As may be seen from Table 1, the mucoprotein fraction of the allantoic fluid, which derives from potential host cells of the virus, contains the same sugars as are present in the virus particle. This observation, taken together with the variation of the carbohydrate content of the virus, raises the question whether or not the mucoprotein present in the virus preparation is a contaminant or an integral part of the particle. The nature of the virus purification process, including specific adsorption to and elution from red cells, eliminates the possibility of a contaminant unassociated with the virus particle. A contaminant associated with the virus by adsorption to its surface seems to be excluded on quantitative grounds; even with a low protein content (e.g. 50%) such a contaminant would entirely cover the particle surface, a situation incompatible with the known enzymic and antigenic properties of the virus. The mucoprotein is therefore considered to be an integral part of the virus particle. It remains to be determined, however, whether the virus exerts a genetic control over the pattern and amount of the mucoprotein or whether its presence in the virus is merely a necessary concomitant of the process by which new virus particles are produced. Whether gene-controlled or not, the general resemblance of the virus mucoprotein to that produced by the non-infected cell would suggest that the same basic mechanism operates in both cases.

SUMMARY

1. The purine-bound pentose of influenza virus nucleic acid has been identified chromatographically as ribose.

2. The remainder of the virus carbohydrate, consisting of galactose, mannose, fucose and glucosamine, is built into a polysaccharide considered to be the carbohydrate moiety of a mucoprotein.

3. Though the proportion of the component non-amino sugars is fairly constant, the total amounts of non-amino sugar and of amino sugar vary appreciably and independently.

4. The potential host cells of the virus were found to produce mucoprotein with a carbohydrate moiety of similar composition to that of the virus.

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Liver Glycerylphosphorylcholine Diesterase

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Recent isotopic experiments have led to the conclusion that free glycerylphosphorylcholine (GPC) and glycerylphosphorylethanolamine (GPE) occur in liver tissue as intermediates in the catabolism of phosphatidylcholine and phosphatidylethanolamine respectively (Dawson, 1955). This being so, it appeared likely that an enzyme would be present in the tissue which was capable of further hydrolysing these phosphodiesters. The present paper describes some of the properties and distribution of such an enzyme, which catalyses the hydrolysis of the diesters into glycerophosphoric acid and the free base. Some of the preliminary observations on the enzyme were made in collaboration with Mr A. Bowyer in 1954.

Contardi & Ercoli (1933) reported that the product obtained by the action of lecithinase B on lecithin was acted on by a further enzyme present in kidney, intestine and pancreas, which liberated choline. The enzyme concerned, however, appears to differ from the present enzyme in having a pH optimum of 4 (cf. 7·5) and in being inhibited by

eserine. Shapiro (1952, 1953) found that extracts from acetone-dried pancreas were able to convert lecithin into GPC but that no further hydrolysis occurred. Recently, Hayaishi & Kornberg (1954) have described the presence of a GPC diesterase in a strain of *Serratia plymuthicum* grown on a lecithin-enriched medium; the enzyme appears to differ from that in liver only by its sensitivity to magnesium ions and by its pH optimum.

The investigation of GPC diesterase in liver necessitated the preparation of the substrate GPC in quantity. As stated by Aloisi & Buffa (1948), earlier published methods for doing this succeeded only in preparing the choline salt of glycerophosphoric acid. In 1948, Baer & Kates published an elegant chemical method for preparing the optically active ester, and recently Uziel & Hanahan (1955) have reported a preparation in which lecithin is treated with a lecithinase present in extracts from *Penicillium notatum*.

Baer & Kates (1950) found that during the alkaline hydrolysis of lecithin the fatty acids were liberated at a faster rate than choline, and deduced from the hydrolysis rates that GPC was an intermediate. This was confirmed chromographically by Dawson (1954), who found that, after a brief alkaline hydrolysis of lecithin in methanolic NaOH, GPC was formed (yield, 84%). In the present investigation these observations have been made the basis of a method for preparing GPC from ovolecithin.

A preliminary report of this work was given to the Second International Congress on Biochemical Problems of the Lipids, Ghent, 1955.

EXPERIMENTAL

Preparation of L-α-*glycerylphosphorylcholine.* Ovolecithin used in the preparation of GPC was prepared by the method of Hanahan (1954) with an activated alumina column to fractionate the crude egg-yolk phospholipids. The ovolecithin (1·4 g.), which consisted of an off-white waxy solid, was dissolved in 8 ml. of carbon tetrachloride, and to the solution was added 63 ml. of methanol and 9 ml. of water. The solution was treated with 20 ml. of M methanolic NaOH and incubated at 37° for 10 min. The hydrolysate was cooled and diluted with 150 ml. of water and the milky liquid rapidly passed (10–15 min.) through a column of Amberlite IRC 50 (H) resin (20 cm. × 3 cm.^{2}; analytical grade; large beads). The Na-free effluent, plus washings, was shaken in succession with equal volumes of carbon tetrachloride, ether, light petroleum, and finally *iso*butanol, which cleared any residual turbidity from the solution. The solution was then filtered and adjusted to pH 7·5 with NH_3 and reduced in volume *in vacuo* to approximately 4 ml. After filtering, the clear solution was treated with 100 ml. of acetone. A milky turbidity immediately formed, and on standing overnight or longer at −10° the acetone could be easily decanted away from an opalescent oil clinging to the walls of the flask. The oil was dissolved in 40 ml. of cold absolute ethanol; the solution was filtered clear of insoluble matter and the GPC recovered by removing the ethanol by distillation under reduced pressure and finally drying the residue in a vacuum desiccator over $CaCl_2$. This gave a colourless viscous oil (yield, 74% based on the lecithin used). {Found: $[\alpha]_D^{23}$, −2·5°; P, 11·1; hydrolysable choline, 42·0 (Entenman, Taurog & Chaikoff, 1944); α-glycerol ester, 100·3% (Voris, Ellis & Maynard, 1940). L-α-GPC requires $[\alpha]_D$, −2·85°; P, 11·27; hydrolysable choline, 44·0; α-glycerol ester, 100%.} It is clear from these results that little migration of the phosphate group could have occurred during the brief incubation with alkali.

Some preparations of the diester showed on paper chromatography in phenol–NH_3 traces of GPE, undoubtedly arising from phosphatidylethanolamine impurity in the lecithin. It was found that this could readily be removed by preparing the $CdCl_2$ complex of GPC (Baer & Kates, 1948) and recrystallizing this from ethanol–water.

The GPC was stored in ethanolic solution and kept at −20°.

Enzyme preparation. GPC diesterase was studied in extracts prepared from acetone-dried rat liver. The preparation of these extracts needed to be carefully done, otherwise low or negligible activities were obtained. The liver from one rat was frozen in a mortar cooled to −20°, and then broken down into small pieces. Ice-cold water (2 vol.) was then slowly added, the mixture being vigorously rubbed with the pestle in order to keep the suspension as homogeneous as possible. The mixture was kept at 0° for 15 min. and then refrozen. It was allowed to thaw while being vigorously rubbed with the pestle. Acetone at −20° (5 vol.) was then added and the suspension immediately filtered under reduced pressure. The filter cake was immediately washed with a further 10 vol. of acetone (−20°) and finally with cold ether. It was dried free from solvent in a vacuum desiccator over liquid paraffin and solid NaOH. The slightly off-white powder was rubbed up in a mortar with 4 vol. of ice-cold water, and dialysed overnight against 100 vol. of distilled water at 0°. The suspension was spun at 8000 *g* for 12 min. and the clear supernatant collected. It could be stored frozen for several days with only a slight loss of activity.

Enzyme assay. GPC-diesterase activity was estimated by measuring the choline liberated in an incubation medium which contained 0·25 ml. of enzyme preparation: 0·5 ml. of Na-glycylglycine buffer (0·1 M, pH 7·8 at 20°); 0·1 ml. of $MgCl_2$ (0·01 M); 0·1 ml. of GPC (0·02 M) and 0·05 ml. of water. Incubation of the enzyme with and without substrate was carried out for 30 min. at 37°. At the end of this period the reaction mixture was cooled in ice and then treated with 0·5 ml. of ice-cold 9% (w/v) perchloric acid. The precipitated proteins were removed by centrifuging and 1 ml. samples of the supernatant assayed for free choline.

Estimation of choline. Choline was estimated in the supernatant by using the periodide method of Appleton, La Du, Levy, Steele & Brodie (1953). The preliminary purification of the sample was omitted and 0·2 ml. of the cold potassium triiodide reagent was added directly to the supernatant. Throughout the entire estimation, including the colorimetry, it was found necessary to keep all solutions at 0°. With dialysed enzyme preparations the blanks were very low, and the recovery of added choline was practically linear up to 32 μg., the maximum tested. Precipitation of the proteins with trichloroacetic acid or

tungstic acid was not found to be as satisfactory as with perchloric acid. With homogenates the recovery of choline added in small concentrations tended to be low.

Miscellaneous. Chromatographic methods used for the examination of phosphate esters have been described in previous papers (Dawson, 1954, 1955). Phenol was estimated by the method formulated by King (1946). Ovolecithin was prepared by the method of Hanahan (1954). A sample of L-α-GPE was kindly provided by Professor E. Baer (Toronto). Lysolecithin was a gift from Drs C. H. Lea and D. N. Rhodes (Cambridge). Sodium diphenylphosphate was a gift from Albright and Wilson Ltd.

RESULTS

In initial experiments it was found that when homogenates of rat liver were incubated with L-α-GPC at 37° there was a rapid liberation of free choline. The activity was practically all located in the supernatant fraction after spinning for 10 min. at 8000 *g*. However, owing to the difficulty of estimating choline in such homogenates the investigation was continued with extracts from acetone-dried powders. When such extracts were freshly prepared, and incubated with GPC according to the procedure described in the Experimental section, approximately 60–80 μg. of choline was liberated per ml. of incubation medium in 30 min. The activity of the enzyme slowly declined on incubation, and it was found that on preincubating the extract for 1 hr. at 37° only about 50% of its former activity was retained.

Reaction products. The accumulation of choline in the incubation medium was confirmed by its precipitation as the reineckate. No liberation of inorganic phosphate occurred when this ion was estimated by the method of Fiske & Subbarow (1925). Chromatography of the phosphate esters in phenol–NH_3 and ethanol–NH_3 showed that the disappearance of α-GPC was associated with an accumulation of α-glycerophosphoric acid in the incubation medium.

Effect of metals. Dialysis of the extract from acetone-dried rat liver overnight caused a small decrease in activity (16–24%). The addition of Mg^{2+} at 10^{-3} M slightly stimulated (16–26%) activity, both in the undialysed and dialysed extracts, but this stimulation became an inhibition with higher concentrations of Mg^{2+} (Fig. 1). Mn^{2+} added at the same concentration (10^{-3} M) slightly reduced the activity, and Ca^{2+} (54%) and Zn^{2+} (90%) were even more inhibitory. Ethylenediaminetetraacetic acid (EDTA) at a concentration of 10^{-3} M virtually abolished activity.

pH *optimum.* Fig. 2 shows that the pH optimum of GPC diesterase, measured in a series of buffers (phosphate, glycylglycine and glycine) was approximately 7·5. Below pH 6·5 or above 9·0 little activity was observed. These results were also confirmed in a series of glycylglycine buffers. It should be noted that the pH of a sodium glycylglycine buffer falls fairly rapidly with temperature, and consequently a buffer showing a pH of 7·8 at 20° was used in the enzyme-assay system.

Effect of inhibitors. Fluoride (10^{-2} M) and iodoacetate ($2·5 \times 10^{-3}$ M) caused no appreciable diminution of enzyme activity. The specific inhibitors of true and pseudocholinesterase, di*iso*propylphosphorofluoridate (DFP, 10^{-5} M) and eserine (2×10^{-5} M) did not inhibit GPC-diesterase activity.

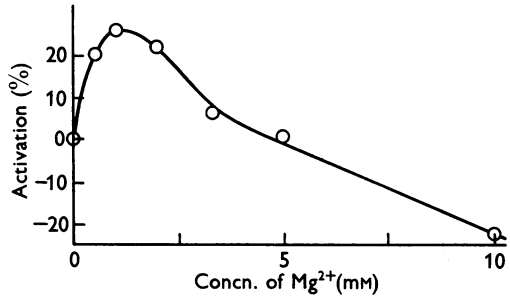

Fig. 1. Effect of Mg^{2+} on the GPC-diesterase activity of an acetone-dried liver extract (dialysed). Activity measured at 37° in 0·045 M sodium glycylglycine buffer (pH 7·8 at 20°).

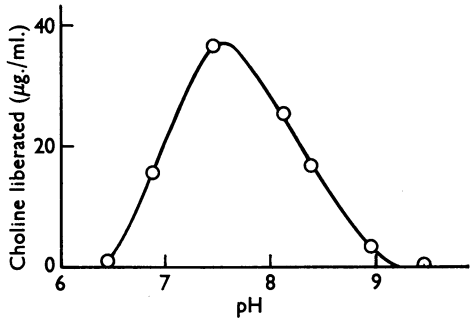

Fig. 2. Variation of GPC-diesterase activity with the pH of the incubation medium. Activity measured at 37° with the following buffers: pH 6·45, sodium phosphate (0·11 M); pH 6·88, 7·45 sodium glycylglycine (0·275 M); pH 8·14, 8·38, 8·96, 9·47 sodium glycine (0·055 M).

The strong inhibitory action of EDTA and Zn^{2+} has been mentioned in the section on the effect of metals.

Substrate specificity. Free choline was not liberated from phosphorylcholine, lysolecithin or lecithin under the conditions used for enzyme assay. Lecithin was not attacked in the presence of ether, which activates the lecithinase that hydrolyses choline from lecithin (Kates, 1953). When L-α-GPE was incubated with the enzyme preparation

hydrolysis of the diester occurred. L-α-GPE was also an inhibitor of GPC hydrolysis and Lineweaver & Burk (1934) plots showed that the inhibition was competitive (Fig. 3). Phenol was liberated from diphenylphosphate at a slow rate (10% of GPC hydrolysis), but the hydrolysis was slightly inhibited by fluoride. The enzyme preparation showed a very small phosphomonoesterase activity measured at pH 7·5 with β-glycerophosphate as the substrate, but this was completely abolished by the addition of fluoride (0·02 M).

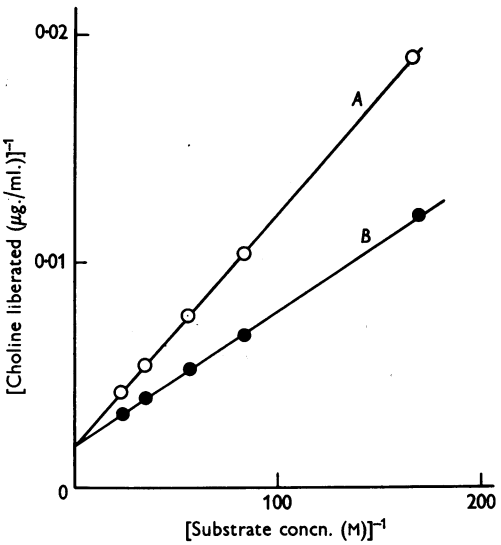

Fig. 3. Influence of substrate concentration on liver-GPC-diesterase activity in the presence of two concentrations of L-α-GPE: (*A*) 0·007 M; (*B*) 0·0035 M. Activity measured at 37° in 0·045 M sodium glycylglycine buffer (pH 7·8 at 20°).

Other tissues. Three samples of liver tissue which were collected from sheep soon after slaughter were found to have very low or negligible GPC-diesterase activities when assayed in a similar manner to that used for rat liver. All other rat tissues which were examined as homogenates showed appreciable ability to hydrolyse GPC (brain, heart, kidney, spleen, intestine, skeletal muscle and whole blood). Four samples of rat plasma and one of ox plasma showed no activity. On the other hand, the corpuscular fraction of rat blood showed appreciable activity.

DISCUSSION

Experiments reported in this paper show that an enzyme is present in rat-liver extracts which hydrolyses L-α-glycerylphosphorylcholine into α-glycerophosphoric acid and free choline. The same liver extract also breaks down L-α-glycerylphosphorylethanolamine, and, as this diester was found to be a competitive inhibitor of GPC hydrolysis, it seems reasonable to assume that a single enzyme is responsible.

It is known that GPC and GPE are not attacked by phosphatases (Schmidt, Greenbaum, Fallot, Walker & Thannhauser, 1955), and in fact the present enzyme preparation showed very little phosphomonoesterase activity, especially in the presence of fluoride, an inhibitor which does not affect GPC diesterase. The ability of the preparation to hydrolyse GPC was not reduced in the presence of eserine or DFP, and therefore the enzyme does not fit the currently accepted definition of a true or pseudocholinesterase. Moreover, highly active preparations of these enzymes isolated from ox plasma by Dr D. C. Hardwick showed no ability to hydrolyse GPC.

A comparison of the present enzyme with the GPC-splitting enzyme recently found to occur in *Serratia plymuthicum* grown on a lecithin-enriched medium (Hayaishi & Kornberg, 1954) indicates two differences: the liver enzyme is stimulated by the addition of Mg^{2+} (10^{-3} M), whereas a similar concentration of this ion almost abolishes the activity of the bacterial enzyme. The bacterial enzyme also has a higher pH optimum (9); at this pH the liver enzyme shows very little activity.

It seems very likely that the physiological importance of GPC diesterase in liver and other tissues of the rat is connected with phosphoglyceride metabolism. Isotopic experiments have shown the liver to be one of the most active phospholipid-synthesizing tissues, and this would suggest that appreciable phosphoglyceride catabolism must also occur in the tissue. One of the pathways of phosphatidylcholine and phosphatidylethanolamine catabolism *in vivo* appears to be their hydrolysis to form fatty acids and GPC or GPE (Dawson, 1955). The present results indicate that these diesters are subsequently hydrolysed in the tissue into glycerophosphoric acid and the free base. Calculation from isotopic results has shown that approximately 23 mg. of phosphatidylcholine and 26 mg. of phosphatidylethanolamine are broken down via this route per hour in 100 g. of normal rat liver (Dawson, 1956).

The apparent lack of GPC-diesterase activity in sheep liver may help to explain the high concentrations of GPC and GPE which are found in the liver of this species (Schmidt, Hecht, Fallot, Greenbaum & Thannhauser, 1952; Schmidt *et al.* 1955).

SUMMARY

1. A method has been developed for the rapid chemical preparation of L-α-glycerylphosphorylcholine (GPC) from ovolecithin.

2. An enzyme has been found in extracts prepared from acetone-dried rat liver which catalyses

the hydrolysis: L-α-GPC + H_2O → α-glycerophosphoric acid + choline.

3. Similar extracts also attacked L-α-glycerylphosphorylethanolamine, and this diester was a competitive inhibitor of GPC hydrolysis.

4. The GPC-splitting activity of such extracts was maximal at pH 7·5 and was stimulated by the addition of Mg^{2+} (10^{-3} M). Similar concentrations of Mn^{2+} and Ca^{2+} were inhibitory and this also applied to higher concentrations of Mg^{2+}.

5. The enzyme was not sensitive to fluoride, iodoacetate, eserine or di*iso*propylphosphorofluoridate. However, ethylenediaminetetraacetic acid and Zn^{2+} were strong inhibitors.

6. Evidence has been obtained for the presence of the enzyme in other tissues of the rat, but it is virtually absent from the liver of the sheep. Activity was not observed in rat plasma, but it was found in the blood corpuscles.

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Studies on the Phosphorylcholine of Rat Liver

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The recent isolation of phosphorylcholine in considerable quantities from aqueous extracts of rat liver (Dawson, 1955*a*) has raised the question of its origin in the tissue *in vivo* and its possible role as an intermediary in lecithin synthesis. Two enzymes are known which can produce phosphorylcholine in tissues: a lecithinase which splits lecithin into phosphorylcholine and a diglyceride, and choline phosphokinase, which catalyses the direct phosphorylation of choline by adenosine triphosphate (Wittenberg & Kornberg, 1953). With regard to its possible role as a lecithin precursor, recent experiments of Rodbell & Hanahan (1955), following the work of Kornberg & Pricer (1952), have convincingly demonstrated that phosphorylcholine can be incorporated as a unit into the lecithin of guinea-pig liver mitochondrial preparations.

Experiments have therefore been performed whereby radioactive phosphate was administered to rats, and a study made of the rate of labelling of both liver phosphorylcholine and lecithin. The results indicate that phosphorylcholine is not formed in the tissue through the action of a lecithinase, and the rapid rate with which it becomes labelled with ^{32}P suggests some process such as the direct phosphorylation of choline. At the same time examination of the specific radioactivity time curves using the Zilversmit, Entenman & Fishler (1943) predictions does not indicate an immediate precursor–product relationship between liver phosphorylcholine and lecithin.

In further experiments, designed to obtain information about the metabolic relationships of phosphorylcholine with other choline-containing compounds, the level of the substance in rat liver has been compared with that of liver glyceryl-