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HYDROLYSIS OF PHOSPHOLIPIDS AND GLYCERIDES BY RAT-LIVER PREPARATIONS

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SUMMARY

1. Rat-liver homogenates were found to hydrolyze phospholipids, giving rise to both the 1-acyl and 2-acyl lysoderivatives. Subcellular fractionation of the homogenate separated the phospholipase A_1 (specific for the 1-acyl ester), the phospholipase A_2 (specific for the 2-acyl ester) and the lysophospholipase(s) to a large extent. The phospholipase A_1 was found to be located mainly in the microsomes, the phospholipase A_2 in the mitochondria and the lysophospholipase(s) in the soluble fraction.

2. Lipase activity, determined using sonicates of a triglyceride and phosphatidyl ethanolamine mixture, was found to be associated with the particulate fractions, mainly the mitochondrial fraction. The triglyceride on hydrolysis gave rise to free fatty acid and diglyceride.

3. Studies using inhibitors and heat inactivation show that the phospholipase A_2 requires metal ions, that the lipase is sensitive to *p*-chloromercuribenzoate and that the lysophospholipase(s) are sensitive to deoxycholate and heating.

INTRODUCTION

The first studies indicating that the two acyl groups of phospholipids undergo independent turnover were reported by HANAHAN[1] in 1960. Later LANDS, MERKL AND HART[2–5], found the 2-acyl glyceryl-3-phosphoryl choline to be acylated preferentially with saturated fatty acids while the 1-acyl glyceryl-3-phosphoryl choline acylated with unsaturated fatty acids by a liver system, suggesting the existence of both isomers in the liver. LLOVERAS, DOUSTE-BLAZY AND VALDIGUIÉ[6] and TATTRIE AND CYR[7] found that the nature of the fatty acid of monoacyl glyceryl-3-phosphoryl choline formed by various systems indicated that it was composed of both the 1-acyl and 2-acyl isomers.

In a study to obtain more direct evidence for the formation of the two lyso derivatives by a liver homogenate, VAN DEN BOSCH AND VAN DEENEN[8] investigated

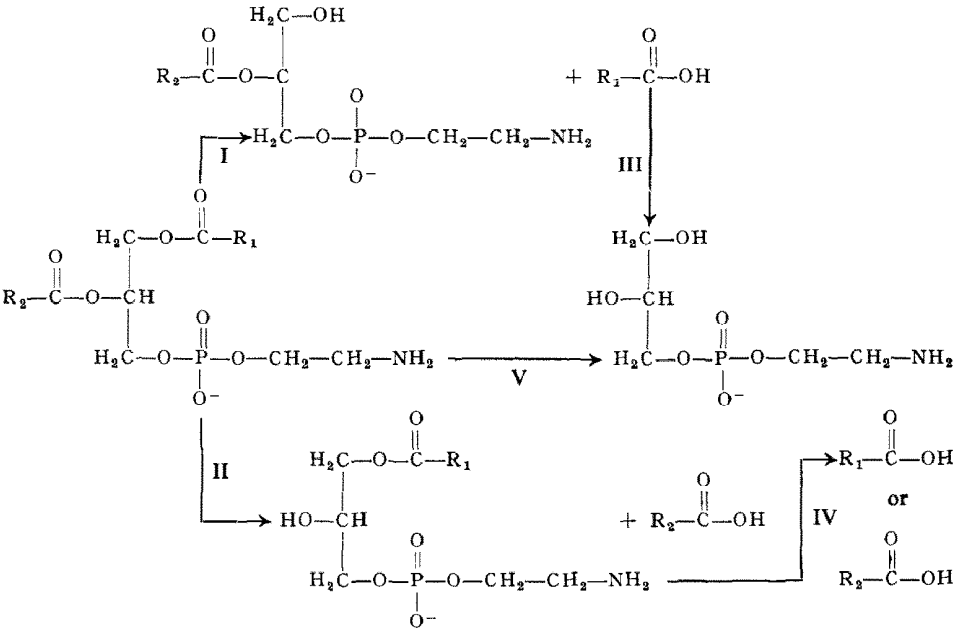
Abbreviation: PCMB, *p*-chloromercuribenzoate.

the breakdown of a mixture of [^{32}P]phosphatidyl choline and 2-[^{14}C]linoleoylphosphatidyl choline. The ^{32}P/^{14}C ratio of the monoacylglyceryl-3-phosphoryl choline produced by the action of the liver enzymes indicated the presence of both the 1-acyl and 2-acyl derivatives, although it could not be ascertained if this was due to the action of one or two enzymes. These conclusions were substantiated in a later work[9] utilizing the breakdown of the product lysoderivatives by phospholipase C (EC 3.1.4.3) forming the 1-acyl and 2-acyl glycerol which were separated and identified by thin-layer chromatography. The results of these studies together with those of LANDS AND MERKL[3] allowed the postulation of a monoacyl–diacyl phospholipid cycle. SCHERPHOF AND VAN DEENEN[10] reported that both [^{32}P]phosphatidyl choline and [^{32}P]phosphatidyl ethanolamine could be broken down by rat-liver mitochondria, the main product of the reaction being the ^{32}P-labeled lyso derivative. They found that phosphatidyl ethanolamine was hydrolyzed at a greater rate than phosphatidyl choline. BJØRNSTAD[11] was able to prepare phospholipids labeled with either [^{14}C]ethanolamine, [^{14}C]methionine, or $^{32}P_i$ in a rat-liver system *in vivo*. It was found that incubation of the microsomes which contained these labeled compounds in the presence of Ca^{2+} gave rise to the corresponding lysoderivative and the glycerylphosphoryl derivative, from which he concluded that both a phospholipase A (EC 3.1.1.4) and a lysolecithin acylhydrolase (EC 3.1.1.5) must be present in the microsomal fraction. This study also showed that phosphatidyl ethanolamine was broken down to a greater extent than phosphatidyl choline. A phospholipase A was partially purified from rat liver mitochondria by ROSSI *et al.*[12] using an acid precipitation procedure. They were able to find a stoichiometric relationship between the amount of phosphatidyl choline which disappeared and the amount of the lyso derivative and free fatty acid which was formed, indicating that their system was devoid of lysophospholipase activity. Neither SCHERPHOF AND VAN DEENEN[10], BJØRNSTAD[11], nor ROSSI *et al.*[12] identified the isomeric form of the monoacyl glycerol-3-phosphoryl ethanolamine or monoacyl glyceryl-3-phosphoryl choline derivative arising from the reaction, however.

The formation of the 2-acyl glyceryl-3-phosphoryl ethanolamine and 2-acyl glyceryl-3-phosphoryl choline was demonstrated by VOGEL *et al.*[13,14] in post-heparin plasma. A recent abstract by these workers indicated that they were unable to separate the enzyme hydrolyzing the 1-acyl fatty acid of the phosphatidyl ethanolamine or phosphatidyl choline from the lipase of the serum[15].

Recently GATT, BARENHOLZ AND ROITMAN[16] reported the presence of an enzyme in a particulate fraction of brain which catalyzed the hydrolysis of the 1-acyl group of phosphatidyl choline, although it was not stated if these preparations had lipase activity. Since DE HAAS, SARDA AND ROGER[17] have found that highly purified pancreatic lipase (EC 3.1.1.3) specifically hydrolyzes the 1-acyl group of phospholipids (although the activity on phospholipids was much lower than that on glycerides), caution must be exercised in determining the nature of the enzyme catalyzing the hydrolysis of the 1-acyl group of phospholipids. In a preliminary communication, SCHERPHOF, WAITE AND VAN DEENEN[18] showed that the activity in rat liver giving rise to the two lysoderivatives could be separated by cell fractionation. The mitochondria were reported to contain a phospholipase A which catalysed the hydrolysis of the 2-acyl fatty acid (Reaction II, Scheme 1) whereas the microsomes were found to bring about the hydrolysis of the 1-acyl fatty acid (Reaction I, Scheme 1). The purpose of this report is to relate further experiments on the phospholipases of the

rat liver, to describe a liver lipase activity which hydrolyses sonicates of a mixture of phospholipid and triglyceride, and to compare some of the properties of the lipolytic activities.



Scheme 1. Possible hydrolytic pathways of phosphatidyl ethanolamine.

MATERIALS AND METHODS

[1-^{14}C]Linoleic acid (specific activity 9.3 mC/mmole) and [9,10-$^{3}H_2$]palmitic acid (specific activity 266 mC/mmole) were purchased from the Radiochemical Centre, Amersham, Great Britain; sodium deoxycholate and PCMIB$_3$ (sodium salt) from Fluka, Buchs, Switzerland; Tris and EDTA from Koch-Light, Colnbrook, Great Britain; and $^{32}P_i$ from Philips-Duphar, Amsterdam, The Netherlands. Triglyceride (mainly triolein) of commercial olive oil was purified by chromatography on thin-layer chromatography plates in the ether–light petroleum (b.p. 60°–80°)–formic acid system (25:75:1.5, v/v). The various phospholipids were isolated from rat livers by extraction according to the method of BLIGH AND DYER[19] followed by chromatography on a column of silicic acid according to the method of HANAHAN AND BROCKERHOFF[20]. The purity of the isolated phospholipids was verified by thin-layer chromatography. Phospholipase A from *Crotalus adamanteus* and phospholipase D (EC 3.1.4.4) were purchased from H. Prenner, Schlangenfarm, Innsbrück, Austria and C. F. Boehringer and Soehne, Mannheim, Germany, respectively. Phospholipase C from *Bacillus cereus* was the generous gift ot L. M. G. van Golde of this department. The [^{32}P]phosphatidyl choline prepared according to the method of VAN DEN BOSCH[8], was kindly furnished by C. T. Bartels of this department. Before use it was repurified by thin-layer chromatography in the chloroform–methanol–water (65:35:4, v/v) system.

The [^{14}C]phosphatidyl choline and phosphatidyl ethanolamine were prepared as follows: 42 mg 1-acyl glyceryl-3-phosphoryl choline or glyceryl-3-phosphoryl ethanolamine (prepared by the hydrolysis of the 2-acyl fatty acid from rat liver phosphatidyl choline or phosphatidyl ethanolamine by phospholipase A), 500 μmoles ATP, and 12.5 μmoles coenzyme A in 12.5 Krebs–Ringer buffer (pH 7.1) were sonicated. To this were added 12.5 ml of 25% rat-liver homogenate in Krebs–Ringer buffer (pH 7.1). After incubating at 37° for 1 h, the lipids were extracted by the method of BLIGH AND DYER[19] and the [^{14}C]phosphatidyl ethanolamine and phosphatidyl choline were separated by thin-layer chromatography first using the chloroform–light petroleum (b.p. 60°–80°)–acetic acid (65:35:2, v/v) system, and then the chloroform–methanol–water (65:35:4, v/v) system. Hydrolysis of the [^{14}C]phosphatidyl choline and phosphatidyl ethanolamine by snake venom phospholipase A followed by isolation of the breakdown products showed that 99% of the [^{14}C]linoleic acid was in the 2-position of the phosphatidyl choline and 98% of the [^{14}C]linoleic acid was in the 2-position of phosphatidyl ethanolamine.

The [^{3}H]palmitoylphosphatidyl choline and phosphatidyl ethanolamine were prepared in the same manner as the [^{14}C]phosphatidyl choline and phosphatidyl ethanolamine except that the 2-acyl lyso derivative was used as the acyl acceptor. The 2-acyl lyso derivative was prepared by the action of pancreatic lipase on the rat-liver phosphatidyl ethanolamine or phosphatidyl choline. The lipase was purified through the first DEAE-cellulose column chromatography step according to the procedure of SARDA *et al.*[21] and was found to be about 90% specific for the 1-position as was found to be about 90% specific for the 1-position as shown by the breakdown of a sample of chemically synthesized 1-[^{3}H]stearoyl-2-[^{14}C]stearoylglyceryl-3-phosphoryl choline. The conditions for the formation of the 2-acyl glyceryl-3-phosphoryl choline or phosphatidyl ethanolamine were as follows: 100 mg of either the rat liver phosphatidyl ethanolamine or phosphatidyl choline was sonicated with 50 mg bovine serum albumin (used in place of deoxycholate since deoxycholate inhibits reacylation), 20 μmoles $CaCl_2$ and 1500 μmoles Tris buffer (pH 7.5) in 10.0 ml final volume. To this were added about 5000 units of the purified lipase and the reaction mixture was incubated for 4.5 h. The lipids were extracted and a sample was chromatographed on micro thin-layer plates which indicated that about 50% of the phosphatidyl ethanolamine or phosphatidyl choline was hydrolyzed. To minimize migration of the acyl group in the 2-position to the 1-position, the mixture of the monoacyl glyceryl-3-phosphoryl choline and phosphatidyl choline or monoacyl glyceryl-3-phosphoryl choline and phosphatidyl ethanolamine was used immediately for the reacylation. The isolation of the [^{3}H]palmitoyl phosphatidyl ethanolamine and phosphatidyl ethanolamine was as described for the ^{14}C-labeled compounds. Hydrolysis of the isolated [^{3}H]phosphatidyl choline and [^{3}H]phosphatidyl ethanolamine showed that 97% of the [^{3}H]palmitate was in the 1-position of the phosphatidyl choline and 88% of the [^{3}H]palmitate was in the 1-position of the phosphatidyl ethanolamine.

The samples of the [^{3}H]phosphatidyl choline and [^{14}C]phosphatidyl choline were mixed to give an isotopic ratio (disint./min) of $^{3}H:^{14}C = 15:1$, as were the samples of phosphatidyl ethanolamine. This corresponds to about $7.5 \cdot 10^5$ disint./min of ^{3}H and $5 \cdot 10^4$ distint./min of ^{14}C per mg phosphatidyl choline and about $1.5 \cdot 10^6$ disint./min of ^{3}H and $1 \cdot 10^5$ disint./min of ^{14}C per mg phosphatidyl ethanolamine. This ratio was varied according to the requirements of the experiment. It has been

shown by VAN GOLDE AND VAN DEENEN[23] that the 1-palmitoyl-2-linoleoyl glyceryl-3-phosphoryl choline (or glyceryl-3-phosphoryl ethanolamine) is one of the major species of these phospholipids in rat liver. The mixed (^{3}H- and ^{14}C-)labeled substrate prepared by the rat liver as described here is considered to be a natural species.

^{3}H, ^{14}C-labeled diglyceride was prepared by the action of phospholipase C from *B. cereus* on a sample of the mixed [^{3}H,^{14}C]phosphatidyl choline (see ref. 22). The diglyceride was purified on preparative thin-layer chromatographic plates developed in the ether–light petroleum (b.p. 60°–80°)–formic acid (25:75:1.5, v/v) system. The ^{3}H,^{14}C-labeled diglyceride prepared in this manner was found to co-chromatograph with chemically prepared 1,2-diglyceride. ^{3}H,^{14}C-labeled triglyceride was synthesized from the ^{3}H,^{14}C-labeled diglyceride in chloroform by reaction with a 20-fold excess of oleyl chloride in the presence of pyridine under anhydrous conditions. The ^{3}H,^{14}C-labeled triglyceride was purified in the same manner as the diglyceride.

[^{3}H,^{14}C]Phosphatidic acid was prepared from [^{3}H,^{14}C]phosphatidyl choline by hydrolysis with phospholipase D according to the procedure of DAVIDSON AND LONG[24]. The products of the reaction were separated by thin-layer chromatography on silica gel G plates prepared in 0.5 M oxalic acid developed in the chloroform–methanol–HCl (87:13:0.5, v/v) system.

Cell fractions were prepared from a 10% rat-liver homogenate in 0.25 M sucrose as follows; the nuclear fraction was precipitated by centrifugation at 1000 × *g* for 5 min, the mitochondria at 4500 × *g* for 10 min (washed once with sucrose and recentrifuged), the composite fraction at 12500 × *g* for 10 min and the microsomes at 100000 × *g* for 1 h. The soluble fraction was the supernatant solution obtained after precipitation of the microsomes. Protein determinations were carried out by the method of LOWRY *et al.*[25].

The reaction mixtures for the assays were prepared by drying the chloroform solution of the lipid under reduced pressure. Unless otherwise stated 0.2 mg phosphatidyl ethanolamine, 0.4 mg phosphatidyl choline, 0.5 mg triglyceride, 0.4 mg diglyceride or 0.4 mg phosphatidic acid was used in each experiment. To the tube containing the lipids were added, per incubation, 125 μmoles Tris buffer (pH 7.4), solubilizing agent (if any) and water to a volume of 0.5 ml. This was then sonicated at a setting of 1 A for 2 min with an MSE sonicator. The tube was kept in an ice bath to minimize hydrolysis during sonication. The sonicate was then incubated with 12.5 mg enzyme protein (unless otherwise stated), together with other compounds indicated in the text, in a total volume of 2 ml for 1 h (unless otherwise stated) at 37°. The reaction was stopped by the addition of methanol and the lipids were extracted according to the method of BLIGH AND DYER[19] followed by 2 additional extractions with chloroform. No ^{3}H or ^{14}C activity was found remaining in the water–methanol layer. In experiments in which comparison of the hydrolysis products was made using both [^{3}H,^{14}C]phosphatidyl choline and [^{32}P]phosphatidyl choline, in order to recover the water-soluble compounds, the mixture was extracted by adding 2 vol. of methanol and 1 vol. of chloroform to 1 vol. of the aqueous reaction mixture. The precipitated proteins were centrifuged from this homogeneous mixture, the lipid-containing supernatant was dried, and hydrolysis products were redissolved in methanol for chromatography. The ^{32}P-labeled compounds were separated on silicic acid-impregnated paper according to MARINETTI[26], whereas the ^{3}H,^{14}C-labeled hydrolysis products were separated by thin-layer chromatography on silica gel G plates (except for the phos-

phatidic acid which required silica gel plates prepared in 0.5 M oxalic acid). Phosphatidyl choline and phosphatidyl ethanolamine were chromatographed first in the chloroform–light petroleum (b.p. 60°–80°)–acetic acid (65:35:2, v/v) system, followed by the chloroform–methanol–water (65:35:4, v/v) system. The triglyceride and diglyceride hydrolysis products were chromatographed in the light petroleum (b.p. 60°–80°)–ether–formic acid (75:25:1.5, v/v) system, and the phosphatidic acid hydrolysis products in the chloroform–methanol–HCl (87:13:0.5, v/v) system. After development of the chromatograms the compounds were located with I_2 vapor and the silica gel containing the compounds was scraped from the plate and put into small columns for elution. Neutral lipids were eluted with 25 ml methanol and the phospholipids with 25 ml of a solution of 0.1% HCl in methanol. The methanol was evaporated under vacuum and the radioactive material dissolved in 0.5 ml methanol. This was quantitatively transferred to a scintillation vial and the radioactivity determined in a Packard Tri-Carb liquid scintillation counter. All values are corrected for quenching and the ^3H counts corrected for the contribution of the ^{14}C counts by use of an external standard. Using known amounts of the lysoderivatives, it was found that 80–90% of the compounds was recovered whereas nearly 100% of the free fatty acid was recovered. For this reason the values for the lysoderivatives must be taken as mininal. Control experiments with enzyme heated in boiling water for 10 min usually showed about 5% breakdown of the added substrate. For reasons still unknown, up to 10–12% of the triglyceride was broken down into monoglyceride upon sonication. All values reported are corrected for this. The percentages presented in the various experiments are calculated from the amount of radioactivity of the compound recovered as compared with the total recovered radioactivity. In experiments with phospholipids 85–90% of the added radioactivity was accounted for, whereas about 95% of the radioactivity was recovered in experiments with glycerides. The exception to this was with mitochondrial hydrolysis of triglyceride in which, in some cases, only 80% recovery was obtained.

RESULTS AND DISCUSSION

The data in Table I indicate the extent of hydrolysis of added mixed-label phospholipids and glycerides. In all cases a much greater formation of free fatty acid can be seen than of the lyso derivative, or monoglyceride or diglyceride, presumably due to the further breakdown of the lyso derivative or diglyceride. Addition of deoxycholate, a known inhibitor of lysophospholipase[27], to the reaction mixture caused an increase in the recovery of the lyso derivative and diglyceride although there was a slight decrease in the overall hydrolytic activity. Even though diglyceride was found to be hydrolyzed to a greater extent than triglyceride, the hydrolysis products of triglyceride usually were in part diglyceride. This possibly could be due to competition between the diglyceride formed and the relatively high amount of triglyceride remaining. Almost no monoglyceride was found to accumulate in these experiments. The recovered lysoderivative is composed of both the ^3H- and ^{14}C-labeled isomers, confirming the observation by VAN DEN BOSCH AND VAN DEENEN[9], in their experiments using a [^{14}C,^{32}P]phosphatidyl choline. Similarly, the diglyceride recovered was found to be composed of both isotopes. In similar experiments, ROBERTSON AND LANDS[28] were unable to detect the formation of

TABLE I

HYDROLYSIS OF VARIOUS PHOSPHOLIPIDS AND GLYCERIDES BY RAT-LIVER HOMOGENATES

Percent compound recovered was calculated as the percent of the compound recovered compared with the total amount of radioactivity recovered.

Substrate	*Deoxycholate*	*Percent compound recovered*							
		*Lyso derivative**		*Free fatty acid*		*Mono-glyceride*		*Di-glyceride*	
		^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C
Phosphatidyl ethanolamine	−	5.4	9.7	23.2	20.1				
	+	6.5	12.3	17.7	14.6				
Phosphatidyl choline	−	1.6	1.5	5.1	5.7				
	+	2.6	2.6	3.6	3.8				
Phosphatidic acid	−	3.3	3.7	85.3	82.3			5.0	5.3**
	+	5.8	6.3	51.2	51.0			2.9	5.2
Triglyceride	−			35.8	35.2	0	1.2	5.5	4.2
	+			22.8	23.6	0	1.9	8.3	5.7
Diglyceride	−			67.1	65.0	0	1.0		
	+			45.9	46.0	0	0		

* Lyso derivative corresponding to the added diacyl compound.

** This amount represents diglyceride, triglyceride and monoglyceride, since they all migrate near the solvent front in the chromatographic system used.

any lysoderivatives. These data indicate that hydrolysis of these added substrates can proceed at both the 1 and 2 positions (Reactions I and II, Scheme 1). If the triglyceride had been hydrolyzed specifically at the 1 or 3 positions a ratio $^{3}H:^{14}C = 1:2$ in the recovered diglyceride would be expected. Since the ratio was found to be closer to unity and since there was an almost equal amount of ^{3}H- and ^{14}C-labeled free fatty acid formed, it seems likely that this system is capable of hydrolyzing ester bonds at the 2 position as well as the 1 and 3 positions. The products of the phosphatidic acid hydrolysis can be seen to include small amounts of diglyceride, monoglyceride or triglyceride, probably due to the action of L-α-phosphatidate phosphohydrolase (EC 3.1.3.4). The high degree of hydrolysis of phosphatidic acid could be due to direct deacylation by phospholipase(s) or, in part, to the combined action of the phosphatidate phosphohydrolase, giving rise to diglyceride, followed by the action of lipase yielding free fatty acid and monoglyceride or glycerol. In these experiments using phosphatidic acid almost no phosphatidyl ethanolamine or phosphatidyl choline was found to be formed. Similarly, with added diglyceride, no more than 3% of the recovered radioactivity was found in the triglyceride, indicating that synthetic reactions were minimal under these conditions. A great deal of variability was found in the degree of hydrolysis and in the relative amount of lyso derivative or diglyceride formed. Possibly this is due to variations in the preparation of the sonicate or due to differences in the animals from which these preparations are made. For these reasons, and since no attempt has been made to ascertain how much of the endogenous lipid is involved in the hydrolytic reactions, caution must be exercised in comparing the breakdown of one substrate with that of another. Generally phosphatidyl ethanolamine was found to be hydrolyzed to a greater extent than phosphatidyl choline, similar to the observations of SCHERPHOF AND VAN DEENEN[10] and of BJØRNSTAD[11].

To show that the non-stoichiometric relationship between the lysoderivative and free fatty acid formed is due to the hydrolysis of the acyl ester of the lysoderiva-

tive (Reactions III and IV of Scheme 1), the hydrolyses of [^{3}H,^{14}C]phosphatidyl choline and of [^{32}P]phosphatidyl choline by the homogenate were compared (Table II). The difference between the ^{3}H,^{14}C-labeled free fatty acid and ^{3}H,^{14}C-labeled-monoacyl glyceryl-3-phosphoryl choline, 10.3%, presumably due to the formation of glyceryl-3-phosphoryl choline, is in good agreement with the amount of the ^{32}P-labeled water-soluble products (glyceryl-3-phosphoryl choline, glycerol phosphate or phosphate) formed, 13.9%. Further, the amount of ^{32}P-labeled water-soluble products plus one-half the amount of ^{32}P-labeled-monoacyl glyceryl-3-phosphoryl choline (since only one fatty acid had been released), 17.1%, is in good agreement with the amount of ^{3}H,^{14}C-labeled free fatty acid released, 16.3%. Both methods of comparison indicate that the further hydrolysis of the lyso derivative accounts for the difference between the lysoderivative formed and the free fatty acid released, but gives no indication whether the hydrolysis to free fatty acid and glyceryl-3-phosphoryl ethanolamine or glyceryl-3-phosphoryl choline is due to the combined action of phospholipase(s) and lysophospholipase(s) (Reactions I and III, and Reactions II and IV of Scheme 1), or to the action of one enzyme which hydrolyzes both fatty acids from a phospholipid (Reaction V, Scheme 1).

TABLE II

STOICHIOMETRY OF THE HYDROLYSIS OF PHOSPHATIDYL CHOLINE BY RAT-LIVER HOMOGENATES

Percent compound recovered was calculated as the sum of the amounts of ^{3}H and ^{14}C recovered divided by 2 so that the amount of the original phosphatidyl choline is 100%.

Substrate	*Percent compound recovered*				*Calculated recovery*	
	Phosphatidyl choline	*Monoacyl glyceryl-3-phosphoryl choline*	*Free fatty acid*	*Glyceryl-3-phosphoryl choline*	*Free fatty acid**	*Glyceryl-3-phosphoryl-choline***
^{3}H, ^{14}C]Phosphatidyl choline	77.7	6.0	16.3	—	—	10.3
^{32}P]Phosphatidyl choline	79.6	6.5	—	13.9	17.1	—

* Calculated as the amount of glyceryl-3-phosphoryl choline recovered *plus* one-half the amount monoacyl glyceryl-3-phosphoryl choline formed, since only one fatty acid had been released from the lyso derivatives.

** Calculated as the difference between the amount of free fatty acid formed and the amount of monoacyl glyceryl-3-phosphoryl choline formed.

By heating the homogenate at various temperatures for 5 min prior to incubation with phosphatidyl ethanolamine, greater amounts of the monoacyl glyceryl-3-phosphoryl ethanolamine were recovered, as seen in Fig. 1. The recovery of ^{3}H-labeled-monoacyl glyceryl-3-phosphoryl ethanolamine increased with temperature up to 65° at which point the recovery was found to be 650% of the 0° control. To a lesser but still appreciable degree there was an increase in the recovery of the ^{14}C-labeled-monoacyl glyceryl-3-phosphoryl ethanolamine up to about 55° or 60°. The amount of both fatty acids, on the other hand, remained constant or decreased with increasing temperature. These results indicate that the lysophospholipase(s) are more heat-labile than the phospholipases. The sum of the various hydrolysis products is found to be constant up to about 60°, giving a further indication of the relative stability of the phospholipase. This experiment clearly demonstrates that there are lysophospholipase(s) which are distinct from the phospholipases. Since it was not possible to show a stoichiometric relationship between free fatty acid and mono-

acyl phosphatidyl ethanolamine at these elevated temperature, it is not possible to rule out the existence of an enzyme attacking both esters (Reaction V). If such an enzyme is present, however, it can only account to a limited extent for the deacylation of the phospholipids.

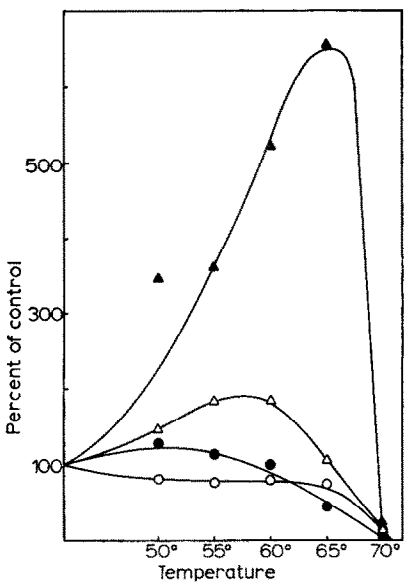


Fig. 1. Effect of temperature on enzymes in a rat-liver homogenate catalyzing the hydrolysis of phosphatidyl ethanolamine. Assay conditions were as described in METHODS except that the homogenate was preheated for 5 min at the indicated temperature. All values are expressed in percent of the values found using enzyme which was not heated; these are, for the ^{3}H-labeled-monoacyl glyceryl-3-phosphoryl ethanolamine (▲) 1.3%, [^{14}C]labeled-monoacyl glyceryl-3-phosphoryl ethanolamine (△) 2.4%. ^{3}H-labeled free fatty acid (●) 21.5%, and ^{14}C-labeled free fatty acid (○), 18.6%.

Examination of the products of hydrolysis catalyzed by the subcellular fractions show that the enzymes catalyzing the different hydrolyses of phosphatidyl ethanolamine are located in distinct parts of the cell, as shown by the results presented in Table III. The homogenate gives rise to both the ^{3}H- and the ^{14}C-labeled lysoderivatives, but a large proportion of this is further hydrolyzed. The nuclear fraction was found to have rather high activity producing large quantities of glyceryl-3-phosphoryl ethanolamine also. Since this fraction contains many particles in addition to the nuclei, it is not known to what this activity is due. The mitochondria were found to be rather specific in catalyzing the hydrolysis of phosphatidyl ethanolamine at the 2 position. In addition, it can be seen that the amount of glyceryl-3-phosphoryl ethanolamine calculated to be formed is rather low in comparison with the amount of the ^{3}H-labeled-monoacyl phosphatidyl ethanolamine recovered. Sonication of the mitochondria prior to their incubation with the phosphatidyl ethanolamine increased the recovery of the ^{3}H-labeled-monoacyl glyceryl-3-phosphoryl ethanolamine by 50%, causing the specificity of hydrolysis to be even greater (ratio ^{3}H:^{14}C in the monoacyl glyceryl-3-phosphoryl ethanolamine fraction changing from 9.1 to 39.2). Sonication seemed to have no effect on the amount of glyceryl-3-phosphoryl ethanol-

ABLE III

YDROLYSIS OF PHOSPHATIDYL ETHANOLAMINE BY SUBCELLULAR FRACTIONS OF RAT LIVER

'action	*Monoacyl glyceryl-3-phosphoryl ethanolamine*			*Free fatty acid*			*Glyceryl-3-phosphoryl ethanolamine*
	^{3}H (%)	^{14}C (%)	Ratio $^{3}H:^{14}C$	^{3}H (%)	^{14}C (%)	Ratio $^{3}H:^{14}C$	Calculated percent formed*
omogenate	0.2	11.3	0.018	19.2	5.2	3.69	6.5
uclear	1.4	15.9	0.088	32.4	13.1	2.46	14.1
itochondria	20.9	2.3	9.1	4.4	29.5	0.15	5.3
itochondria, sonicated	30.6	1.2	39.2	6.1	36.3	0.17	5.3
ɔmposite	1.5	7.5	0.20	9.2	7.5	1.22	3.8
icrosome	0.3	14.4	0.021	25.3	8.7	3.44	9.6
icrosome, sonicated	0.9	5.9	0.15	9.4	4.2	2.24	1.9
ɔluble	0	2.4	0	19.4	8.6	2.32	13.0

Calculated as the difference between the monoacyl glyceryl-3-phosphoryl ethanolamine and free fatty acid ivided by 2.

amine calculated to be formed. The microsomal fraction, in contrast to the mitochondrial fraction, was found to be rather specific in catalyzing hydrolysis at the 1 position, although the initial site of hydrolysis is somewhat difficult to ascertain since relatively large amounts of lysophospholipase present caused the further hydrolysis of the lysoderivatives formed. Another characteristic of the microsomal activity which differs greatly from that of the mitochondria is the response to sonication. In this case sonication caused a 3-fold decrease in the recovery of the ^{14}C-labeled-monoacyl glyceryl-3-phosphoryl ethanolamine. The calculated amount of glyceryl-3-phosphoryl ethanolamine also decreases but this in in part due to the decrease in the lysoderivative formed. The composite fraction, presumably containing microsomes, mitochondria and lysosomes, was found to have rather low activity and not to be specific in comparison with the mitochondrial and microsomal fractions. It would seem reasonable to assume that the activity in this fraction under these conditions is due to contamination with mitochondria and microsomes. The soluble fraction accumulated neither lysoderivative due to the high lysophospholipase activity, in agreement with the findings of ERBLAND AND MARINETTI[29]. It is impossible therefore to ascertain the initial site of hydrolysis in this fraction on the basis of this experiment. The sum of the activities found in the cell fractions (on a per mg basis) is nearly equal to the activity found in the total homogenate showing that cell fractionation did not cause loss of activity.

In Table IV data are presented concerning the specificity of the mitochondria and microsomes based on the percent of the ^{3}H-labeled- and ^{14}C-labeled-monoacyl derivatives formed. The sonicated mitochondrial fraction catalyzed the formation of monoacyl glyceryl-3-phosphoryl ethanolamine of which 93% was the ^{3}H-labeled form. If the mitochondria contained only phospholipases specific for the 2 position, the resulting monoacyl glyceryl-3-phosphoryl ethanolamine would be expected to be 98% the ^{3}H- and 2% the ^{14}C-labeled derivative. It is possible that the mitochondrial preparations are nearly as specific for the 2- position of phospholipids as the *C. adamanteus*[30] or heat-stable pancreatic[31] phospholipase A. The presence of the small amounts of lysophospholipase activity causes difficulty in precisely determining the degree of specificity.

The action of the microsomes gives rise to the formation of monoacyl glyceryl-

TABLE IV

MONOACYL ISOMER FORMED BY MITOCHONDRIA AND MICROSOMES

Fraction	*Number of experiments*	*Average percent of the lysophosphatidyl ethanolamine isomer**	
		^{3}H-labeled-monoacyl glyceryl-3-phosphoryl ethanolamine	*^{14}C-labeled-monoacyl glyceryl-3-phosphoryl ethanolamine*
Mitochondria	11	88.7	11.3
Mitochondria sonicated	6	93.7	6.3
Microsome	8	12.5	87.5
Microsome sonicated	4	20.3	79.7

* Calculated as amount of ^{3}H-labeled-monoacyl phosphatidyl ethanolamine or ^{14}C-labeled-monoacyl phosphatidyl ethanolamine divided by the sum of monoacyl phosphatidyl ethanolamine × 100.

3-phosphoryl ethanolamine which is up to about 87% the ^{14}C-labeled derivative. Since 12% of the ^{3}H is in the 2 position of the substrate phosphatidyl ethanolamine, this represents the expected percent of the ^{14}C-labeled derivative for an enzyme specific for the 1 position. Due to the large amounts of the monoacyl glyceryl-3-phosphoryl ethanolamine which were further degraded, it is impossible to ascertain the specificity of the initial site of hydrolysis. As can be calculated from the data presented in Table III, up to 35% of the ^{14}C released can be found as free fatty acid, indicating that the microsomal phospholipase may be minimally 65% specific for the 1 position. Since cell fractionation provides a means by which the activities catalyzing the hydrolysis of phospholipids at the 1 and 2 positions can be separated to a large extent, the microsomal activity is herein referred to as phospholipase A_1 (specific for the 1 position) and the mitochondrial activity referred to as phospholipase A_2 (specific for the 2 position), as proposed by VAN DEENEN AND DE HAAS[32].

The data in Table III indicate that the soluble fraction contains an enzyme or enzymes capable of catalyzing the hydrolysis of phosphatidyl ethanolamine even though the initial site of attack could not be ascertained. Similar experiments were then performed comparing the activity of the soluble fraction with and without added deoxycholate. The results of this experiment, presented in Table V, show

TABLE V

EFFECT OF DEOXYCHOLATE ON HYDROLYSIS OF PHOSPHATIDYL ETHANOLAMINE BY THE SOLUBLE FRACTION OF RAT LIVER

Conditions	*Percent compound recovered*						
	^{3}H-labeled compound		*^{14}C-labeled compound*		*Sum*		*Calculated recovery of glyceryl-3-phosphoryl ethanolamine**
	Monoacyl glyceryl-3-phosphoryl ethanolamine	*Free fatty acid*	*Monoacyl glyceryl-3-phosphoryl ethanolamine*	*Free fatty acid*	*Monoacyl glyceryl-3-phosphoryl ethanolamine*	*Free fatty acid*	
Without deoxycholate	0.7	34.5	0.6	37.9	1.3	72.4	35.5
With deoxycholate	14.6	19.0	15.2	17.8	29.8	36.8	3.5

* Calculated as the difference between monoacyl glyceryl-3-phosphoryl ethanolamine and free fatty acid divid by 2 so that amount of the original phosphatidyl ethanolamine is 100%.

that the lysophospholipase activity of the soluble fraction is 90% inhibited by the addition of deoxycholate, while the overall activity (measured as the change in phosphatidyl ethanolamine disappearance) is only 20% inhibited. Under these conditions about 15% of each isotope recovered is now found as the monoacyl glyceryl-3-phosphoryl ethanolamine, indicating the presence of both phospholipase A_1 and A_2 in the soluble fraction. It cannot be ascertained from these experiments if these two activities are due to one or two enzymes.

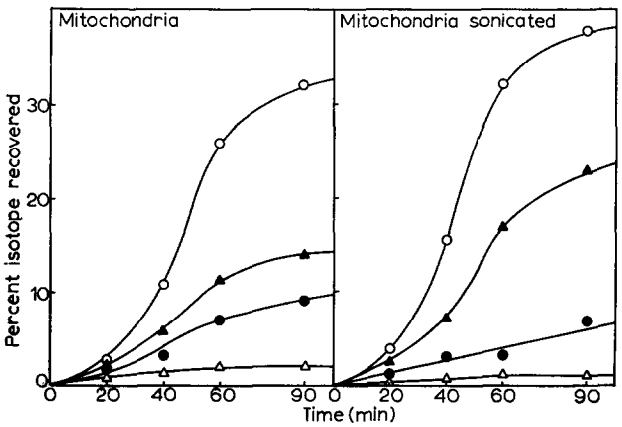


Fig. 2. Effect of time on the formation of the various hydrolysis products from phosphatidyl ethanolamine by mitochondria and sonicated mitochondria. Assay conditions were as described in METHODS except for the variation in time of the incubation. The preparations of the sonicated mitochondria were prepared with an MSE sonicator set at 1 A for 2–3 min, the mitochondrial preparation being kept in an ice bath during sonication. The designations of the compounds formed are the same as in Fig. 1.

The activity of the phospholipase A_2 was studied as a function of time. As can be seen in Fig. 2 there is an increase in the formation of the various hydrolysis products up to about 1 h, after which there is a decrease in the activity. Also, a lag period of 20 min can be observed. This was thought to be due to an initial impermeability of the mitochondrial membrane to the added phospholipid. Even though there is an overall increase in activity upon sonication of the mitochondria, the lag period is still found. It is possible that some physical interaction of the enzyme and phospholipid must occur before the enzymatic catalysis can take place. As shown above, the formation of the ^{3}H-labeled free fatty acid and ^{14}C-labeled-monoacyl glyceryl-3-phosphoryl ethanolamine is reduced by sonication of the mitochondria. Fig. 3 shows the effect of sonication of the mitochondria on the hydrolysis at each position (calculated as the sum of ^{3}H-labeled-monoacyl glyceryl-3-phosphoryl ethanolamine and ^{14}C-labeled free fatty acid for the 2 position and ^{3}H-labeled free fatty acid and ^{14}C-labeled-monoacyl glyceryl-3-phosphoryl ethanolamine for the 1 position). Here again it can be seen that sonication increases the activity at the 2 position and decreases the activity at the 1 position.

Increasing concentrations of mitochondrial protein cause increasing degrees of hydrolysis at the 2 position up to a concentration of nearly 20 mg/ml as seen in Fig. 4. The formation of ^{3}H-labeled free fatty acid and ^{14}C-labeled-monoacyl glyceryl-

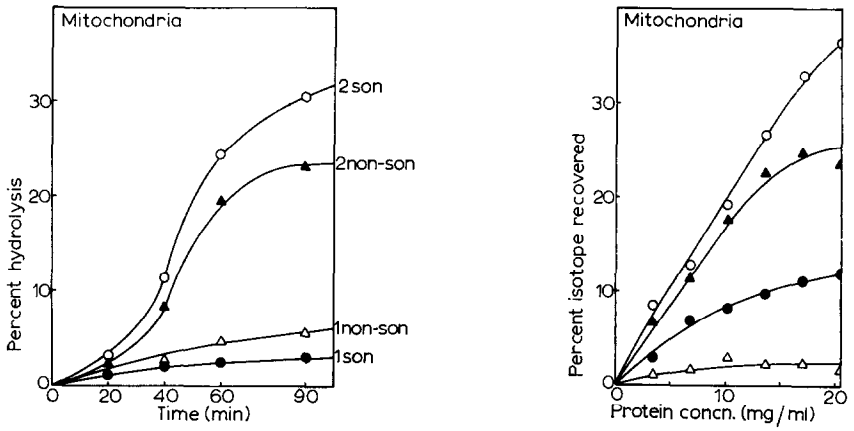


Fig. 3. Effect of sonication of the mitochondria on the hydrolysis of phosphatidyl ethanolamine at each position. Hydrolysis at the 2 position (calculated as the sum of ^{14}C-labeled free fatty acid and ^{3}H-labeled-monoacyl glyceryl-3-phosphoryl ethanolamine from Fig. 2) by sonicated mitochondria is designated ○, and that by non-sonicated mitochondria, ▲. Hydrolysis at the 1 position (calculated as the sum of ^{3}H-labeled free fatty acid and ^{14}C-labeled-monoacyl phosphatidyl ethanolamine) by sonicated mitochondria is designated ●, and that by non-sonicated mitochondria, △.

Fig. 4. Effect of the sonicated mitochondrial protein concentrations on the formation of hydrolysis products of phosphatidyl ethanolamine. Conditions were as described in METHODS except for the variation in protein concentration. The designations of the compounds formed are the same as for Fig. 1.

3-phosphoryl ethanolamine (hydrolysis at the 1 position) was found to increase only up to a protein concentration of about 10 mg/ml.

Time studies done with microsomes show a proportionate increase in the formation of ^{3}H-labeled free fatty acid and ^{14}C-labeled-monoacyl glyceryl-3-phosphoryl ethanolamine up to about 40 min (see Fig. 5) after which the reaction proceeds at a lower linear rate. No lag period was found with the microsomal phospholipase A_1 activity, a characteristic different from that found with the phospholipase A_2 of the mitochondria. Another striking difference is the effect of sonication, as already seen in Table III. Sonication of the microsomes lowered the recovery of ^{3}H-labeled free fatty acid and ^{14}C-labeled-monoacyl glyceryl-3-phosphoryl ethanolamine. The hydrolysis at the 1 position (see Fig. 6), catalyzed by microsomes which have been sonicated, is found to proceed at a rate equal to that found for the non-sonicated microsomes after the high rate of the first 40 min. It is possible that there are two enzymes catalyzing the hydrolysis at the 1 position, one of which is inactivated by sonication and loses activity upon incubation for periods greater than 40 min. There is little effect on hydrolysis at the 2 position due to sonication, as seen in Fig. 6, even though a greater amount of ^{14}C-labeled free fatty acid is recovered than ^{14}C-labeled-monoacyl glyceryl-3-phosphoryl ethanolamine (see Fig. 5).

Fig. 7 shows the relationship between activity and the concentration of non-sonicated microsomal protein. The recovery of each compound is proportional to the concentration up to about 10 mg/ml. In this experiment 2.5 μmoles EDTA per ml reaction mixture was used, otherwise there was almost no increase in activity with increasing amounts of protein. This possibly indicates the presence of a metal inhibitor in the microsomal preparations.

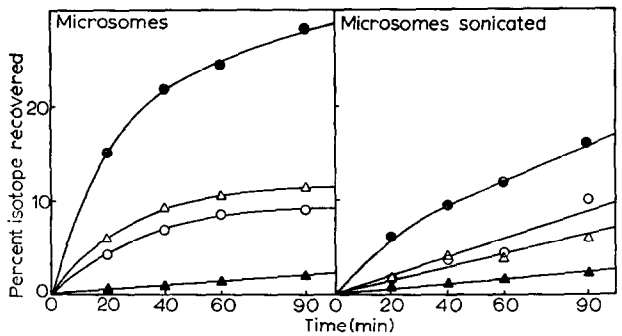
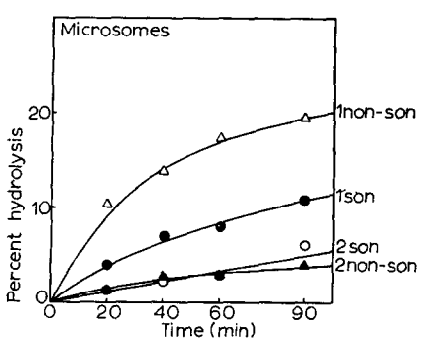


Fig. 5. Effect of time on the formation of the various hydrolysis products from phosphatidyl ethanolamine by microsomes and sonicated microsomes. Conditions and designations are as described for Fig. 2.

The finding of DE HAAS, SARDA AND ROGER[17] that pancreatic lipase can catalyze the hydrolysis of the 1-acyl group of phospholipids indicates the possibility of the phospholipase A_1 activity being due in part to a lipase. In order to investigate this possibility, experiments were designed to assay lipase activity in the rat liver and to compare some properties of this enzyme with those found for the phospholipases. Plasma[33], deoxycholate[34,35] and phospholipids were examined as emulsifying agents for the substrate ^3H,^{14}C labeled triglyceride. Both plasma (either citrated or normal) and deoxycholate failed to produce emulsions on sonication which were readily hydrolyzed by the liver homogenate. Under the conditions of these experiments, the extent of triglyceride hydrolysis was less than 5%. The addition of factors known to stimulate lipoprotein lipase such as heparin or albumin caused no stimulation. When sonicates were made of mixtures of either phosphatidyl ethanolamine and the ^3H,^{14}C-



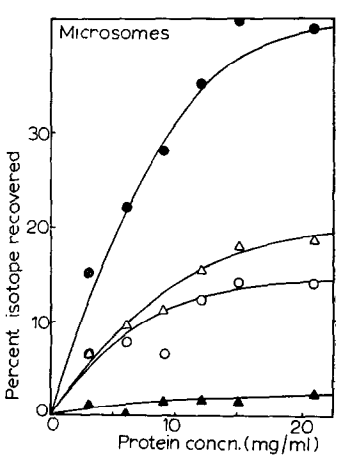


Fig. 6. Effect of sonication of the microsomes on the hydrolysis at each position. Calculations and designations are as described for Fig. 3.

Fig. 7. Effect of the non-sonicated microsomal protein concentration on the hydrolysis products of phosphatidyl ethanolamine. Conditions were as described in METHODS except for the variation in protein concentration and for the addition of EDTA to a concentration of $2.5 \cdot 10^{-3}$ M. The designations of the compounds formed are the same as for Fig. 1.

labeled triglyceride in the ratio 2:1, or of phosphatidyl ethanolamine, phosphatidyl choline and ^{3}H,^{14}C-labeled triglyceride in the proportions 1:1:1, up to 30% of the triglyceride was hydrolyzed by the liver homogenates. This made possible comparative studies of phospholipases and lipase using sonicates which had the same ratio of phosphatidyl ethanolamine to triglyceride. These varied only in the lipid containing the isotope and in the nature of the triglyceride used with the radioactive phosphatidyl ethanolamine (triolein was used in place of the radioactive triglyceride which contained mainly 1-palmitoyl, 2-linoleyl, 3-oleyl groups; the same degree of unsaturation).

When the breakdown of these two substrates was studied in the various cell fractions, a pattern such as that presented in Fig. 8 was found. The lipase activity, represented by the left-hand bar, is found in all fractions except the soluble one, the greatest activity being in the mitochondrial fraction. These findings are not in accordance with those found by OLSON AND ALAUPOVIC[34,35] (using deoxycholate as the emulsifying agent) or those of HIGGINS AND GREEN[36] (using labeled chylomicrons), although neither group correlated the production of free fatty acid with the disappearance of triglyceride. It must be pointed out, however, that quite different assay conditions were used in these experiments, indicating the possibility of more than one enzyme being present in the liver which can act on glycerides.

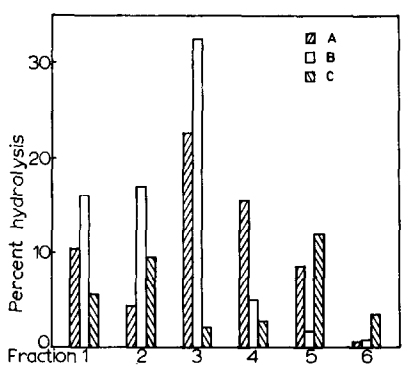


Fig. 8. Hydrolysis of mixed lipid sonicates by rat-liver cell fractions, which are designated as follows; 1, homogenate; 2, nuclear; 3, mitochondrial; 4, composite; 5, microsomal; 6, soluble. Assay conditions were as described in METHODS except that the sonicates were prepared from either a mixture of 0.5 mg ^{3}H, ^{14}C-labeled triglyceride and 1.0 mg non-radioactive rat-liver phosphatidyl ethanolamine (for lipase), or a mixture of 0.5 mg non-radioactive triglyceride (mainly triolein) and 0.2 mg [^{3}H, ^{14}C]phosphatidyl ethanolamine diluted with 0.8 mg non-radioactive rat-liver phosphatidyl ethanolamine (for phospholipases). Lipase activity, measured as the disappearance of triglyceride (equal to the formation of diglyceride *plus* free fatty acid) is designated by Bars A; phospholipase A_2 activity, measured as the formation of ^{3}H-labeled-monoacyl glyceryl-3-phosphoryl ethanolamine is designated by Bars B. Phospholipase A_1 activity, measured as the formation of ^{14}C-labeled-monoacyl glyceryl-3-phosphoryl ethanolamine is designated by Bars C.

The phospholipase A_2 activity (represented by the middle bars) was found in the same fractions as the lipase although there is variation in the relative magnitude of the activities in the fractions. Such a variation might be expected since any ^{3}H-labeled-monoacyl glyceryl-3-phosphoryl ethanolamine (taken as the measure of the phospholipase A_2 activities) formed in the composite and microsomal fractions is

possibly hydrolyzed further. The phospholipase A_1 activity (represented by the right-hand bars), as already shown in Table III, is found mainly in the microsomal fraction, very little being found in the mitochondrial fraction. This experiment shows that under the conditions used in this experiment, there is no correlation between the intracellular distribution of the lipase and phospholipase A_1 activities. Further, on a per mg hydrolyzed basis, the phospholipase A_1 activity was found to be greater than the lipase activity. The pancreatic lipase, on the other hand, is much more active with triglyceride than phospholipid*. In these experiments with mixed lipid sonicates, the specific activity of the phosphatidyl ethanolamine was one-fifth of that used in experiments with phosphatidyl ethanolamine alone. The percent recovery of both lyso derivatives was as high or higher using mixed sonicates, however, indicating that the level of substrate used is not saturating, or possibly that the mixed lipid sonicate is a better substrate than the phosphatidyl ethanolamine alone.

The lipase activity in the mitochondrial and microsomal fractions is attributed to hydrolysis of triglyceride as shown by the relationship between the substrate triglyceride and products seen in Table VI. This shows that the increase in free fatty acid and diglyceride is correlated with the decrease in triglyceride containing long-chain acyl groups, a true measure of lipase activity. These values were corrected for the hydrolysis of the few percent of monoglyceride formed during sonication of the triglyceride. Further, no accumulation of monoglyceride could be demonstrated. As was found with the homogenate (see Table I), considerable amounts of diglyceride were found to accumulate. The recovery of radioactivity was somewhat low with the mitochondrial system, especially when a high degree of hydrolysis had occurred. This is probably due to the oxidation of the free fatty acid liberated by the mitochondria. For this reason percent recovery of free fatty acid with the mitochondria is considered a minimal value.

TABLE VI

HYDROLYSIS OF TRIGLYCERIDE BY RAT-LIVER MITOCHONDRIA AND MICROSOMES

	Average compound recovered*					
	Triglyceride		*Diglyceride*		*Free fatty acid*	
	^3H	^{14}C	^3H	^{14}C	^3H	^{14}C
Mitochondria	58.0	61.3	10.9	14.5	31.1	24.2
Microsomes	90.4	90.3	3.0	3.7	6.6	6.0

* Average of 8 experiments.

To obtain further information on the nature of the three lipolytic activities, assays with both substrates were performed using both the mitochondrial and microsomal fractions in the presence of possible inhibitors. Table VII contains the results of an experiment in which EDTA was added to the reaction mixture. The phospholipase A_2 activity of the mitochondria was found to be completely inhibited by EDTA, indicating the requirement for metal ions, presumably Ca^{2+}. The microsomal phospholipase A_1 and lipase activities were stimulated by the addition of EDTA. The mitochondrial lipase activity is clearly not affected by the addition of EDTA, even

* Personal communication, G. H. DE HAAS.

at a level of $5 \cdot 10^{-3}$ M. It seems, therefore, that this liver lipase does not require Ca^{2+} in contrast to the pancreatic lipase[37] and lipoprotein lipase[33].

TABLE VII

EFFECT OF EDTA ON RAT-LIVER PHOSPHOLIPASE AND LIPASE ACTIVITIES

Enzyme activity	*Addition*		*Percent* of control*
	None (control)	*EDTA ($2.5 \cdot 10^{-3}$M)*	
Phospholipase A_2**	37.8	0	0
Phospholipase A_1**	18.9	32.1	170
Lipase (mitochondria)***	43.1	42.2	97.9
Lipase (microsomes)***	11.0	16.3	148

* Calculated as activity found in samples containing EDTA divided by activity found in sample without EDTA × 100.
** Taken as the amount of the appropriate lysoderivative formed.
*** Taken as the sum of diglyceride and free fatty acid formed.

TABLE VIII

EFFECT OF PCMB ON PHOSPHOLIPASE AND LIPASE ACTIVITIES

Enzyme activity	*Activity (percent of control)**		
	25 μM PCMB	*50 μM PCMB*	*250 μM PCMB*
Phospholipase A_2	73.7	129	117
Phospholipase A_1	98.9	97.1	113
Lipase mitochondrial	73.4	65.9	50.8
Lipase microsomal	91.0	69.1	67.0

* Calculation as for Table VII.

The effect of increasing amounts of PCMB in these reaction mixtures on the lipase and phospholipase activities can be seen in Table VIII. Neither phospholipase activity is affected by the presence of PCMB in concentrations up to $2.5 \cdot 10^{-4}$M. In contrast, the lipase activity of the mitochondria and microsomes is decreased up to 50% by the addition of PCMB. Recovery of the radioactive compounds is much higher when PCMB is present in the assay mixture for the lipase of the mitochondria. This is probably due to the inactivation of the fatty acid-oxidizing system. The degree of inhibition is therefore a minimal value since the percent inhibition is determined by comparison with the amount of hydrolysis products formed without addition of PCMB, in which a part of the released fatty acid is further metabolized. The inhibition of the lipase by PCMB suggests that a sulfhydryl group on the protein is involved in the activity, although this level of PCMB is rather high. The explanation for this may be similar to that offered by WILLS[37] for the effect of PCMB on pancreatic lipase, postulating that the sulfhydryl group reacting with the PCMB is not actually in the active site but close enough to cause a partial blocking of the site.

CONCLUSIONS

The data presented here demonstrate that various phospholipids or glycerides are hydrolyzed by rat-liver homogenates with the accumulation of both lysoisomers

or diglyceride. No monoglyceride was found to accumulate in these experiments, presumably due to the presence of a monoglyceride lipase as reported by SENIOR AND ISSELBACHER[38], and by BELFRAGE[39]. The non-stoichiometric relationship between the amount of free fatty acid and the lyso derivative formed could be attributed to the lysophospholipase present mainly. Our experiments gave variable ratios between the amounts of lyso derivative and free fatty acid formed (lysophospholipase activity) indicating a lack of understanding of the factors controlling the activity of these enzymes. Both heat treatment and addition of deoxycholate to the reaction mixture specifically inhibited the lysophospholipase activity, inproving the recovery of the lysoderivative. These experiments indicate that, under the conditions employed, the activity causing the release of both acyl groups is due to a great extent to the combined activities of the phospholipases and lysophospholipases (Reactions I and III, and Reactions II and IV of Scheme 1, respectively).

This indication was further substantiated by experiments with subcellular fractions of the liver homogenate. Using this technique it was possible to separate the phospholipase acting at the 1 position from that acting at the 2 position (phospholipases A_1 and A_2, respectively) and the lysophospholipases. The microsomal fraction was shown to catalyze the hydrolysis at the 1 position mainly, whereas the mitochondrial fraction was found to be highly specific in attacking the 2 position. The soluble fraction, as demonstrated before, was found to contain lysophospholipase activity, although it is possible that this activity, some of which is found in the particulate fraction, is solubilized by the isolation procedure. It has not been demonstrated thus far whether this lysophospholipase activity is due to one enzyme which can attack either the 1 or the 2 position, or to two enzymes, each specific in its site of action. Using deoxycholate to inhibit lysophospholipase activity in the soluble fraction, both lysoisomers were found to accumulate. Further, there is a nearly stoichiometric relationship between the amounts of monoacyl glyceryl-3-phosphoryl ethanolamine and free fatty acid recovered. From this it can be concluded that under these conditions the phospholipase A_1 and A_2 of this fraction have little lysophospholipase activity, if any, and that the activity of the soluble fraction can be accounted for as the combined Reactions I and III, and II and IV of Scheme 1, rather than by a single phospholipase, Reaction V. It is not clear what the relationship is between the mitochondrial and soluble phospholipase A_2 and the microsomal and soluble phospholipase A_1.

Recently, it has been shown that highly purified pancreatic lipase is capable of specifically attacking the 1-acyl ester of phospholipids[21]. This has raised the question as to the possibility of other enzymes acting at the 1 position of phospholipids not being true phospholipases but having both lipase and phospholipase activity. Experiments were devised to obtain information concerning this problem. It was found that the greatest lipase activity could be demonstrated using sonicates of a mixture of triglyceride and phosphatidyl ethanolamine. The findings here show that the lipase activity is located mainly in the mitochondrial fraction, whereas the phospholipase A_1 activity is found mainly in the microsomal fraction. The emphasis of this study is the comparison of lipase and phospholipase A_1 activities under similar conditions and should not be interpreted as an exhaustive study of liver lipases.

Comparison of the responses of the mitochondrial and microsomal phospholipase and lipase activities to the presence of EDTA demonstrates the requirement

of phospholipase A_2 for a metal. Since other known phospholipase A_2 activities require Ca^{2+} (ref. 40), it is reasonable to assume this requirement to be similar*. The microsomal activities undergo a marked stimulation in the presence of EDTA which may be attributed to the removal of a heavy-metal inhibitor. Neither the lipase nor the phospholipase A_1 seems to require metal ions for activity. Both pancreatic lipase[37] and lipoprotein lipase[33] have been shown to require Ca^{2+}. Neither phospholipase activity was inhibited by PCMB whereas the lipase found in both the microsomes and mitochondria is partially inhibited by $2.5 \cdot 10^{-4}$M PCMB.

Various approaches in the studies comparing phospholipase and lipase activities support the concept that these activities are catalyzed by separate and distinct enzymes although it is possible that the lipase may have some phospholipase A_1 activity. It also seems quite possible that at least part of the lipase activity in the microsomal fraction is due to contamination from the mitochondrial or composite fraction.

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* Since the preparation of this manuscript, a paper by P. BJØRNSTAD has been published which also reports a requirement of the mitochondria phospholipase for Ca^{2+}.

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