5. Adenosine diphosphate (ADP) was less inhibitory than ATP when it replaced part of the latter; inosine triphosphate had little inhibitory effect. Over a similar range, increasing concentration of uridine triphosphate produced progressively increasing inhibition.

6. No inhibitory action on the magnesium-activated myofibrillar ATPase could be demonstrated with myokinase preparations.

7. Ethylenediaminetetraacetate strongly inhibited the magnesium-activated myofibrillar ATPase in concentrations which were only one-twentieth of that of the added magnesium chloride. Calcium activation of the ATPase was much less sensitive to ethylenediaminetetraacetate.

8. These findings are discussed in relation to the relaxation studies by other workers on glycerated muscle fibres.

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The Phospholipase B of Liver

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Previous isotopic experiments have indicated that the free glycerylphosphorylcholine (GPC) and glycerylphosphorylethanolamine (GPE) present in liver tissue are intermediates in the metabolic breakdown of phosphatidylcholine and phosphatidylethanolamine respectively (Dawson, 1955). In support of this conclusion an enzyme, GPC diesterase, has been found in liver tissue which specifically hydrolyses GPC and GPE into glycerophosphoric acid and the free base (Dawson, 1956*a*). The present paper reports some observations on a phospholipase B (nomenclature of Zeller, 1952) which occurs in liver, forming GPC and probably GPE by the splitting of the single saturated fatty

acid from lysolecithin or lysophosphatidylethanolamine.

The enzyme phospholipase B has a comparatively wide distribution in nature. Contardi & Ercoli (1933), studying rice bran, were probably the first to recognize that it was a specific enzyme which did not attack lecithin until one fatty acid had been removed by phospholipase A. The enzyme has also been found to occur in moulds (Fairbairn, 1948) and bacteria (Hayaishi & Kornberg, 1954). Schmidt, Hershman & Thannhauser (1954) and Schmidt, Greenbaum, Fallot, Walker & Thannhauser (1955) observed the accumulation of the ethanol-soluble GPC in autolysing pancreas and intestine, but not

in liver. Consistent with this is the demonstration by Shapiro (1953) of an active phospholipase *B* in glycerinated extracts of ox pancreas. The properties of the phospholipase *B* of liver were found to resemble most closely those of this pancreatic enzyme.

EXPERIMENTAL

Enzyme preparation

Phospholipase *B* activity was mainly studied in extracts from acetone-dried rat liver, which was prepared by a method previously described (Dawson, 1956*a*). The acetone-dried liver (1 g.) was extracted first with 7.5 ml. of 50% (v/v) aqueous glycerol, to which was then added 7.5 ml. of 0.1% NaHCO_3 according to the method of Shapiro (1953). After centrifuging at 10 000 *g* for 20–30 min., the slightly turbid supernatant was removed and either used for experiments directly or dialysed overnight at 0° against a solution of 25% (v/v) glycerol and 0.05% NaHCO_3 . The extracts only slowly lost activity when stored at –20°.

Materials

Ovolecithin was prepared by the method of Hanahan (1954), in which the crude phospholipids are fractionated on an alumina column. Lysolecithin was prepared essentially by the action of cobra (*Naja naja*) venom on lecithin dissolved in diethyl ether according to the general directions of Hanahan (1954) and Hanahan, Rodbell & Turner (1954). The gelatinous precipitate which formed was evaporated almost to dryness in a rotary drier *in vacuo*, and then dissolved in the minimum volume of CHCl_3 . The lysolecithin was precipitated by the addition of 10 vol. of ether and recovered by centrifuging. The precipitation was repeated twice, and the lysolecithin stored in CHCl_3 solution which was centrifuged or filtered to remove any turbidity. It had a fatty acid:P ratio of 1.05:1, and was completely water-soluble. According to Hanahan (1954) the fatty acid remaining after the attack of venom on ovolecithin is on the β -carbon atom, and consists of stearic acid, although Levene, Rolf & Simms (1924) and Rhodes (private communication) have found that other saturated fatty acids are also present.

L- α -GPC was prepared by a previously described method (Dawson, 1956*a*). Lysophosphatidylethanolamine was a gift from Dr D. N. Rhodes. Diphenylchloroarsine and bromoacetophenone were kindly supplied by Sir Rudolph Peters, F.R.S., and the *Naja naja* (common Indian cobra) venom by the Zoological Society of London.

Measurement of GPC liberation

The activity of the enzyme was usually measured by estimating the GPC liberated during the incubation. The usual incubation medium consisted of 0.25 ml. of $\text{Na}_2\text{HPO}_4\text{--KH}_2\text{PO}_4$ buffer (0.3M), pH 6.4, 0.2 ml. of 50% (v/v) glycerol, 0.2 ml. of the enzyme preparation, and 0.1 ml. of lysolecithin (0.03M). After incubation at 37° the reaction mixture was placed in ice and 0.25 ml. water added, followed by 0.15 ml. of 5% serum albumin (Armour) solution to assist in the precipitation of lysolecithin. The mixture was treated with 0.5 ml. of 10% trichloroacetic acid solution, and centrifuged. A 1.1 ml. sample of the supernatant was mixed with 0.25 vol. of 5N-HCl and hydrolysed at 100° for 20 min. to hydrolyse the GPC. The hydrolysate was centrifuged and a 1.0 ml. sample removed from the precipitate

which developed during the hydrolysis. Choline was then estimated by the procedure of Appleton, La Du, Levy, Steel & Brodie (1953), 0.3 ml. of the cold triiodide reagent being added directly to the hydrolysate. It was found necessary to keep all solutions in ice throughout the estimation. The choline periodide was made up in ethylene dichloride to a final volume of 10 ml. On reading at 365 m μ , 120 μ g. of GPC gave an optical density of approximately 0.64. It was observed that the recovery of GPC added to reaction mixtures tended to be low, especially with small concentrations of GPC. Moreover, the lysolecithin substrate blanks were, although constant, reasonably high, presumably due to the incomplete precipitation of the lysolecithin by the trichloroacetic acid, resulting in its hydrolysis to free choline and estimation as GPC. Fortunately, however, the recovery of GPC from reaction mixtures containing lysolecithin was linear between 0 and 240 μ g. Consequently, by adding lysolecithin after the incubation to the tubes to which it had not previously been added, it was possible to obtain a reasonable estimate of the GPC produced by the enzymic action. It was observed that many other compounds, e.g. adenosine triphosphate (ATP) and eserine, when present in the incubation medium gave precipitates with iodine which absorbed at 365 m μ , and consequently careful control experiments were necessary.

Free fatty acid estimation

The reaction mixture (0.75 ml.) was treated with an equal volume of ethanol and then with 0.1 ml. of 5N-HCl. The mixture was shaken mechanically for 5 min. with 3 ml. light petroleum (b.p. 60–80°), centrifuged, and 2 ml. of the upper layer carefully withdrawn without touching the walls of the tube. To the sample was added 3 ml. of acetone, followed by 0.15 ml. of 0.04% cresol red indicator. The fatty acid was then titrated with 0.01N-KOH in methanol using a stream of N_2 to stir the solution. Recovery of a stearic acid standard added to the reaction mixture was linear between 0 and 5 μ moles, the maximum tested. Failure to add ethanol resulted in practically no extraction of fatty acid from the reaction mixture. The method is a far less sensitive measure of phospholipase *B* activity than the estimation of GPC liberation, a titration difference of 0.1 ml. corresponding to a breakdown of 1 μ mole of substrate.

Miscellaneous estimations

In a few experiments, disappearance of acyl ester in the reaction mixture was measured by a modified hydroxamic acid method (Stern & Shapiro, 1953).

The liberation of water-soluble amino N from lysophosphatidylethanolamine was measured by using the Moore & Stein (1948) ninhydrin colorimetric method, after the reaction mixture had been treated with serum albumin and trichloroacetic acid as for the GPC estimation. The trichloroacetic acid in the supernatant was removed by ether extraction before the estimation. In all these experiments the enzyme was dialysed to reduce its free amino N content. Acid-soluble phosphorus was estimated by the procedure of Fiske & Subbarow (1925).

RESULTS

Although the conditions of the enzyme preparation were standardized as far as possible, the preparations from individual animals showed considerable

variation in activity. However, activity was always detectable, and at a substrate level of $4 \times 10^{-3} \text{M}$, 10–30 % of the lysolecithin was broken down in the first hour. During this time the production of GPC in a standard reaction mixture occurred at a constant rate, suggesting that during this period the enzyme was not inhibited by its reaction products. Dialysis of the enzyme preparation against 25 % (v/v) glycerol–0.05 % (w/v) NaHCO_3 overnight at 0° resulted in a loss of some activity (35 %), but dialysis overnight against water resulted in an even greater loss of activity (80 %). The activity of the enzyme preparation dialysed against water was not improved by the addition of glycerol to the incubation medium. The stabilizing effect of glycerol on enzyme preparations is a well-recognized phenomenon (Bayliss, 1919; Krebs & Eggleston, 1944; Fairbairn, 1948; Shapiro, 1953).

Nature of the enzyme-catalysed reaction

The acid-labile choline liberated during activity was associated with the production of an equivalent amount of acid-soluble phosphorus. This must represent GPC, as phosphorylcholine is very stable to acid hydrolysis (Plimmer & Burch, 1937). No free choline could be detected among the reaction products at pH 6.4, presumably because at this pH GPC-diesterase activity of the extract would be negligible (Dawson, 1956*a*). The liberation of acid-labile choline was associated with the formation of free fatty acid and a decrease of acyl ester bonds. These observations are consistent with phospholipase *B* activity, i.e. $\text{lysolecithin} + \text{H}_2\text{O} \rightarrow \text{GPC} + \text{fatty acid}$.

Effect of substrate concentration

An example of the variation of enzyme activity with the concentration of lysolecithin in the incubation media is shown in Fig. 1. In three enzyme preparations tested, maximal activity was observed

Fig. 1. Effect of substrate concentration on the hydrolysis of lysolecithin by liver phospholipase *B*.

with substrate levels between 2 and $3.5 \times 10^{-3} \text{M}$. Activity was reduced at higher concentrations of lysolecithin. A similar inhibition at high substrate concentrations has been observed for the phospholipase *B* of pancreas (Shapiro, 1953).

pH optimum

Maximal activity of the liver enzyme, measured in a series of phosphate buffers, was observed at about pH 6.2 (Fig. 2). This is similar to the optimum activity of ox-pancreatic enzyme (pH 6.0) (Shapiro, 1953), but distinct from that of phospholipase *B* in *Penicillium notatum* (pH 3.8–4.4) (Fairbairn, 1948), and that for the corresponding enzyme of *Aspergillus oryzae* (pH 3.5) (Contardi & Ercoli, 1933).

Effect of inhibitors, metals, etc.

With a few preparations a small stimulation of the activity was observed on the addition of cysteine (10^{-2}M). The effect, however, could not be made reproducible, and in fact most preparations showed

Fig. 2. Variation of liver phospholipase *B* activity with pH. Value at pH 4.4 obtained in sodium acetate–acetic acid buffer, other activities in Na_2HPO_4 – KH_2PO_4 buffers.

Table 1. Effect of substances reacting with sulphhydryl groups on liver phospholipase *B* activity

Inhibitor	Concn. (mm)	Inhibition (%)
Hg^{2+}	0.1	37
	1.0	94
Cu^{2+}	0.1	38
	1.0	75
Zn^{2+}	1.33	74, 65
<i>p</i> -Chloromercuribenzoic acid	0.1	2, 4
Iodoacetic acid	5.0	2, 0
Bromoacetophenone	5.0	40*, 38*
Diphenylchloroarsine	0.5	42, 46*

* Pre-incubated 5 min. with enzyme preparation.

negligible cysteine activation. The effect of various substances reacting with sulphhydryl groups is given in Table 1. Although some of these inhibitors were active against the enzyme, the high concentrations needed suggest that any sulphhydryl groups on the enzyme play only a minor role in the formation of the active centre. Fairbairn (1948) reported that mould phospholipase *B* was inhibited by copper, but its sensitivity appears to be much less than that of the liver enzyme. The mould enzyme is also different in its sensitivity to cyanide; whereas liver phospholipase *B* is not inhibited by 0.01 M cyanide, suppression of the mould enzyme is virtually complete (97 %) (Fairbairn, 1948).

Calcium, magnesium, manganese and ferrous ions all produced a slight inhibition of the enzyme when added at a concentration of 1.33×10^{-3} M. Three enzyme preparations tested in the presence of 10^{-3} M ethylenediaminetetraacetic acid all showed a stimulation of activity (13, 73, 4 %) which is probably dependent upon the removal of contaminating traces of heavy metals. It confirms that the enzyme activity is not dependent on the presence of bivalent metal ions, e.g. Ca^{2+} (cf. Contardi & Ercoli, 1933).

Fluoride (0.02 M) produced a small inhibition of activity (34 %); histidine (5×10^{-3} M), diisopropyl phosphorofluoridate (1.33×10^{-5} M), eserine (2×10^{-4} M) and 2-acetamido-1:3:4-thiadiazole-5-sulphonamide (10^{-4} M) did not diminish the activity. Francoli (1937) reported the almost complete inhibition of phospholipase *B* in wasp venom by eserine at about one-third of the concentration used in the present experiments. Saturation of the incubation medium with diethyl ether, or the addition of 6 % (v/v) ethanol, gave large inhibitions of activity. This is in contrast to the behaviour of phospholipases *A*, *C* and *D*, where activity appears to be enhanced by the presence of ether (Hanahan, 1952; Hanahan *et al.* 1954; Kates, 1954).

When the acetone-dried liver extract (pH 7.1) was kept at 55° for 10 min. activity was virtually abolished, whereas when kept at 50° for 10 min. there was no appreciable diminution of activity. Noguchi (1944) has observed that a preparation from lung containing phospholipase *B* was inactivated at 60°. This heat lability of phospholipase *B* may be contrasted with the outstanding heat resistance of phospholipase *A*.

Specificity

When the enzyme preparation was incubated with emulsions of lecithin for long periods at varying pH values it was found that no liberation of fatty acid or decrease of acyl ester bonds occurred. Furthermore, on incubating lecithin with the enzyme preparation at pH 6.4, no liberation of GPC occurred, although the presence of small quantities

of lysolecithin added to the system could readily be detected by this method.

The preparation possessed a small amount of lipase activity as measured with triolein as substrate, but this was readily distinguished from phospholipase *B* by its complete thermostability on keeping at 55° for 10 min.

When the enzyme preparation was incubated with a sample of lysophosphatidylethanolamine for 90 min. at varying pH values no liberation of water-soluble amino N or P was detectable. However, it was thought that this lack of activity might be due to the insolubility of the lysophosphatidylethanolamine in the aqueous medium. Consequently, a sample of mixed lysophosphoglycerides was prepared by allowing *Naja naja* venom to act on a crude egg-phospholipid preparation dissolved in ether. The ether-insoluble material was purified in the same way as has been described for the preparation of lysolecithin. This material gave a clear solution in water, and on incubation with a dialysed enzyme preparation there was a slow liberation of acid-soluble amino N. It was also found that mixtures of lysophosphatidylethanolamine and lysolecithin (1:2.75 mol.prop.) were soluble in water, and again on incubation with the dialysed enzyme acid-soluble amino N was produced. It can be concluded, therefore, that the liver extract will attack lysophosphatidylethanolamine as well as lysolecithin, although it cannot be assumed that the same enzyme is responsible. However, it is significant that added lysophosphatidylethanolamine will inhibit lysolecithin breakdown.

Distribution of the enzyme in animal tissues

Appreciable liberation of GPC was observed when glycerinated extracts of acetone-dried rat kidney, spleen, brain and blood were incubated with lysolecithin at pH 6.4. Little activity could be detected in similar extracts from heart or skeletal muscle. Phospholipase *B* was also found in sheep liver, kidney and thyroid, but very little activity was detectable in whole blood.

DISCUSSION

The present report of an active phospholipase *B* in rat liver adds to the evidence already obtained that the pathway of phosphatidylcholine and phosphatidylethanolamine catabolism in this tissue is via the glycerylphosphoryl esters (GPC and GPE) (Dawson, 1955, 1956*a*). Of the other possible routes for the breakdown of these phosphoglycerides in the tissue, it has been shown isotopically that the large amounts of phosphorylcholine present in the liver are not formed by phospholipase *C* activity (Dawson, 1956*b*), nor has it been possible to obtain in our experiments any evidence for the presence of

a phospholipase *D*. Consequently, the 'glyceryl-phosphorylester intermediate' route probably represents the major if not the sole pathway of phosphoglyceride catabolism in the liver.

Although lysolecithin would be an intermediate in the route

its presence has never been demonstrated with certainty in fresh mammalian tissues. However, an accumulation of this intermediate may be undesirable for the animal because of its well-known property of disrupting cell membranes. Consequently, an active phospholipase *B* would be a necessity, especially in tissues which can rapidly catabolize phosphoglycerides.

SUMMARY

1. The presence of an active phospholipase *B* has been demonstrated in glycerinated extracts of acetone-dried rat and sheep liver, with lysolecithin as a substrate.

2. The liver extracts can also break down lysophosphatidylethanolamine when this is added in a soluble form.

3. Maximal hydrolysis of lysolecithin was obtained at pH 6.2 and a substrate concentration between 2 and 3.5×10^{-3} M.

4. The enzyme is resistant to most enzyme inhibitors. However, Hg^{2+} , Cu^{2+} and Zn^{2+} ions are inhibitory, as is saturation of the incubation medium with ether.

5. Lysolecithin breakdown has also been observed to occur with extracts from acetone-dried rat kidney, brain, heart, spleen and whole blood, but not with skeletal muscle. Phospholipase *B* is also present in sheep kidney and thyroid.

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Protection of Succinic Dehydrogenase Thiol Groups by Fluoride and Phosphate

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The biological oxidation of succinate is inhibited by fluoride (Batelli & Stern, 1911). Several explanations have been proposed for fluoride action, e.g. competition of fluoride with succinate for succinic dehydrogenase [D. J. O'Kane, unpublished data quoted by Potter & Schneider (1942)]. Phosphate is necessary for the inhibitory action of fluoride (Slater & Bonner, 1952), and kinetic deductions led these authors to postulate the formation of a

slightly dissociated inactive complex made up of fluoride, dehydrogenase and phosphate in which fluoride and phosphate compete with succinate for the enzyme active centre.

Succinic dehydrogenase is protected from thiol reagents by succinate and competitors of succinate, e.g. malonate (Hopkins, Morgan & Lutwak-Mann, 1938; Potter & DuBois, 1943; Barron & Singer, 1945), oxaloacetate, suramin and 1-naphthylamine