The Distribution of Phospholipase B in Mammalian Tissues

By ELIZABETH A. MARPLES AND R. H. S. THOMPSON

Department of Chemical Pathology, Guy's Hospital Medical School, London, S.E. 1

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The breakdown of phospholipids in animal tissues has for long been regarded as involving the removal of the fatty acid residues by the successive action of phospholipases A and B, followed, in lecithin breakdown, for example, by the degradation of the glycerylphosphorylcholine so formed by means of a specific diesterase. The initial removal of a single fatty acid from the phospholipid molecule results in the formation of a lysophosphatide. Because of the marked cytolytic properties of these compounds, together with the fact that lysolecithin has recently been shown to be present in lipid extracts of animal tissues (Marinetti, Witter & Stotz, 1957; Hadju, Weiss & Titus, 1957; Phillips, 1957, 1958), a comparison of the ability of different tissues to degrade the lysophosphatides by means of phospholipase B is of physiological interest.

Although this enzyme was first recognized in rice bran by Contardi & Ercoli in 1933, and was found also to occur in certain moulds (Fairbairn, 1948) and bacteria (Hayaishi & Kornberg, 1954), little detailed work appears to have been done on its distribution in animal tissues. Noguchi (1944) studied a number of rabbit tissues, and found considerable phospholipase B activity in pancreas and lung. Shapiro (1953) obtained active preparations from glycerol extracts of ox pancreas, and Dawson (1956) has carried out a careful study of the properties of this enzyme in glycerol extracts of acetone-dried rat and sheep liver. Dawson also states that he obtained liberation of glycerylphosphorylcholine when glycerol extracts of rat kidney, spleen, brain and blood were incubated with lysolecithin, but no quantitative assessment of enzymic levels was given for these tissues.

While this paper was in preparation a comparison of some of the properties of the phospholipase B in rat intestinal mucosa with those of the lecithinase also present in this tissue has been published by Epstein & Shapiro (1959).

EXPERIMENTAL

Tissue preparations. Homogenates (1 g. of tissue in 4 ml. of water) of the various tissues were prepared by using a modified Potter-Elvehjem homogenizer (Webster, 1954). In some cases the tissue was finely minced with sharp scissors before homogenization. Rat skeletal muscle was homogenized for 5 min. in a macerator (M.S.E., Ltd.), after first mincing finely with scissors.

Rat tissues were removed from freshly decapitated animals, and rabbit tissues from animals freshly killed with pentobarbital sodium (60 mg./kg. body wt.). Human brains were obtained from cadavers as soon as possible after death; the areas of brain to be investigated were dissected, washed with aq. 0.9% NaCl and homogenized as above.

Estimation of enzymic activity. Activity of the homogenates was estimated by determination of both free fatty acid release and of the glycerylphosphorylcholine (GPC) formed after incubation with lysolecithin.

Samples (2.0 ml.) of homogenates prepared as above were incubated at 38° with 2.5 ml. of 0.2 M-KH₂PO₄-Na₂HPO₄ buffer (pH 6.5) and 6.2 mM-lysolecithin in a final volume of 5.0 ml.

Estimation of free fatty acids. For the estimation of free fatty acid release, a 2.0 ml. sample was removed from the reaction mixture immediately after the addition of the lysolecithin, and a further 2.0 ml. sample removed after incubation for 60 min. at 38°. Estimations were also carried out on samples from control tubes from which either tissue or lysolecithin had been omitted. The method of van der Vies (1955) was used; this involves extraction with light petroleum (40–60°) and, after removal of the light petroleum, titration with 0.01 N-NaOH by using a Conway horizontal burette.

Estimation of glycerylphosphorylcholine. The estimation of GPC involves precipitation of protein with trichloroacetic acid followed by determination of free choline before and after hydrolysis by heating in N-HCl at 100° for 20 min. (Schmidt, Hecht, Fallot, Greenbaum & Thannhauser, 1952). Since Dawson (1956) had pointed out that lysolecithin is also hydrolysed under these conditions, and since lysolecithin is only partially precipitated with the protein, the fraction remaining in solution would interfere with the estimation of GPC. To overcome this difficulty, lysolecithin was added to the control tubes (tissue only) at the end of the 60 min. incubation period and immediately before the addition of the trichloroacetic acid. As a further precaution against interference from unprecipitated lysolecithin the samples, after the addition of trichloroacetic acid, were frozen solid and centrifuged immediately after thawing (this procedure was found to increase the proportion of lysolecithin precipitated); after removal of the precipitated protein, the still cloudy solutions were incubated at 38° with bovine γ -globulin and reprecipitated with trichloroacetic acid; by this procedure clear supernatants were obtained.

GPC was determined by estimating free choline, before and after hydrolysis (see above), with the periodide method of Appleton, La Du, Levy, Steele & Brodie (1953), as modified by Webster (1956).

A 2.0 ml. sample of the reaction mixture was pipetted into 1.0 ml. of 15% (w/v) trichloroacetic acid solution. After mixing, the contents of the tubes were frozen solid

and, after being allowed to melt, were immediately centrifuged for 10 min. at 1700 g. A sample of the supernatant (2.0 ml.), which varied from clear to very cloudy, was extracted twice with ether; after removal of the last traces of ether by bubbling through nitrogen, the solution was incubated for 5 min. at 38° with 0.4 ml. of a 5% (w/v) suspension of bovine γ -globulin (Armour). Trichloroacetic acid solution (1.2 ml. of 15%, w/v) was added and the precipitated proteins were removed by centrifuging. A portion (0.5 ml.) of the clear supernatant was used for estimation of free choline, and 1.5 ml. was heated in a firmly stoppered tube for 20 min. at 100° with 0.5 ml. of 4N-HCl. After cooling, 0.5 ml. of the hydrolysate was used for estimation of total (free + acid-labile) choline.

Standards and blanks were set up as follows: after incubation of homogenate with buffer, GPC and lysolecithin were added to the standard tubes, and lysolecithin only to the blank tubes, trichloroacetic acid being added to each immediately after these additions.

Lysolecithin. This was prepared from ovolecthithin by the method of Long & Penny (1957). The crude lysolecithin was crystallized from hot ethanol (Saunders & Thomas, 1958), which removed phospholipase A precipitated with the lysolecithin during the preparation (Marples & Thompson, 1958).

Glycerylphosphorylcholine. The crystalline CdCl_2 complex of GPC was prepared by Dr G. R. Webster in this Laboratory according to the method of Tattrie & McArthur (1955). Aqueous solutions of free GPC were freshly prepared from this by removal of the cadmium on an ion-exchange resin (Zeo-Karb 225, Na form).

Buffers. For the pH-activity curve, sodium acetate-acetic acid buffers at pH 5.0 and 5.5, and KH_2PO_4 - Na_2HPO_4 buffers at pH 6.0, 6.5, 7.0 and 7.4, were used, all at a final concentration of 0.1 M.

RESULTS

Although Dawson (1956) has described the properties of the phospholipase B in glycerol extracts of acetone-dried preparations of rat liver, it was thought advisable to characterize briefly some of the properties of this enzyme in fresh homogenates of whole liver. When rat-liver homogenate and lysolecithin were incubated together at pH 6.5, there was a release of free fatty acid amounting to 29.4 $\mu\text{moles/g./hr.}$, together with a production of GPC of 31.5 $\mu\text{moles/g./hr.}$, but no formation of free choline. The reaction proceeded rapidly for at least 1 hr., though the rate fell off slowly throughout the course of the reaction.

A series of buffers with pH values between 5.0 and 7.4 were used to determine the optimum pH for the enzyme. Rat-liver homogenate was incubated for 60 min. at 38° with 6.2 mM-lysolecithin by using these buffers, and the release of free fatty acid was determined. The results are given in Fig. 1 and show that the optimum pH is at 6.5, although there is considerable activity at the physiological pH.

The effect of enzyme concentration and substrate concentration on the rate of reaction was

studied. The rate of reaction increased linearly with increasing weights of liver between 100 and 400 mg. of tissue/5 ml. of reaction mixture; the rate also increased with increasing substrate concentration up to a maximum at 6.2 mM (Fig. 2). No evidence was found of any falling-off of activity at the higher substrate concentrations studied. From the substrate concentration-activity curve a Michaelis constant of 2.3–2.6 mM was calculated.

Fig. 1. pH-Activity curve of rat-liver phospholipase B.

Fig. 2. Effect of substrate concentration on the phospholipase B activity of whole rat-liver homogenates.

Table 1. *Effect of some inhibitors on phospholipase B activity of homogenates of whole rat liver*

Inhibitor	Concn. (mM)	Activity (μ moles of fatty acid/g./hr.)		Inhibition (%)
		Normal	Inhibited	
EDTA	1	16.6	16.9	0
NaF	20	16.6	9.6	42
KCN	10	16.6	13.3	20
Cysteine	10	16.6	17.2	0
Hg ²⁺	1	22.4	0	100
Zn ²⁺	1	30.7	19.8	33
Cu ²⁺	1	30.3	2.8	90
Mg ²⁺	1	20.9	18.1	13
Iodoacetate	1	15.6	15.4	0

Table 2. *Distribution of phospholipase B in homogenates of whole rat tissues*

Here and in Table 3 activities expressed as μ moles of free fatty acid or of GPC + free choline formed/g./hr. and the number of experiments is given in parentheses.

Tissue	Activity (μ moles/g./hr.)			
	Fatty acid	Total choline	Free choline	GPC
Lung	57.2 (2)	51.6 (2)	7.1 (2)	44.5
Ileum	71.2 (3)	72.3 (2)	2.6 (2)	69.7
Spleen	52.3 (2)	46.7 (4)	3.2 (4)	43.5
Liver	29.4 (5)	31.5 (4)	0 (3)	31.5
Pancreas	27.2 (3)	—	—	—
Kidney	9.4 (2)	9.6 (2)	4.4 (2)	5.2
Testis	6.7 (2)	5.3 (2)	0 (2)	5.3
Skeletal muscle	4.4 (2)	5.6 (2)	0.1 (2)	5.5
Heart	—	2.1 (2)	0 (2)	2.1
Brain	—	1.7 (3)	0 (3)	1.7
Spinal cord	—	0.8 (2)	0 (2)	0.8
Adipose tissue	0 (2)	—	—	—

Table 3. *Phospholipase B activity of some areas of human brain*

	Activity (μ moles/g./hr.)			
	Fatty acid	Total choline	Free choline	GPC
Grey matter	3.9 (2)	6.4 (2)	0 (2)	6.4
White matter	0.7 (2)	0.3 (2)	0 (2)	0.3
Cerebellum	4.8 (3)	3.9 (2)	0 (2)	3.9
Caudate nucleus	5.0 (2)	5.4 (2)	0.8 (2)	4.6
Corpus callosum	1.9 (2)	0.8 (2)	0 (2)	0.8

The phospholipase B activity of whole-liver homogenates is sensitive to heat; incubation of a water homogenate of rat liver for 15 min. at 60° completely abolished the enzymic activity.

Effect of inhibitors. Solutions of the inhibitors tested were added to the liver homogenate and buffer, and the mixture was incubated for 10 min. at 38° before the addition of lysolecithin. Unlike the phospholipase A of cottonmouth-moccasin venom (Long & Penny, 1957), the liver phospholipase B was unaffected by mM-ethylenediamine-tetra-acetic acid (EDTA); Dawson (1956) had found that the phospholipase B of an extract of acetone-dried rat liver was slightly activated by this concentration of EDTA. The effect of various

metal ions on the enzymic activity was tested and the results are shown in Table 1. Ca²⁺ and Mn²⁺ ions (mM) had no significant effect. The enzyme was unaffected by cysteine (10 mM) and by iodoacetate (mM); 20 mM-NaF and 10 mM-KCN produced inhibitions of 42 and 20 % respectively.

Distribution of enzyme. The distribution of phospholipase B has been studied in a variety of rat tissues and in some areas of human brain. The results are presented in Tables 2 and 3, where the amounts of free fatty acid, GPC and choline formed/g. of tissue/hr. are given. Intestine (ileum), lung and spleen were the most active of the rat tissues tested, while nervous tissue and heart had little activity. No values have been given for free fatty acid release from brain (rat), spinal cord and heart, as the activity of these tissues was too low for accurate estimation by the method used; it was not possible to estimate the GPC and free choline formed in rat pancreas, because this tissue contains a substance which forms a copious precipitate with the iodine reagent, and though this was only slightly soluble in chloroform it had a high extinction at 365 m μ . The results with human brain (Table 3) suggest that the activity in this tissue is associated with the grey rather than with the white matter. Human blood showed only very low levels

of activity, serum and packed red cells releasing 0.51 and 0.83 μ mole of fatty acid/ml./hr. respectively.

Noguchi (1944) has described the phospholipase B activity of extracts of rabbit lung and pancreas, but found no activity in extracts of rabbit kidney, spleen, intestine or liver. With fresh preparations, however, measurable activity was found in all these rabbit tissues; the activity was of the same order as that of rat tissues, with the exception of intestine, which had a relatively low activity, and brain, which was more active than rat brain. Rabbit grey matter was more active than rabbit white matter.

It has been mentioned above that there was no formation of free choline during the incubation of lysolecithin with rat-liver homogenate at pH 6.5. This finding agrees with that of Dawson (1956), who noted that rat-liver GPC-diesterase was inactive at pH 6.5. As can be seen in Table 2, however, when rat kidney, intestine, spleen and lung were incubated with lysolecithin at pH 6.5 there was a measurable production of free choline; with rat kidney almost half the total acid-soluble choline produced was present as free choline. This could be explained if rat kidney contained a GPC-diesterase with activity at pH 6.5. Rat-kidney homogenate was therefore incubated at 38° and pH 6.5 with 0.3 mM-GPC (the concentration of GPC formed from lysolecithin by rat kidney in 60 min.). Free choline was formed at the rate of 7.6 μ moles/g./hr., and the diesterase activity is therefore probably sufficient to account for the production of choline from the GPC formed by the phospholipase B. With 0.02 M-GPC, the GPC-diesterase activity of rat kidney (32.3 μ moles of choline produced/g./hr.) was about four times as much at pH 6.5 as that of rat brain (Webster, Marples & Thompson, 1957).

DISCUSSION

The results described above indicate that phospholipase B activity is readily demonstrable in simple water homogenates of a number of mammalian tissues. Titration of the fatty acid released, or estimation of the glycerylphosphorylcholine formed, after incubation of the homogenate with lysolecithin provides a satisfactory method for the estimation of enzymic activity, and the agreement between the estimates obtained by these two analytical methods is reasonable (Table 2).

A comparison of our findings on whole ileum (i.e. mucosa and muscle layers) with those of Epstein & Shapiro (1959) on intestinal mucosa suggests that the greater part of the intestinal activity is associated with the mucosa. Hajdu *et al.* (1957) have recently identified the substance with digitalis-like activity, which occurs in mammalian blood, as β -palmitoyl-lysolecithin; rat-heart muscle

contains even less phospholipase B than skeletal muscle.

In general, the properties of the phospholipase B, as displayed in whole-tissue homogenates (liver), appear to agree well with those described by Dawson (1956) for the partially purified enzyme in the glycerol extracts of acetone-dried rat liver which he used. The only major difference which we have found is in the effect of substrate concentration. Dawson found maximal activity with substrate levels between 2 and 3.5 mM, with a reduction of activity at higher substrate concentrations; using whole-tissue homogenates we have found maximal activity with about 6 mM-lysolecithin, and no fall-off in the rate with increases of substrate concentration up to 10 mM. Epstein & Shapiro (1959), using aqueous preparations of rat intestinal mucosa, have also found no fall-off in the rate with concentrations up to 10 mM-lysolecithin, although the glycerol extracts of acetone-dried ox pancreas used earlier by Shapiro (1953) showed an effect comparable with that found by Dawson (1956).

SUMMARY

1. A study has been made of the distribution of phospholipase B in certain mammalian tissues, by using whole homogenates of the different tissues.
2. Enzymic activity has been estimated by determining the rate of release of both free fatty acids and glycerylphosphorylcholine.
3. The properties of the enzyme in whole rat-liver homogenates agree closely with those described by Dawson (1956) in glycerol extracts of acetone-dried preparations of this tissue.
4. High levels of activity were found in ileum, lung, spleen, liver and pancreas. The activity was low in skeletal muscle, heart muscle, kidney, testis, brain and blood. The grey matter of the central nervous system contains considerably more activity than the white matter.

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The Incorporation of ^{15}N into Adenine Nucleotides and their Formation from Inosine Monophosphate by Skeletal-Muscle Preparations

By ALISON A. NEWTON AND S. V. PERRY*

Department of Biochemistry, University of Cambridge

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Most vertebrate skeletal muscles contain an active enzyme specific for the deamination of adenosine 5'-monophosphate (Schmidt, 1928). When skeletal muscle is repeatedly stimulated or damaged this enzyme is responsible for production of the ammonia that was the subject of detailed investigations by the Parnas and Embden schools in the 1920's [see Needham (1932) for a review of this work]. Both groups of workers considered that resynthesis of the adenosine monophosphate from inosine monophosphate occurred in muscle and Parnas (1929) in particular favoured the idea of aerobic reamination at the expense of the tissue amino acids, although he failed experimentally to demonstrate this reaction (Parnas, 1933). Subsequent studies by Kalckar & Rittenberg (1947) confirmed that reversible deamination of the 6-amino group of adenine nucleotides could occur in living muscle. These workers showed that after the administration of [^{15}N]ammonium citrate to rats, significant incorporation of the isotope could be detected in the adenosine triphosphate isolated from skeletal muscle. With systems *in vitro*, Kleinzeller (1942) was, however, unable to demonstrate in muscle homogenates the reamination either of inosine diphosphate or of inosine triphosphate by incubation with amino acids. Not surprisingly, in view of the thermodynamical considerations (Tarver & Morales, 1951), Kalckar (1947*a*) failed to demonstrate the reversibility of the 5'-adenylic deaminase reaction. More recently, however, Nekhorocheff & Wajzer (1952) and Nekhorocheff (1955) have provided evidence that, in muscle, creatine can act as a precursor of the 6-amino group

of adenosine triphosphate, whereas Kometiani & Klein (1956) propose, on the basis of their experimental findings, β -alanine for this function.

The present paper is concerned with a study of the mechanism of reamination of inosine monophosphate in muscle systems. A partly purified enzyme preparation is described which catalyses the transfer of the amino group of aspartic acid to inosine monophosphate to give adenine nucleotides. Evidence is provided which indicates that the system may be similar to that described by Carter & Cohen (1955) for the synthesis of adenosine monophosphate in yeast.

A preliminary account of some aspects of this work has already been published (Newton & Perry, 1957).

METHODS

Whole-animal experiments. A total of 100 ml. of ammonium citrate solution containing 280 mg. of NH_3 nitrogen (62 atoms % excess of ^{15}N) was injected subcutaneously into adult rabbits in three equal doses at 2 hr. intervals. Animals were not fed but could move freely in their cages during the experiment. They were killed for the extraction of the muscle nucleotides 4 hr. after the final injection.

Preparation of homogenates. Back and leg muscles from a freshly killed normal rabbit were removed and chilled in 0.16 M-KCl; they were freed from adherent moisture, minced and briefly homogenized with 4 vol. of a medium so that final concentrations in the homogenate were: 0.1 M-KCl, 50 mM-glycylglycine-HCl, pH 7.5, mM- MgCl_2 , mM-adenosine triphosphate (ATP), 10 μg . of pyridoxal phosphate/ml. and 11 mg. of $^{15}\text{NH}_4\text{Cl}$ or the equivalent amount of nitrogen as [^{15}N]aspartic acid/100 ml. The homogenate was stirred at room temperature for 30 min., the reaction was stopped by the addition of 60% (w/v) trichloroacetic acid to give a final concentration of 7% and treated at 0°

* Present address: Department of Biochemistry, University of Birmingham.