroform-methanol treatment, a similar behaviour by other lipid materials cannot be assumed.

Purification of phospholipase A

Post-mortem human pancreas was minced with scissors and freed from blood vessels and fat as completely as possible. The minced tissue was washed briefly with aqueous 0.9% NaCl, blotted dry and frozen (-10°). While still in the frozen state the tissue was further subdivided by slicing with a scalpel.

Stage 1. A sample of the minced pancreas was homogenized for 10 min. in 10 vol. of acetone in a Waring Blender. The tissue suspension was centrifuged and the supernatant discarded. The precipitate was returned to the Blender and homogenized for 10 min. in 10 vol. of butan-1-ol (Morton, 1955). After centrifuging, the precipitate was resuspended in a further 10 vol. of acetone. During these operations the temperature of the solvents was not permitted to rise above 0°. The precipitate was again recovered by centrifuging, thoroughly mixed with 5 vol. of

diethyl ether and allowed to stand at room temperature for 15 min. with occasional stirring. After centrifuging, the supernatant was discarded, the precipitate broken up with a spatula and quickly dried *in vacuo*. The dried material is referred to as fraction A. It appears to be stable for months in the dry state.

Stage 2. Fraction A (1 g.) was homogenized in 25 ml. of aqueous 50% (v/v) glycerol for 15 min. in a tight-fitting all-glass homogenizer. After the addition of 25 ml. of 1% NaHCO_3 , homogenization was continued for a further 15 min. After centrifuging at 10 000g for 15 min., the slightly cloudy supernatant was decanted and either used directly or subjected to the series of solvent fractionations described below. The phospholipase A contained in the glycerol-treated preparation is remarkably stable, and was unaffected by storage for up to 2 weeks at room temperature.

Stage 3. The glycerol-treated preparation was adjusted to pH 4, and cooled to 0°. Acetone (3 vol., at a temperature of -5° or less) was added slowly and with constant stirring. The preparation was allowed to stand in an ice bath for a short time and was centrifuged. The supernatant was discarded and the rather gummy precipitate treated in cold (-5°) acetone by kneading with a stirring rod. After centrifuging, the acetone was poured off, diethyl ether added and the kneading procedure repeated. The solid, referred to as fraction B, was recovered by centrifuging and dried *in vacuo* over P_2O_5 .

Stage 4. Fraction B (0.5 g.) was dissolved in 50 ml. of water containing a trace of NaOH, so that the final pH of the solution was in the range 7.5-8.0. The pH was next adjusted to 4.5 and the preparation was heated to 75°, kept at this temperature for 5 min. and then cooled rapidly to room temperature. After centrifuging, the clear supernatant was decanted from the underlying layer of denatured protein. The pH of the supernatant was checked, readjusted if necessary to 4.5, and the solution was cooled to 0°. The addition of 3 vol. of cold (-5°) acetone brought down a precipitate, which was collected by centrifuging and washed once with acetone as described in stage 3. The solid material (fraction C) was dried *in vacuo*.

Stage 5. Fraction C (0.1 g.) was dissolved in 20 ml. of mm-EDTA, and the solution was dialysed against several changes of distilled water over a period of 24 hr. After dialysis, the now cloudy preparation was transferred quantitatively to a centrifuge bottle. The pH was adjusted to 4, the preparation cooled to 0° and 6 vol. of cold (-5°) acetone added slowly with constant stirring. The preparation was stored at -15° for 1 hr. before centrifuging. The supernatant was discarded and the solid washed once with acetone as described above. The final precipitate was dried *in vacuo* over P_2O_5 . The white solid is referred to as fraction D.

Fractions B, C and D are stable in the dry state at room temperature. They are water-soluble, particularly in a slightly alkaline medium.

RESULTS

Assay system. An aqueous assay system has been described which contained 2,4,6-collidine buffer and diethyl ether (Magee & Thompson, 1960). Snake venom was used as a convenient source of the enzyme in this study, and it was hoped that the method could be used as a basis for a survey of phospholipase A activity in tissues. This has proved to be impossible, since the mammalian enzyme possesses properties which appear to necessitate solubilization of the substrate. A satisfactory assay system, development of which is discussed below, contains ovolecithin solubilized with sodium deoxycholate in glycylglycine buffer.

A direct comparison of the activity of cottonmouth-moccasin-venom phospholipase A in the two assay systems is made in Table 1. Although incubation was carried out at a higher temperature with the glycylglycine-deoxycholate system, the phospholipase A activity of the venom was greater in the 2,4,6-collidine-ether system.

Table 1. *A comparison of phospholipase A activities in various reaction systems*

Reaction mixture contained 1 mg. of ovolecithin in 1 ml. of either 2,4,6-collidine (C) or glycylglycine (G) buffer (0.05M, pH 7.3), activated by either 0.1 ml. of diethyl ether (E) or 2 mg. of sodium deoxycholate (D).

Source of phospholipase A	Reaction system			Specific activity*
	Buffer	Activator	Temp.	
Cottonmouth-moccasin venom	C	E	30°	7500
Cottonmouth-moccasin venom	G	D	38	5920
Human pancreas (water homogenate of fraction A)	C	E	30	2.5
	G	E	30	2.3
	G	D	30	29.0
	G	D	38	46.9
Human pancreas (glycerol-treated fraction A)	G	D	38	67.5
Commercial pancreatin (water homogenate)	C	E	30	0.44
Commercial pancreatin (glycerol-treated)	G	D	38	0.72

* Ester linkage hydrolysed ($\mu\text{m-equiv./min./mg.}$).

With a water homogenate of solvent-extracted pancreas (fraction A) as the source of enzyme, there was little difference between the 2,4,6-collidine- and glycylglycine-buffered reaction systems with diethyl ether as activator. The slightly lower specific activity obtained with the latter buffer was observed in all experiments. At the same temperature (30°), replacement of ether by deoxycholate brought about a tenfold increase in the activity of the enzyme preparation. Increase of the incubation temperature to 38° produced the expected increase in reaction rate. However, a much higher activity was observed when the same pancreatic preparation was treated with glycerol before assay.

Commercial pancreatin contained relatively little phospholipase A (Table 1), and the activity was not strikingly increased in the presence of deoxycholate, as was the case with fraction A. Although prior treatment of the pancreatin with glycerol, and incubation in a system containing deoxycholate rather than ether, almost doubled the specific activity, this difference could be explained entirely on the basis of the temperature differential between the two reaction systems. It is possible that the enzyme present in the pancreatin does not respond to solubilization in the same way as the solvent-extracted pancreas tissue. However, a much more probable explanation would be that the pancreatin, which is prepared from a soluble extract of the tissue, contains only the phospholipase A that was in soluble form in the original pancreas. Pancreatin is not prepared with the object of phospholipase A recovery, and much of the enzyme could well be discarded in the tissue residue.

The very large increase in specific activity produced by activating with sodium deoxycholate rather than ether suggests that the deoxycholate itself is capable of some solubilization of the phospholipase A in fraction A. Discussion of this point is complicated by the fact that sodium deoxycholate also solubilizes the substrate. The amount of deoxycholate added to the system was quite critical and depended on substrate concentration. It was deemed preferable therefore to solubilize the enzyme with glycerol so that the effect of deoxycholate on the substrate could be investigated independently.

In preliminary experiments it was observed that autolysis of a water homogenate of fraction A from human pancreas could, under appropriate conditions, partly solubilize the phospholipase A. The effect was variable, however, whereas the glycerol treatment gave reproducible results and higher activities.

A quantitative comparison of specific activities in the two reaction systems described above by using glycerol-treated preparations from human pan-

creas was not possible. Glycerol-containing extracts could not be added to the 2,4,6-collidine-ether system without causing a precipitation of the emulsified ovoidlecithin, and sodium deoxycholate reacted with 2,4,6-collidine to form an insoluble complex.

With regard to the solubilization of pancreatic phospholipase A, Rimon & Shapiro (1959) reported that they were unable to extract an active enzyme from fresh ox pancreas, and that no phospholipase A activity could be detected in homogenates of fresh tissue. They made the unusual observation that, upon storage of frozen pancreas for at least 2 weeks, phospholipase A activity appeared. It was decided to investigate this phenomenon with frozen human pancreas. Each pancreas was minced and blended before storage in the frozen state, so that reproducible samples could be obtained as required. After storage periods of from 1 hr. to 100 days, samples of the tissue were treated and glycerol extracts prepared and assayed; all the enzyme preparations had very similar phospholipase A activities.

The effect of sodium deoxycholate at various concentrations on the time-course of hydrolysis of ovoidlecithin by a glycerol-treated fraction A preparation is shown in Fig. 1. Without deoxycholate in the reaction system, lecithin was not hydrolysed. The initial rate of hydrolysis was maximal in the presence of 2 mg. of sodium deoxycholate, at least for the conditions employed in these experiments. Higher concentrations were inhibitory. With 1 mg. (1.2 μ moles) of ovoidlecithin in the reaction system, 2 mg. (4.8 μ moles) of sodium deoxycholate represented an amount slightly in excess of that required

Fig. 1. Effect of sodium deoxycholate on the time-course of hydrolysis of ovoidlecithin by pancreatic phospholipase A. The reaction system contained 1 mg. of ovoidlecithin, 0–3 mg. of sodium deoxycholate and 1 mg. of glycerol-treated fraction A in 1 ml. of 0.05 M-glycylglycine buffer, pH 7.3. Temp., 38°. Concn. of sodium deoxycholate (mg./sample): ▲, 0; ○, 1; ●, 2; ■, 3.

to produce a completely clear solution of the ovolcithin in the buffered medium. Sodium taurocholate could replace sodium deoxycholate to a limited extent, but was much less effective.

Table 2. *Effect of enzyme concentration on initial reaction velocity*

Reaction mixtures contained 1 mg. of ovolcithin, 2 mg. of sodium deoxycholate and 0.5–1.5 mg. of glycerol-treated fraction A in 1 ml. of 0.05M-glycylglycine buffer, pH 7.3. Incubation temp., 38°.

Concn. of enzyme (mg. of glycerol- treated fraction A)	Initial reaction velocity (μ m-equiv. of ester linkage hydrolysed/min.)	
	Expt. A	Expt. B
0.5	41	40
1.0	84	80
1.5	119	107

Fig. 2. Hydrolysis of ovolcithin, lysolecithin and tributyrin by glycerol-treated fraction A. Reaction system contained the selected substrate, 2 mg. of sodium deoxycholate and 1 mg. of glycerol-treated fraction A in 1 ml. of 0.05M-glycylglycine buffer, pH 7.3. Temp., 38°. Substrate: ●, ovolcithin (1.2 μ moles/sample); ■, lysolecithin (2.4 μ moles/sample); ▲, tributyrin (0.8 μ mole/sample).

The optimum molecular relationship (4:1) between solubilizing agent and substrate noted above was also observed with higher substrate concentrations. It would be difficult to study the effect of substrate concentration on reaction velocity with this method, because high concentrations of sodium deoxycholate in the medium reacted with the glycylglycine to form an insoluble complex and an unstable ovolcithin solution. In addition, excessive deoxycholate tended to interfere with the ester-linkage determination.

The results in Table 2 show that the initial reaction velocity was doubled when the amount of enzyme in the reaction system was increased from 0.5 to 1.0 mg. The response was almost linear with further increase to 1.5 mg. of enzyme.

Glycerol-treated preparations of fraction A were capable of hydrolysing both lecithin and tributyrin (Fig. 2). The conversion of lecithin into lysolecithin proceeded to completion under appropriate experimental conditions. In contrast, the ester linkage of lysolecithin was not attacked (Fig. 2).

Investigation of purification procedure. The phospholipase A and tributyrinase activities of enzyme fractions obtained from human pancreas are shown in Table 3. Owing to the water-insoluble nature of most of the phospholipase A present in pancreatic tissue the specific activity in the original tissue had to be calculated assuming that the total activity assayed in glycerol-treated preparations of fraction A represented a complete recovery of the original enzyme. It can be seen that there was little loss of either phospholipase A or tributyrinase during the preparation of fraction B. In the heat-denaturation stage leading to the isolation of fraction C the tributyrinase was almost completely destroyed, the activity remaining representing approximately 0.1 % of that originally present. Although the conditions used were selected specifically to preserve phospholipase A activity (see below), a considerable loss occurred during heat-treatment, possibly because of the occlusion of active enzyme

Table 3. *Phospholipase A and tributyrinase activities of preparations of human pancreas*

Enzyme estimations were carried out in the glycylglycine–deoxycholate reaction system. Specific activities are expressed as μ m-equiv. of ester linkage hydrolysed/min./mg.

Enzyme source	Phospholipase A (pancreas no. 1)			Phospholipase A (pancreas no. 2)			Tributyrinase (average values)†		
	Fraction wt. (mg.)	Total activity	Specific activity	Fraction wt. (mg.)	Total activity	Specific activity	Fraction wt. (mg.)	Total activity	Specific activity
Original tissue	1 000	—	24.7*	1 000	—	9.4*	1 000	—	31.8*
Fraction A	168	24 700	147	139	9 390	67.5	159	31 800	200
Fraction B	55	24 200	440	58	9 160	158	57	29 000	510
Fraction C	18	12 300	685	22	6 710	305	20	20	<1
Fraction D	12	12 000	1 000	4	5 600	1 400	—	—	—

* Calculated values. † Average of values obtained in three separate experiments with three pancreases.

by denatured protein; the specific activity of fraction C, however, was greater than that of fraction B. Fraction D of pancreas no. 1 contained 49% of the original total phospholipase A activity, representing a 40-fold purification of the enzyme. The phospholipase A activity of fraction D from pancreas no. 2 represented a 60% recovery and a 150-fold purification of the enzyme.

Lowering the pH of an aqueous solution of fraction B to 4.0 resulted in the formation of a precipitate, only 42% of the phospholipase A and 2% of the tributyrinase remaining in the supernatant. Evans & Stansfield (1960) have also noted that the lipase of pig-pancreas homogenates is insoluble at low pH.

Effects of heating. Pancreatic phospholipase A was unaffected by heating for 5 min. at pH 5 at temperatures of up to 75°. Boiling for 10 min. at pH 5.0 inactivated it by 42%. Like snake-venom phospholipase A (Levene, Rolf & Simms, 1924; Hughes, 1935), pancreatic phospholipase A becomes increasingly unstable to heat with increasing pH, although after heating for 5 min. at 75° at pH 10 about 50% of the activity is still found in the supernatant. Boiling for 10 min. at pH 10.0 almost completely precipitates the enzyme. The tributyrinase of human pancreas is readily destroyed by heat.

Fig. 3. Effect of pH on the specific activity of pancreatic phospholipase A. Reaction system contained 1 mg. of ovolecithin and 2 mg. of sodium deoxycholate in 1 ml. of 0.05M-glycylglycine buffer. Incubation temp., 38°. Enzyme source: ○, 1 mg. of glycerol-treated fraction A; ●, 50–100 μg. of fraction D.

Effect of pH. Fig. 3 shows that for both fraction D and glycerol-treated fraction A the phospholipase A of human pancreas was optimally active at pH 9.0.

Inhibitors and activators. The effect of certain enzyme inhibitors and other reagents on the activity of pancreatic phospholipase A is shown in Table 4. The enzyme does not appear to be SH-dependent, since iodoacetate, cysteine, glutathione and Hg^{2+} ions (see Table 5) have little effect. Although fluoride also was without effect the results obtained with EDTA are complex; thus

Table 4. *Effect of certain enzyme inhibitors and other reagents on the hydrolysis of ovolecithin by phospholipase A from human pancreas*

Reaction mixtures contained 1 mg. of ovolecithin and 2 mg. of sodium deoxycholate in 0.8 ml. of a glycylglycine-buffered medium. Other reagents, and the enzyme solution, were added in a volume of 0.1 ml. The enzyme (100 μg. of fraction C) was added after temperature equilibration. The complete reaction system had a volume of 1 ml., a buffer concentration of 0.05M and pH 7.3. Incubation was at 38°, with a reaction period of 10 min.

Reagent added	Concn. (mM)	Activity (as percentage of control)
Iodoacetate	1	102
F^- ions	20	95
Diethyl <i>p</i> -nitrophenyl phosphate (E 600)	0.1	109
Glutathione	1	79
Ascorbic acid	1	80
Cysteine	1	126
EDTA {	0.001	121
	0.01	172
	0.1	22
	1	0
KCN {	0.1	86
	1	123
	10	163
Bovine serum albumin (1 mg./ml.)	—	106
Bovine serum albumin (10 mg./ml.)	—	160

Table 5. *Effect of metal cations on the hydrolysis of ovolecithin by phospholipase A from human pancreas*

Experimental conditions were as described in Table 4.

Cation added	Concn. of added cation (mM)			
	0.001	0.01	0.1	1.0
	Activity (as percentage of control)			
K^+	—	—	—	100
Hg^{2+}	—	—	93	86
Fe^{3+}	—	98	—	86
Zn^{2+}	—	81	27	20
Ag^+	—	102	75	0
Pb^{2+}	104	72	15	0
Al^{3+}	—	96	94	182
Ca^{2+}	—	—	85	55

0.01 mM-EDTA showed a marked stimulatory action on fraction C, which was drastically reduced when a dialysed preparation (fraction D) was used. Higher concentrations of EDTA were strongly inhibitory. The almost identical increases in enzyme activity produced by 0.01 mM-EDTA, 10 mM-potassium cyanide and bovine serum albumin (10 mg./ml.) suggest, however, the possibility that an inhibitory metal cation may be present in the reaction system.

The marked inhibition by high concentrations of EDTA (0.1–1.0 mM) could be caused by the removal of some cofactor required by the enzyme or because of some direct action of EDTA on the enzyme itself. With regard to the latter supposition Zieve & Vogel (1961) found that priorsaturation of the EDTA with calcium did not alter its inhibitory effect.

The stimulatory effect of cyanide on pancreatic phospholipase A can be contrasted with its action on phospholipase B. Fairbairn (1948) observed that the phospholipase B of *Penicillium notatum* was inactivated by cyanide. Marples & Thompson (1960) noted that 10 mM-potassium cyanide produced a 20% inhibition of rat-liver phospholipase B.

The effect of certain metal cations on the hydrolysis of ovoidicithin by phospholipase A from human pancreas is shown in Table 5; mM- K^+ ions had no effect. In marked contrast with phospholipase B from mammalian sources (Dawson, 1956; Marples & Thompson, 1960), phospholipase A was almost unaffected by the presence of Hg^{2+} ions. Zn^{2+} , Ag^+ and Pb^{2+} ions were strongly inhibitory. The stimulation produced by mM- Al^{3+} ions has been repeatedly found but is unexplained. In confirmation of the work of Zieve & Vogel (1961), Ca^{2+} ions were inhibitory when added to a reaction system containing sodium deoxycholate.

In most of the experiments summarized in Table 5 the cations under investigation were added as solutions of the appropriate chloride. In certain cases this was not possible because of the solubility characteristics of the salt concerned. However, the nature of the accompanying anion appears to be of little relevance here, as millimolar concentrations of Cl^- , NO_3^- , SO_4^{2-} and acetate ions had no effect on the rate of enzymic hydrolysis.

DISCUSSION

The lack of agreement in the earlier literature (see Ercoli, 1940) about the presence of phospholipase A in pancreas preparations could well be explained by the fact that the enzyme requires special treatment before becoming fully water-soluble, as well as by the inadequacy of the assay systems employed. It was found in this investigation that water homogenates of acetone- and butanol-extracted human tissue exhibited con-

sistently low phospholipase A activities. The full activity of such preparations was realized only after solubilization of the phospholipase A by treatment with aqueous glycerol. The method employed differed slightly from that outlined by Shapiro (1952) in that the tissue powder was homogenized in a larger volume of liquid and 1% sodium hydrogen carbonate was used instead of 0.1%. The more concentrated hydrogen carbonate solution raised the pH of the final homogenization medium to approximately 8. Both modifications improved the recovery of phospholipase A. Complete solubilization of the enzyme by this technique is suggested by the fact that the phospholipase A activity of such preparations was unaffected by centrifuging at 10 000g.

Storing human pancreas in the frozen state had no effect on the phospholipase A content of the tissue in either unsolubilized or glycerol-treated preparations. This is in contrast with the observations of Rimon & Shapiro (1959), who were unable to detect the enzyme in aqueous extracts of fresh ox pancreas, but who found activity after the frozen tissue had been stored for at least 2 weeks. The same authors stated that autolysis of the tissue at higher temperatures produced a variable activation of the enzyme. This suggests that some mechanism other than autolysis, perhaps alternate freezing and thawing, had solubilized or activated the ox-pancreas enzyme in these experiments. With the method outlined here, we have had no difficulty in demonstrating a considerable phospholipase A activity in fresh rat pancreas.

The phospholipase A of human pancreas did not attack ovoidicithin readily unless the substrate had been solubilized previously by sodium deoxycholate. It was found (Fig. 1) that enzymic hydrolysis proceeded optimally in a system in which a 4:1 molecular relationship existed between solubilizing agent and substrate. Whereas the phospholipase A of cottonmouth-moccasin venom was more active in the 2,4,6-collidine-ether system than in the glycylglycine-deoxycholate system (Table 1), the reverse was true of the pancreatic enzyme. In fact, with increasing purity the pancreatic enzyme became less and less active in the 2,4,6-collidine-ether system. Fraction D (Table 3), with a specific activity approaching that of the snake venom (Table 1), in the deoxycholate-containing system, was inactive in the 2,4,6-collidine-ether-reaction system. Attempts to activate the mammalian phospholipase A in the 2,4,6-collidine-buffered medium by the addition of bovine serum albumin and other reagents were unsuccessful. Zieve & Vogel (1961) reported that diethylether slightly inhibited phospholipase A, but we were unable to demonstrate any effect of 0.1 ml. of ether/ml. of reaction mixture on pancreatic

phospholipase A activity in the glycylglycine-deoxycholate-reaction system. In this regard, however, it was found that fraction D was completely inactive in the ethereal system used by Hanahan (1952) and Long & Penny (1957).

Although human pancreas contains an active tributyrinase (Table 3; Fig. 2), the presence of this enzyme did not interfere with the phospholipase A estimations. For example, solutions of fraction B could be heated to 75° for 5 min. at pH 5.0, tributyrinase being thereby inactivated, without changing the rate at which ovoidlecithin was hydrolysed. In the presence of an active tributyrinase the complete hydrolysis of lecithin by phospholipase A took place (Fig. 2), but the lysolecithin produced was completely stable. It is concluded therefore that pancreatic tributyrinase is unable to act on either lecithin or lysolecithin.

The absence of phospholipase B activity in glycerol-solubilized pancreas preparations was surprising, as the enzyme has been clearly demonstrated in pancreas (Shapiro, 1952; Marples & Thompson 1960), and Dawson (1956) prepared phospholipase B by glycerol-solubilization of acetone-dried liver of the rat and sheep. Recent experiments in this Laboratory have shown that pancreatic phospholipase B is inhibited by sodium deoxycholate. The stability of lysolecithin in the deoxycholate-containing system makes possible therefore the estimation of phospholipase A by direct ester-linkage determinations.

Like phospholipase A from other sources, the pancreatic enzyme is remarkably heat-stable, particularly in solutions of pH 5 or less. Hanahan (1952) reported that pancreatic phospholipase A was 80% inactivated by heating for 10 min. at 70° at pH 5.5–6.8. Vogel & Zieve (1960) found that the phospholipase A present in the duodenal contents of man was unaffected by incubation for 30 min. at temperatures of 50–55° at pH 8.4, although there was a rapid inactivation of the enzyme at this pH by temperatures in excess of 80°.

The phospholipase A of human pancreas was optimally active at pH 9.0. Similar pH optima for phospholipase A preparations from mammalian sources have been reported. Ogawa (1936), using horse pancreas, found that the enzyme was most active at pH 9.4. In a study with pig pancreas, Noguchi (1944) reported an optimum of pH 10.1. Vogel & Zieve (1960) noted that the phospholipase A of human duodenal contents was optimally active at pH 8.4–8.5 in the presence of deoxycholate. Omission of deoxycholate from their system lowered the optimum to pH 7.4.

Most of the experiments reported in this paper have been carried out, not at the pH optimum, but at pH 7.3. Although this results in a decreased reaction velocity, the choice of the lower pH was

made for several reasons. The assay method was devised with a view to carrying out a phospholipase A-tissue survey. As the pancreas is probably the richest mammalian source of the enzyme, the assessment of phospholipase A in certain other tissues will require much longer incubation periods than were necessary in the present investigation. Under these conditions the stability of both the substrate and the enzyme would be greater at pH 7.3 than at pH 9.0. Also, the pH optimum may vary from tissue to tissue in the same species as well as from species to species. With the proposed tissue survey in mind, therefore, it was decided to use a system that was as physiological as possible. In addition, the study of cofactor requirements and enzyme inhibitors was simplified in a medium buffered at pH 7.3; at pH 9.0 the formation of insoluble hydroxides would be troublesome.

The inhibition produced by Ca^{2+} ions was unexpected; Ca^{2+} ions stimulate the phospholipase A activity of snake venoms (Long & Penny, 1957; Magee & Thompson, 1960), and Rimon & Shapiro (1959) observed a Ca^{2+} ion requirement with their pancreatic phospholipase A preparations. However, a similar inhibition of pancreatic phospholipase A by Ca^{2+} ions has been reported by Zieve & Vogel (1961). As the reaction system employed by the last-mentioned authors also contained sodium deoxycholate, one suspects that the Ca^{2+} ion effect might not be a direct inhibitory action, but an indirect consequence of an interference with the delicate balance between deoxycholate and substrate concentrations. A similar interference must be borne in mind when the action of other inhibitors is considered. It is possible that sodium deoxycholate can take over the usual role of calcium in deoxycholate-free systems.

A quantitative comparison of relative reaction velocities with other substrates has not been attempted as yet. A study of this type will require more information than we have available at the present time about the distribution of other phospholipids in the biphasic system and the colour yields of the lipids and their reaction products by the ester-linkage method. Preliminary experiments have shown, however, that phosphatidylethanolamine and phosphatidylserine are readily attacked by pancreatic phospholipase A. The ethanolamine plasmalogen of human white matter (kindly supplied by Dr G. R. Webster of this Department) is hydrolysed more slowly.

SUMMARY

1. A system containing ovoidlecithin solubilized with sodium deoxycholate in a glycylglycine-buffered medium is described that is suitable for the estimation of pancreatic phospholipase A.

2. Phospholipase A has been shown to occur in fresh or aged frozen human pancreas. The tissue requires special treatment to render the enzyme fully water-soluble.

3. A relatively simple and rapid method for the purification of phospholipase A from human pancreas is described.

4. Some of the properties of the pancreatic enzyme have been investigated, including heat-stability, pH optimum and the effect of certain inhibitors.

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Biochem. J. (1962) **83**, 25

The Absorption of Calcium, Strontium, Barium and Radium from the Gastrointestinal Tract of the Rat

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(Received 8 September 1961)

The comparative metabolism of calcium and strontium has been widely studied and it is now established that calcium is utilized in preference to strontium in many species of animals, including man (Comar, Whitney & Lengemann, 1955; Comar, Wasserman & Nold, 1956; Comar, Russell & Wasserman, 1957).

However, little attention has been paid to the metabolism of the two heavier members of the alkaline-earth series, namely barium and radium; and there are few reports in the literature on the comparative metabolism of the four elements.

In the present paper comparative studies of the effect of age, and other factors, on the absorption of calcium, strontium, barium and radium from the gastrointestinal tract of the rat are described.

In order to reduce biological variation, and to allow ready inter-comparison of results, absorption has been measured by means of double radioactive-tracer techniques with the following pairs of isotopes: ^{45}Ca and ^{85}Sr ; ^{85}Sr and ^{140}Ba ; ^{85}Sr and ^{226}Ra .

EXPERIMENTAL

Animals. The animals used were female rats of a highly inbred, brown-hooded August strain, aged between 14 days and 70 weeks. Animals less than 28 days old were suckled by the mother up to the start of each experiment; all other animals were maintained on M.R.C. diet no. 41 B, both food and water being allowed *ad libitum*.

This diet contains about 0.8% by wt. of calcium.

Radioactive materials. Solutions of $^{45}\text{CaCl}_2$, $^{140}\text{BaCl}_2$ and $^{226}\text{RaCl}_2$ in dilute HCl were obtained from The Radiochemical Centre, Amersham, Bucks. The specific activity of the ^{45}Ca was from 5 to 10 mc/mg. of Ca. The ^{140}Ba was carrier-free; ^{85}Sr was prepared as a carrier-free solution of $^{85}\text{SrCl}_2$ from RbCl bombarded with deuterons in a cyclotron (Overstreet, Jacobson, Scott & Hamilton (1951)).

The activity administered, as a single dose, to each animal was approx. $1\mu\text{C}$ of ^{45}Ca or ^{85}Sr , $10\mu\text{C}$ of ^{140}Ba and approx. $0.01\mu\text{g.}$ of ^{226}Ra .

Measurement of absorption. Appropriate amounts of the pair of isotopes being studied, contained in 0.2–0.5 ml. of 0.01N-HCl, or in some experiments in cow's milk, were administered by intragastric intubation. The activity was