Positional Specificities in Phospholipid Hydrolyses*

ALEX F. ROBERTSON† AND WILLIAM E. M. LANDS

From the Department of Biological Chemistry, the University of Michigan, Ann Arbor, Michigan

Received February 26, 1962

Lecithin containing a radioactive acid located specifically in either the β- or α′-position was synthesized enzymatically. Incubating these labeled lecithins with *Crotalus adamanteus*, *Crotalus atrox*, and *Bothrops atrox* venoms demonstrated the selective β-esterase activity of the venoms. The lecithinase of heat-treated pancreatin was also a β-esterase. In addition, lecithinase activity was demonstrated in homogenates of rat intestine, spleen, lung, heart, liver, and brain. In all tissue homogenates tested no radioactive lysolecithin accumulated, so that the lysolecithinase activity appeared to be at least as great as the lecithinase activity.

Experiments have repeatedly demonstrated that saturated and unsaturated fatty acids tend to occupy stereospecifically distinct locations in the lecithin molecule (Hanahan, 1954). Furthermore, Hanahan and Blomstrand (1956) have shown that the incorporation of a specific fatty acid varies depending upon its structure and whether it is esterified at the α′- or β-position. These results led Hanahan to describe the α′-position as a metabolically active site and to suggest that the fate of a fatty acid may be a function of its location on the phospholipid molecule (Hanahan, 1960). The positional assignments were reversed at that time, and it is now known that the reactions studied involved the β-position.

We have been interested in studying the turnover of fatty acids in the lecithin molecule in order to find how selection of the different acids occurs. One possibility, described by Weiss *et al.* (1960), involves the specificity of cytidine diphosphate choline diglyceride transferase. These authors showed that this lecithin-synthesizing enzyme fails to act on some diglycerides, so that lecithins containing certain combinations of fatty acid radicals might not be formed. Another manner by which selection of fatty acids may occur would simply involve the hydrolysis of lecithin and its re-esterification at either the α′- or β-position. An enzyme from rat liver microsomes has been described that acylates lysolecithin (Lands, 1960). This enzyme has been shown to esterify preferentially unsaturated fatty acids at the β-position and saturated fatty acids at the α′-position to form lecithin (Lands, 1961). Thus the combined effect of a phospholipase with such an acylating system could produce a selective turnover of fatty acids at either the α′- or β-position.

Phospholipases have been found in many tissues (Zeller, 1951), but their relative activity or position of attack has in most cases not been determined, with the exception of the phospholipase from snake venom, which is now considered to be a β-esterase (Tattrie, 1959; Hanahan *et al.*, 1960; de Haas and van Deenen, 1961). The presence in tissues of such an enzyme could explain fatty acid turnover at the β-position. In contrast to this result, we know of no lecithinase initially hydrolyzing the α′-ester. The discovery of such an enzyme would help explain fatty acid turnover at the α′-position in phospholipids.

To study the presence of phospholipases and their position of attack we have prepared stereospecifically labeled lecithins (diacyl-GPC[1]). Using different isomers of lysolecithin (acyl-GPC) with a rat liver acylating system we were able to produce diacyl-GPC containing radioactive fatty acid in either the α′- or β-position.

Experimental Procedures

Materials.—Diacyl-GPC was isolated from chicken egg yolks by the method described by Hanahan (1951), and the lecithin fraction from beef heart lipids was isolated as previously described (Warner and Lands, 1961).

Dried snake venoms were purchased from Ross Allen's Reptile Institute. They were prepared for use by adding 25 mg of venom to 1 ml of 0.5 M Tris buffer at *p*H 7.5 and 0.02 ml of 1 M calcium chloride solution. This solution was diluted to 5 ml with deionized water to give a solution containing 0.5 mg of venom per 0.1 ml of solution.

Rat liver microsomes were prepared by ultracentrifugation in sucrose solutions as previously described (Warner and Lands, 1961). Heat-treated pancreatin was prepared by the method of Hanahan (1952).

* This work was supported in part by a grant (A-5310) from the National Institutes of Health. A preliminary report has been presented (Robertson and Lands, 1961).

† Supported by United States Public Health Service Postdoctoral Training Grant [2A-5278 (C1)] awarded to the Department of Pediatrics, The University of Michigan, Ann Arbor.

[1] The abbreviations used are: GPC, α-glycerylphosphorylcholine; GPE, α-glycerylphosphorylethanolamine.

All solvents used were reagent grade, the silicic acid used was 100–200 mesh (Mallinckrodt), and 1-C^{14}-oleic acid was purchased from the Volk Radiochemical Company.

Analytical Methods.—Phosphorus determinations were obtained by a modification of the method of Subba Row and Fiske (Bartlett, 1959). The concentration of plasmalogen in the lecithin fractions was determined by the iodometric analysis for vinyl ether (Norton, 1960). For chromatographic separation of lipids on silicic acid, three different solvent systems were used.

(*A*) To separate the fatty acids, phosphatidic acids, diacyl-GPE, and diacyl-GPC, the sample, containing less than 30 μmoles of phospholipid in a diethyl ether solution, was placed on a 5-g silicic acid column moistened with diethyl ether (Lands, 1958). Neutral lipids and fatty acids were then eluted with 50 ml of diethyl ether, and 5-ml fractions were collected. This was followed by establishing a gradient (G1) of 50% ether in ethanol[2] into ether (100 ml in the mixing chamber). During gradient elution, 10-ml samples were collected. When 100 ml of solvent had passed through the mixing chamber, the eluant was approximately 30% ethanol in ether (Warner and Lands, 1960). A second gradient (G2) was then begun by changing to 100% ethanol in the reservoir. When 100 ml of this gradient had left the mixing chamber, the eluant was approximately 75% ethanol in ether. A third gradient (G3) was then begun by changing to 100% methanol in the reservoir. After 150 ml of this gradient the eluant consisted of 77% methanol, 17% ethanol, and 6% ether.

(*B*) Fatty acids, diacyl-GPC, and acyl-GPC were eluted by the following stepwise procedure (Lands, 1960). The sample containing less than 30 μmoles of phospholipid was dissolved in 10% methanol in chloroform and placed on a 5-g silicic acid column moistened with the same solution. The lipids were then eluted with 50 ml of 10% methanol in chloroform, 250 ml of 40% methanol in chloroform, and 50 ml of methanol. The eluate was collected in 10-ml samples.

From the fractions collected in *A* ahd *B*, 0.1 ml was plated on a planchet, allowed to evaporate, and counted to ± 5% accuracy in a Nuclear-Chicago end-window gas-flow counter.

(*C*) For later experiments, involving less radioactivity, the sample, containing less than 30 μmoles of phospholipid, in 10% methanol in chloroform was placed on a 4-g silicic acid column moistened with the same solution. The lipids were eluted with 50 ml of 10% methanol in chloroform, 140 ml of 40% methanol in chloroform, and 50 ml of methanol, with the last 10 ml of each solvent collected separately. Each effluent solution was collected in a separate beaker and evaporated to dryness. The walls of the beakers were rinsed down with 50% methanol in chloroform and reevaporated. The lipid was then dissolved in 2 ml of 50% methanol in chloroform and a 0.1 ml sample plated as before for counting. The radioactivity of these fractions was then expressed as per cent of total radioactivity added to the incubation mixture.

[2] All mixed solvents are reported as percentage composition on the basis of their volumes.

Procedures

(*A*) *Preparation of β-Labeled Lecithin.*—α′-Acyl-GPC was produced from egg lecithin in diethyl ether solution by treatment with *Crotalus adamanteus* venom (Long and Penny, 1957) and purified by silicic acid chromatography. In a typical synthesis, 180 μmoles of α′-acyl-GPC and 150 μmoles of oleic acid (containing 6 μc of 1-C^{14}-oleic acid) were evaporated to dryness in a 500-ml Erlenmeyer flask. The residue was then dissolved in 150 ml of sodium phosphate buffer (0.1 M, *p*H 7.5). To this mixture was added 200 μmoles of ATP (the disodium salt dissolved in water and adjusted to *p*H 6.5 with sodium hydroxide), 200 μmoles of $MgCl_2$, 2 μmoles of coenzyme A (in aqueous solution adjusted to *p*H 6.5 with sodium bicarbonate), and the microsomal fraction from 3 g of rat liver. This mixture was stirred by a motor-driven glass rod. The flask was left unstoppered in a 37° water bath, and at the end of 30 and 60 minutes of incubation additional aliquots of ATP, Mg^{++}, CoA, and microsomes were added in amounts equal to those added originally. At the end of 3 hours of incubation the reaction was stopped by addition of 100 ml of 80% methanol in chloroform. Three hundred ml of 20% methanol in chloroform and 100 ml of H_2O were added. The mixture was inverted 10 times in a separatory funnel, and the chloroform-methanol solution was removed and evaporated to dryness. The lipids were then dissolved in 10% methanol in chloroform and placed on a 20-g silicic acid column and eluted with 100 ml of 10% methanol in chloroform, 500 ml of 40% methanol in chloroform, and 200 ml of methanol. The eluate obtained with 40% methanol in chloroform was evaporated to dryness and the residue brought up to a 50 ml volume in 50% methanol in chloroform. Aliquots of this material were then diluted for determination of phosphorus and radioactivity, and for treatment with snake venom.

(*B*) *Preparation of α′-Labeled Lecithin.*—Sixty-five μmoles of beef heart lecithin, containing 21 μmoles of plasmalogen, was dissolved in 3 ml of 40% methanol in chloroform. To this was added an ethanolic solution of iodine (23 μmoles) and 2 ml of water, and the mixture was stirred with a magnetic stirrer. After 2 minutes, 3 ml of absolute alcohol was added and the excess iodine was titrated with sodium thiosulfate to its potentiometric end-point. The alkenyl ether of plasmalogen reacts quantitatively under these conditions, which represent merely a large scale reaction of the assay procedure of Norton (1960). The β-acyl-GPC which is formed may be separated

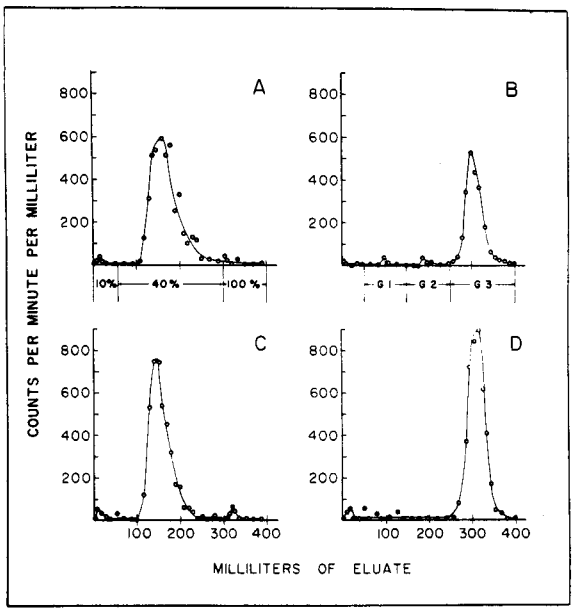


FIG. 1.—Silicic acid chromatography of synthesized C^{14}-diacyl-GPC. A and C represent the stepwise elution with 10%, 40%, and 100% methanol in chloroform. B and D represent elution with 50 ml diethyl ether followed by increasing proportions of ethanol in ether (G1 and G2) and finally by increasing proportions of methanol in ethanol (G3) as described in the analytical methods. A and B are chromatographs of β-1-C^{14}-diacyl-GPC, while C and D are chromatographs of α'-1-C^{14}-diacyl-GPC.

from the iodoaldehyde and contaminating diacyl-GPC by the use of silicic acid columns. This procedure is not advisable if the preparation of the β-isomer is to be completely free of the α'-acyl-GPC, since migration of the ester from the β- to α- position can occur during silicic acid treatment. Thus, to get labeling only in the α'-position, the complete reaction mixture, after titration of the iodine, was used as quickly as possible in the following manner. The solution was evaporated to dryness, brought up in 33% methanol in chloroform, and washed with water, and the water layer was removed and the lipid evaporated to dryness in an Erlenmeyer flask, along with 30 μmoles of oleic acid (containing 2 μc of 1-C^{14}-oleic acid). Phosphate buffer (50 ml), ATP (100 μmoles), $MgCl_2$ (100 μmoles), CoA (2 μmoles), and microsomes (2 ml) were added, and from this point on the two procedures were identical.

(*C*) *Treatment with Snake Venom.*—Suitable amounts of the synthesized diacyl-GPC (containing 10-20,000 cpm) were evaporated to dryness in a 10-ml volumetric flask. To this was added 3 ml of diethyl ether and 0.1 ml of the snake venom solution. The mixture was shaken vigorously at intervals and incubated at 20° for 3 hours. Ten ml of 40% methanol in chloroform was then added and the solution washed with one fifth volume of water.

(*D*) *Preparation of Substrate for Incubation with Tissue.*—An amount of radioactive diacyl-GPC sufficient for several enzymatic assays was evaporated to dryness in a screw-cap test tube. The lipid was then dissolved in 1 ml of diethyl ether. To this was added enough histidine buffer (0.1 M, *p*H 6.5) so that 1 ml of the mixture contained about 15,000 cpm. The mixture was shaken vigorously and the ether blown off with nitrogen. The mixture was again shaken and then "sonicated" in a 250-watt Raytheon sonicator for 15 minutes. Material so prepared will be referred to as the "sonicated diacyl-GPC."

(*E*) *Tissue Incubation.*—Tissue homogenates were prepared by homogenizing 5 g of tissue in 10 ml of histidine buffer (0.1 M, *p*H 6.5) with a Virtis "23" homogenizer for 1 minute. The incubation mixture contained 2 ml of this homogenate, 1 ml of a 0.28 M $CaCl_2$ solution, 0.9 ml of the "sonicated diacyl-GPC," and 2 ml of histidine buffer. Incubation was carried out in a shaking incubator at 37°. The reaction was stopped by the addition of 5 ml 80% methanol in choloroform. The lipids were extracted with 15 ml of 20% methanol in chloroform and washed with one fifth volume of water. The water layer was then removed.

RESULTS

Characterization of Specifically Labeled Lecithins.—When α'-acyl-GPC is incubated with 1-C^{14}-oleic acid in the presence of the microsomal acylating system, the expected product is β-1-C^{14}-diacyl-GPC. After purification the radioactive product is eluted from silicic acid by 40% methanol in chloroform (Fig. 1A); this shows that the isotope is in the diacyl-GPC fraction and not in the free fatty acid or acyl-GPC fraction. Figure 1B shows the radioactivity migrating in the methanol gradient of the ether-ethanol-methanol solvent system. This behavior proves that the isotope is not in the phosphatidic acid or diacyl-GPE fractions. These results show that the radioactive acid is incorporated into a component migrating with the diacyl-GPC fraction but give no information as to which position within the molecule the radioactive acid occupies. Incubation of the enzymatically synthesized diacyl-GPC fraction with *Crotalus adamanteus* venom containing a specific β-esterase (Tattrie, 1959; Hanahan *et al.*, 1960; de Haas and van Deenen, 1961) resulted in the release of C^{14}-fatty acids as shown in Table I. The amount of radioactivity appearing in the acyl-GPC fraction was 11.5%; this fact indicates that only a small amount of isotope is located at the α'-position. These results show that incubating α'-acyl-GP with 1-C^{14}-oleic acid in the presence of the microsomal acylating system yielded diacyl - GPC labeled predominantly at the β-position.

When β-acyl GPC is incubated with 1-C^{14}-oleic acid, the expected product is α'-1-C^{14}-diacyl-GPC. Similarly, this radioactive product was eluted

TABLE I

HYDROLYSIS OF LABELED LECITHIN BY *Crotalus adamanteus* VENOM

Lipid Fraction	Per Cent of Total Radioactivity			
	β-1-C^{14}-Diacyl-GPC		α′-1-C^{14}-Diacyl-GPC	
	Zero Time[a]	3 Hours	Zero Time	3 Hours
Fatty acids	16 (1)	80	0.5	0.5
Diacyl-GPC	75.5 (106)	1.5	97	2.3
Monoacyl-GPC	8.5 (5)	11.5	2.5	105

[a] Results from a control without added enzyme are shown in parentheses which indicate some enzymatic action occurred in this "zero-time" sample.

TABLE II

HYDROLYSIS OF LABELED LECITHIN BY *Crotalus atrox* and *Bothrops atrox* VENOMS

Lipid Fractions	Per Cent of Total Radioactivity					
	β-1-C^{14}-Diacyl-GPC			α′-1-C^{14}-Diacyl-GPC		
	No Enzyme	*Crotalus atrox*	*Bothrops atrox*	No Enzyme	*Crotalus atrox*	*Bothrops atrox*
Fatty acids	9	91	69	0	6	4
Diacyl-GPC	80	2	3	90	1.5	1.5
Monoacyl-GPC	11	3	11	4.5	119	89

TABLE III

HYDROLYSIS OF LABELED LECITHINS BY DIFFERENT PREPARATIONS OF PANCREATIC TISSUE

Lipid Fraction	Per Cent of Total Radioactivity[a]					
	Ox Pancreas Homogenate		Pancreatin		Heat-Treated Pancreatin	
	β-1-C^{14}-Diacyl GPC	α′-1-C^{14}-Diacyl GPC	β-1-C^{14}-Diacyl GPC	α′-1-C^{14}-Diacyl GPC	β-1-C^{14}-Diacyl GPC	α′-1-C^{14}-Diacyl GPC
Fatty acids	56 (2)	75 (4)	90	84	76 (3)	6 (8)
Diacyl-GPC	36 (95)	18 (91)	2	7	9 (91)	31 (91)
Monoacyl-GPC	8 (3)	2 (5)	2	7	2 (6)	52 (1)

[a] Values for "zero-time" controls are in parentheses.

only with the diacyl-GPC fraction by methanol-chloroform (Fig. 1C) and by ether-ethanol-methanol (Fig. 1D). Hydrolysis by *Crotalus adamanteus* venom resulted in no significant release of 1-C^{14}-acid. The per cent of the total radioactivity in the acyl-GPC fraction increased, however, from an initial 2.5% to a final 105%, proving that the isotope had been incorporated into the α′-position.

Specificities of Phospholipases from Other Venoms.—Table II shows the results of 3-hour incubations of the β- and α′-labeled diacyl-GPC with *Crotalus atrox* and *Bothrops atrox* venoms. In both instances, when β-1-C^{14}-diacyl-GPC was the substrate the isotope was found in the fatty acid fraction after incubation, indicating hydrolysis at the β-position. When α′-1-C^{14}-diacyl-GPC was used, the isotope was found predominantly in the acyl-GPC fraction, showing that little or no hydrolysis had occurred at the α′-position. These results prove that these venoms, like *Crotalus adamanteus* venom, contain a β-esterase.

Specificity of Phospholipases from Pancreas.—Homogenates of ox pancreas showed both β- and α′-esterase activity when incubated with labeled diacyl-GPC. As shown in Table III, with both β- and α′-labeled diacyl-GPC, the isotope was found predominantly in the free fatty acids, and the radioactivity of the acyl-GPC fraction did not increase significantly. The α′-esterase activity might have been lysolecithinase activity which has been described previously in this tissue (Shapiro, 1953). To investigate the possibility that released 1-C^{14}-acid might have been incorporated into the acyl-GPC fraction, thereby randomizing the stereospecific labeling, 1-C^{14}-oleic acid and "cold" diacyl-GPC were incubated with the ox pancreas homogenate. No significant radioactivity was found in either the diacyl- or acyl-GPC fraction after a 3-hour incubation.

When commercial Pancreatin was used as the enzyme source, the radioactivity was again predominantly in the 1-C^{14}-acid fraction from both substrates, showing both β- and α′-esterase activity (Table III)

Heat-treated pancreatin (Hanahan, 1952) resembles snake venoms in its β-esterase activity. Table III shows an increase in 1-C^{14}-acid after

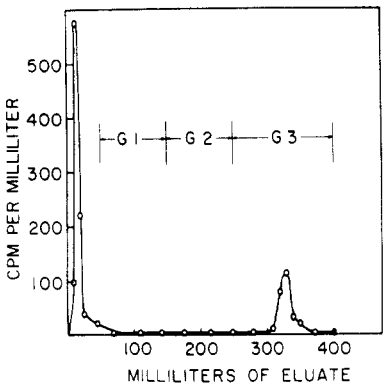


FIG. 2.—Silicic acid chromatography of lipids after incubation of β-1-C^{14}-diacyl-GPC with homogenate of rat spleen. Lipids were eluted with 50 ml of diethyl ether followed by increasing proportions of ethanol in ether (G1 and G2) and finally by increasing proportions of methanol in ethanol (G3) as described in the analytical methods.

incubation with β-1-C^{14}-diacyl-GPC and no increase in radioactive acyl-GPC. In contrast, incubation with α′-1-C^{14}-diacyl-GPC produces C^{14}-acyl-GPC and no significant increase of 1-C^{14}-acid. This result proves that heat treatment has selectively destroyed the α′-esterase activity of pancreatin.

Demonstration of Tissue Phospholipases.—A number of rat tissue homogenates were studied to determine what phospholipases were present in the various tissues. Table IV gives the percentage of isotope added which was recovered in the fatty acid fraction after 4 hours incubation. The percentage is corrected for hydrolysis occurring in the control incubation containing no enzyme. Intestine, spleen, and lung showed the most active hydrolysis, while kidney, heart, brain, and liver were less active; no hydrolysis was demonstrated in washed red blood cells or serum. With lung homogenates, 1-C^{14}-acid was released from both the β- and α′-labeled diacyl-GPC, indicating that both β- and α′-esterase activity were present. In addition, none of the reaction systems with rat tissues showed an accumulation of radioactive monoacyl-GPC. Furthermore, the addition of NaCN, NaF, or diethyl ether to the crude homogenate did not allow radioactive acyl-GPC to accumulate.

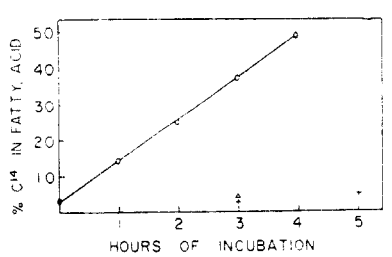


FIG. 3.—Incubation of β-1-C^{14}-diacyl-GPC with homogenate of rat lung. Δ represents incubation with no enzyme added. X, incubation with boiled enzyme.

TABLE IV

HYDROLYSIS OF LABELED LECITHIN (β-1-C^{14}-DIACYL-GPC) BY RAT TISSUE HOMOGENATES

Tissue	% C^{14} in Fatty Acid Fraction
Intestine (small)	77
Spleen	48
Lung	37
Kidney	13
Heart	13
Brain	11
Liver	10
Red blood cells (whole)	0
Serum	0

The lipids in the 10% methanol in chloroform fraction were rechromatographed to prove that the radioactivity was actually in the fatty acids and not in the form of a phosphatidic acid. Figure 2 shows the distribution of the isotope with the ether-ethanol-methanol solvent system used after incubation of β-1-C^{14}-diacyl-GPC with rat spleen homogenate. The two peaks represent fatty acid and diacyl-GPC, and no significant amount of radioactivity was found in any other fraction.

The progress of hydrolysis with increased incubation times was studied in lung homogenates. Figure 3 shows that approximately 50% of the added diacyl-GPC was hydrolyzed in 4 hours. In the absence of tissue no hydrolysis occurred in the incubation medium. Also, no significant hydrolysis occurred in five hours on incubation with boiled tissue homogenates, showing the heat-sensitive nature of the agent responsible for hydrolysis.

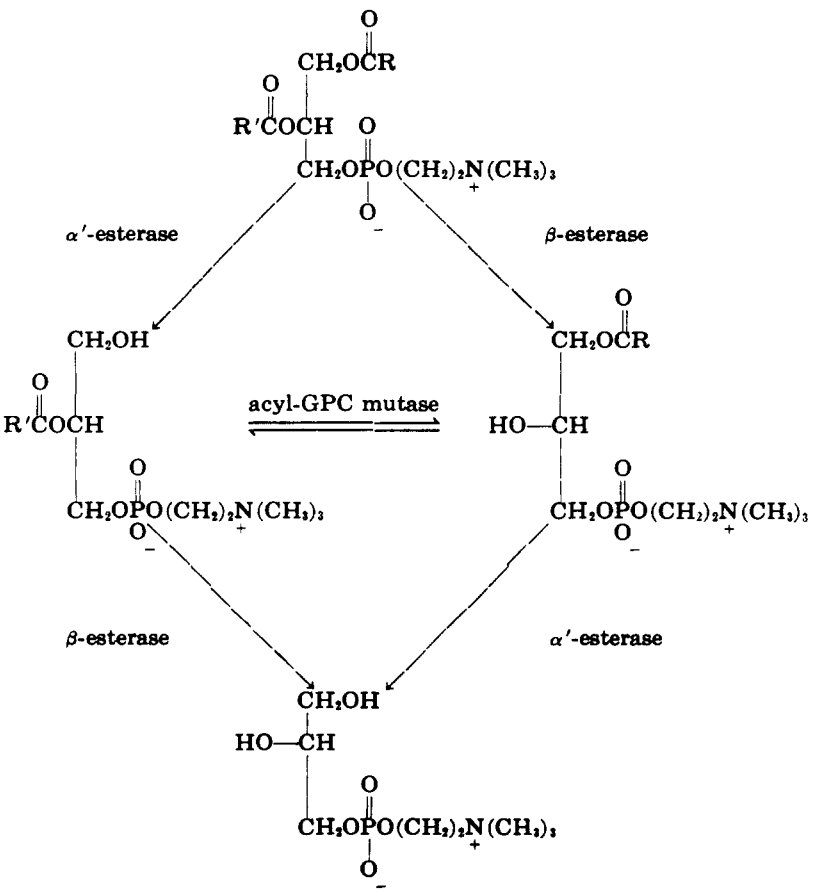
DISCUSSION

Other workers have determined the activity of tissue phospholipases by titrating the fatty acids released (Fairbairn, 1945) or by measuring the decrease in ester groups (Long and Penny, 1957) as hydrolysis proceeds. These methods, however, give no information about the position of attack of the phospholipase, but measure only the extent of hydrolysis.

Several direct and indirect methods have been used to indicate the positional specificity of snake venom phospholipases. Marinetti *et al.* (1958) and Debuch (1959) showed that the alkenyl ether group of plasmalogen was attached to the α′-position of glycerophosphatides. Since snake venom removes a fatty acid from plasmalogen (Franzl and Rapport, 1955) its specificity appears to be for the β-position. In this paper we have similarly used the known structure of plasmalogen to indicate the positional specificity of snake venom phospholipase. The results with β-labeled

lecithin show only that there is no net rearrangement of the molecule during venom hydrolysis and reacylation and that the venom consistently attacks the same position to release the radioactive acid. However, the α'-labeled lecithin is prepared from the β-acyl-GPC from plasmalogen and in this case no radioactive acid is released with venom, but instead, radioactive acyl-GPC is formed. These results show that, even with a diacyl-GPC derived from plasmalogen, the venom phospholipase attacks only the ester adjacent to the position that contained the alkenyl ether (*i.e.*, hydrolyzes the β-ester). Tattrie (1959) demonstrated by using gas chromatography that unsaturated fatty acids are released from egg lecithin with venom treatment, and that these fatty acids occupy the β-position. Using chemically synthesized lecithins with different fatty acids in the β- and α'-position, de Haas and van Deenen (1961) have shown directly that the fatty acids at the β-position are released by snake venom, whereas those in the α'-position remain esterified. In crude tissue homogenates many different fatty acids are present in the phospholipids and neutral lipids, and sometimes the same type of fatty acid may be found at both the β- and α'-positions (Dittmer and Hanahan, 1959a). For this reason gas chromatography of the released fatty acid will not prove conclusively the position or extent of attack of a phospholipase in the crude system.

If, however, in an experiment the added phospholipid contains at a specific position a fatty acid not found to any great extent in the tissue, its release can measure both the position as well as the extent of hydrolysis. We preferred to use radioactive acids in our experiments so that the synthesized substrate would most closely resemble the endogenous substrate. This objective seemed desirable since even acids as similar as palmitic and stearic show metabolic differences (Dittmer and Hanahan, 1959b). Even with such substrates, however, the absolute activity of the enzymes is difficult to determine since the relative availability of the exogenous and endogenous lecithin to the enzyme may vary. The difficulty of solubilizing lecithin, and its known affinity for proteins, are probably important factors controlling the rate of reaction. The relatively low solubility of lecithin in water solutions may explain the incomplete hydrolysis generally found in crude homogenates (Magee and Thompson, 1960). In some enzyme systems this difficulty may be avoided by using ether solutions (Hanahan, 1952) or ether-saturated water solutions (Magee and Thompson, 1960), but of course this method can be applied only to enzymes not denatured by this solvent.



Since all crude tissues that we have studied yielded C^{14}-fatty acids from both the β- and α'-positions, hydrolysis must occur at both points in the molecule. The apparently rapid removal of the second fatty acid from the lecithin molecule made it impossible to prove the stereospecificity of the tissue lecithinases.

The results may indicate the presence of both α'- and β-types of lecithinase or of a β-lecithinase followed by an α'-lysolecithinase. It is interesting that although these two enzymes have been demonstrated (Zeller, 1951), neither the α'-lecithinase or β-lysolecithinase have been shown to exist. Whenever there is any combination of lecithinase activity at one position and lysolecithinase activity at the other position, there will be produced GPC and two fatty acids, making it impossible to determine the original site of attack. This might be further complicated by the presence of an acyl-GPC mutase. However, inhibiting the lysolecithinase activity should cause monoacyl-GPC to accumulate. Then if we use β-labeled diacyl-GPC and there is present an α'-lecithinase, C^{14}-monoacyl-GPC will accumulate. So far we have not been able to demonstrate this with attempts to inhibit lysolecithinase with fluoride or cyanide or by diluting with nonradioactive acyl-GPC. For this reason, methods are badly needed for reducing the lysolecithinase activity in tissue homogenates without altering the lecithinase activity. Inactivation by high temperatures (Rimon and Shapiro, 1959) and high *p*H values (Dawson, 1956) have been proposed, but such treatment may also destroy lecithinases. If an α'-lecithinase exists, careful protein fractionation would permit its activity to be separated from that of the lysolecithinases to allow its identification.

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