Fractionation of the Nitrogenous Water-soluble Constituents of Liver

1. THE ISOLATION OF GLYCERYLPHOSPHORYLETHANOLAMINE AND OF TAURINE

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Any theory of protein synthesis *in vivo* has to take into account the apparent absence from the cell of the obvious intermediates in the synthesis of protein from amino-acids, namely peptides. Linderström-Lang (1949) has pointed out that thermodynamic considerations would render unlikely the accumulation of appreciable quantities of peptides of low molecular weight; nevertheless, such compounds might be traced by the improved methods now available for the fractionation of amino-acids and peptides.

It was considered worth while to re-open this question by applying to the water-soluble constituents of liver the methods of fractionation used by us in the isolation of the toxic factor from agenzized flour (Campbell, Work & Mellanby, 1951). Liver was chosen as an organ with a rapid rate of metabolism and a considerable capacity for protein synthesis. This paper presents some preliminary results on the fractionation of liver extracts during which we have isolated and characterized glycerylphosphorylethanolamine, (I), a substance not

(I)

α -Glycerylphosphorylethanolamine

previously known as a natural metabolite. We have also isolated taurine and have identified various amino-acids as normal constituents of liver cells. A preliminary account of this work has already been published (Campbell, Simmonds & Work, 1951).

EXPERIMENTAL

Chromatography. Paper-partition chromatography was carried out as described by Consden, Gordon & Martin (1944). The following solvents were used: phenol/ NH_3 with KCN (Dent, 1948); 'collidine' (Dent, 1948); butanol/acetic acid/water in proportions given by Campbell, Work & Mellanby (1951); pyridine/amy alcohol/water, 35:35:30% (v/v) as described by Edman (1945); and propanol/ NH_3 (Hanes & Isherwood, 1949).

Liver extracts. Pig liver was placed in ice immediately after removal from the animal and was homogenized at 0° in a blender with the minimum quantity of water. The pH of the homogenate was adjusted to about 5 by addition of 3N-HCl and the whole homogenate (from 3012 g. liver) poured into 40 l. of boiling distilled water. The temperature was held at 100° for 30 min. and the coagulated protein then removed by filtration. The filter cake was resuspended in 10 l. of boiling water and again separated and the process repeated twice with 5 l. of water. The combined extracts were concentrated to 5600 ml. at 50–60° and filtered. The filtrate contained 11.35 g. N (Kjeldahl).

The concentrated extract was dialysed against 20 l. of distilled water for 24 hr. and then against further lots of about 7 l. The combined diffusates contained 6.45 g. N.

PRELIMINARY FRACTIONATION

Zeo-Karb. A column of Zeo-Karb 225 (The Permutit Co. Ltd., London, W. 4), diameter 2.25 in., was prepared with 200 g. (dry wt.) of size-graded resin (200 mesh/in. to 250 mesh/in. grid). The column was activated with 5N-HCl as described by Partridge & Westall (1949) for Zeo-Karb 215. The liver diffusate (3 g. N) was added to the column and the effluent (fraction A) collected (total N = 1090 mg.). The diffusate was washed into the column with distilled water and washing continued until a drop of the effluent failed to give a blue colour on paper sprayed with ninhydrin. The material still adsorbed on the column was displaced with an aqueous solution of NH_3 (0.2M) and the effluent collected in bulk (fraction B, total N = 1430 mg.). This fraction was then applied to filter paper and chromatographed in phenol/ NH_3 and collidine. After development with ninhydrin the substances present were identified by comparison with Dent's map (Dent, 1948).

Mercury precipitation. Fraction A (3440 ml.) was found by paper chromatography to contain at least three substances giving coloured products with ninhydrin. These three substances were all precipitated as their mercury salts by the method of Neuberg & Kerb (1912). To 100 ml. of A was added, in 1 ml. portions alternately, a 25% (w/v) solution of mercuric acetate (25 ml.) and 10% (w/v) Na_2CO_3 (25 ml.). Absolute ethanol (250 ml.) was then added and the mixture left at 0° for 2 hr. The precipitate was collected in a centrifuge and washed twice with 66% ethanol (60 ml. total). The combined washings were added to the supernatant (fraction C, 14.5 mg. N). The precipitate was suspended in water (400 ml.) and decomposed with H_2S (fraction D, 11.2 mg. N in 150 ml.). Paper chromatography of fraction C showed that it was almost free of ninhydrin-positive material, whereas fraction D (50 $\mu\text{l.}$), run on paper

chromatograms and sprayed with ninhydrin, showed three well defined spots which we shall refer to as α , β and γ , in the order of increasing R_F values in amyl alcohol/pyridine.

Properties of three acidic compounds, α , β and γ . As the three compounds, in fraction *D* all passed through a Zeo-Karb 225 column they must be strong acids, but they also gave a blue colour with ninhydrin and presumably possessed primary NH_2 groups. A sample of *D* was hydrolysed with 6*N*-HCl for 16 hr. at 110° and amino N before and after hydrolysis measured by the Van Slyke HNO_3 method. There was no increase in amino N. Samples of *D* before and after hydrolysis were run on two-dimensional paper chromatograms using phenol/ NH_3 and 'collidine' as solvents. Spots α and β disappeared on hydrolysis, spot γ was unaffected. In the hydrolysed material a new spot appeared in the position normally occupied by ethanolamine. As *D* contained phosphorus, a sample of ethanolamine phosphoric acid was synthesized by the method of Outhouse (1937) and compared with the three unknown substances. In no solvent could α be separated from authentic ethanolamine-*O*-phosphoric acid.

The position of γ on a two-dimensional chromatogram suggested that it might be taurine, and addition of an authentic sample of taurine to a chromatogram showed that it could not be separated from γ . This preliminary identification of γ as taurine was confirmed by isolation and analysis (see p. 452).

Substance β appeared (phenol/collidine) in the position reported by Dent (1948) as occupied by citrulline, but it was unaffected by pretreatment of the paper with basic copper carbonate (Crumpler & Dent, 1949) and disappeared on acid hydrolysis.

Isolation of acid-labile substance β from liver

Since the nature of β could not be deduced from its behaviour on chromatograms it became necessary to isolate a pure sample. Fraction *D* containing taurine, ethanolamine-phosphoric acid and β was a suitable starting material for this project; ethanolaminephosphoric acid was a minor constituent as compared to the two other substances.

Precipitation with heavy metals. Attempts were made to separate substance β from fraction *D* as the Cu salt, but none of the methods tried was successful. The uranium acetate method of Colowick & Cori (1939) for the precipitation of phosphate esters also failed. Au and Pt salts were also tried without success. Separation by the Cd salt technique of Baer & Kates (1948*a*) also failed.

Fractionation on anion-exchange resin. A column was prepared with 0.5 g. of Deacidite F (The Permutit Co. Ltd.), a strongly basic anion-exchange resin, and a sample of fraction *D* added. The effluent from the column failed to give any reaction with ninhydrin, i.e. all three substances present in *D* were adsorbed. The column was eluted with 0.1*N*-HCl but taurine, ethanolamine phosphate, and β all appeared as a single band. It was concluded that β must be strongly acidic. Later experiments using the method of Cohn & Carter (1950) suggest that separation on an anion-exchange column might be successful, but as other methods of separation had already been found this method was not developed.

Electrodialysis. A three-compartment electrodialysis cell was used, similar in construction to, but smaller (250 ml./cell) than that previously described (Campbell, Work & Mellanby, 1951). A sample fraction of *D* (250 ml.) con-

taining 125 mg. N was placed in the centre compartment (pH 6.5), the anode compartment was filled with water and the pH adjusted to about 3 with dilute H_2SO_4 , and the cathode compartment was brought to pH 9 with dilute NH_3 . At 210 V. the maximum current was 1.5 amp., and after 3 hr. the current was 220 ma. Two-thirds of the contents of the anode and cathode compartments were withdrawn after the first 30 min. and replaced by distilled water. In three similar runs 434 mg. N (fraction *D*) were fractionated in this way. The final distribution of N in the three compartments was anode 45, centre 33 and cathode 22 %. A sample of the material from the centre compartment (fraction *E*) gave excellent two-dimensional chromatograms and showed the presence of taurine and the unknown substance β in approximately equal amounts. Practically all the ethanolamine phosphate was found in the anode compartment.

Paper columns. A paper column (3 × 55 cm.) was prepared with washed Solca Floc (100 g.) as described by Campbell, Work & Mellanby (1951). The acetone was replaced by a pyridine/amyl alcohol mixture made by mixing redistilled technical pyridine (1870 ml., b.p. 128–140°), amyl alcohol (1000 ml., pure technical), and water (940 ml.). A sample of fraction *E* (35 mg. N) was evaporated to dryness and mixed with the pyridine/amyl alcohol solvent (50 ml.). Not all the material dissolved and after some hours 41 mg. of crystals were collected and later identified as taurine. The solution of *E* (now about 30 mg. N) in pyridine/amyl alcohol was transferred to the column and the effluent collected in 5 ml. fractions on a fraction collector. The nature of the material, if any, in each fraction was determined by paper chromatography in pyridine/amyl alcohol. The taurine appeared in tubes 120–150, the unknown β in tubes 245–305, and a small quantity of ethanolamine phosphate in tubes 450–500.

Solvent was removed from the combined β fractions and samples of the residual oil were run on paper chromatograms in pyridine/amyl alcohol, phenol/ NH_3 , butanol/acetic acid and collidine. In each case the substance gave a single well defined spot after spraying with ninhydrin, and it was therefore assumed to be pure; nevertheless, all attempts at crystallization from various solvents failed.

Tubes 120–150 were worked up also and 19 mg. of crystalline taurine was isolated.

Characterization of substance β as glycerylphosphorylethanolamine

Despite our failure to crystallize substance β , we felt, from the chromatographic evidence, that we were justified in assuming we had isolated a pure specimen. This assumption was justified by subsequent experiment.

Acid hydrolysis. A sample was hydrolysed in a sealed tube at 110° for 16 hr. in 6*N*-HCl and the hydrolysate analysed by two-dimensional chromatography on paper, using phenol/ NH_3 followed by collidine. There was no ninhydrin-positive spot in the position normally occupied by substance β , but instead a single spot approximately in the position normally associated with ethanolamine (Dent, 1948). A second sample of substance β was dissolved in 6*N*-HCl and left at room temperature for 2 hr. Acid was removed by leaving in a vacuum over KOH and the residue analysed by two-dimensional chromatography as before. Two spots were visible after spraying with ninhydrin, one corresponding to the original substance β and the second to ethanolamine.

Alkaline hydrolysis. A sample of substance β was hydrolysed with 0.5*N*-Ba(OH)₂ for 70 min. at 100°. The only

ninhydrin-positive substance produced was in the position of ethanolamine on a two-dimensional chromatogram.

Identification of ethanolamine derived from β . Although the ninhydrin-positive hydrolysis product from substance β followed ethanolamine in a two-dimensional chromatogram, this was not considered to be conclusive evidence as to its identity. Authentic samples of 3-amino- and 2-amino-propanol and of ethanolamine were mixed with samples of the hydrolysis product and run on paper in various solvents. No solvent could be found which could separate ethanolamine from our degradation product, but it could be separated from 3-amino- or from 2-amino-propanol in butanol/acetic acid.

As an additional check on the identity of the degradation product from substance β , a sample was oxidized by the method of Cooley, Ellis & Petrow (1950) by which ethanolamine is oxidized to glycine. The product of this oxidation in our case was also found to be glycine.

The identification of ethanolamine as a product of mild acid or alkaline hydrolysis suggested that we were dealing with an ethanolamine ester, and tests were accordingly made for an acid moiety involved in this ester linkage.

Absence of fatty acid from substance β . A sample of substance β was hydrolysed with HCl, and the hydrolysate tested for fatty acids by paper chromatography and by vapour phase chromatography (James & Martin, 1951). These experiments were kindly made for us by Dr A. J. P. Martin, F.R.S. No evidence could be found for the presence of a fatty acid.

Test for phosphorus. Two samples of substance β were run side by side on a paper sheet using propanol/ NH_3 as solvent. One strip was sprayed with ninhydrin and the other was developed for P as described by Hanes & Isherwood (1949). The P reagent gave a strong blue spot in a position which corresponded exactly with the blue ninhydrin spot of the other chromatogram.

Ratio N:P. The absence of fatty acid and the presence of P, coupled with the instability towards alkali, suggested that we were dealing with a phosphate ester of ethanolamine and the N:P ratio was therefore determined by comparison with an authentic sample of ethanolamine phosphate. The NH_2 equivalent of substance β was determined by application of the ninhydrin colorimetric method of Harding & MacLean (1915) scaled down to 10 ml. total volume in place of the original 100 ml. Colour was measured in a Spekker absorptiometer. A standard curve was prepared from suitable dilutions of a solution of ethanolamine phosphate. P was also determined colorimetrically by the method of Allen (1940). A quantity of substance β containing 16 μg . P gave by the ninhydrin method a colour intensity equivalent to 9.0 μg . N and it was therefore concluded that the N:P atomic ratio was probably 1:1.

Glycerophosphoric acid, a degradation product of substance β . Samples of α - and β -glycerophosphate and of substance β were run on the same sheet of paper with propanol/ NH_3 as solvent. The substances were located by the method of Hanes & Isherwood (1949). Substance β ran faster than either α - or β -glycerophosphate. A sample of substance β was then hydrolysed with 0.5 N-Ba(OH) $_2$ and after removal of Ba^{++} the product was again run on paper in parallel with α - and β -glycerophosphate. Colour development by the Hanes & Isherwood method revealed that substance β had been degraded to give α -glycerophosphate and β -glycerophosphate. This appearance of both glycerol esters is explained in the Discussion (p. 453). When substance β was

hydrolysed with 5 N-HCl and chromatograms were run in the same way, a spot was found corresponding with inorganic phosphate as well as one in the glycerophosphate position.

The presence of a glycol grouping in substance β was confirmed by the method of Buchanan, Dekker & Long (1950). A sample of substance β was dissolved in water and a few drops of a dilute solution of NaIO_4 were added. The mixture was heated at 100° for 3 min. and water saturated with SO_2 added to destroy excess periodate. Finally, Schiff's reagent was added and the solution warmed again. A pink colour was obtained, indicating formation of an aldehyde from substance β .

Comparison of synthetic glycerylphosphorylethanolamine and substance β . A sample of synthetic glycerylphosphorylethanolamine (supplied by Prof. E. Baer) was mixed with an equal quantity of substance β , and attempts were made to separate the two compounds on paper chromatograms. The following solvent systems were tried, pyridine/amy alcohol, butanol/acetic acid, collidine, phenol/ NH_3 . In no case was there any sign of separation; we therefore conclude that substance β is glycerylphosphorylethanolamine.

Glycerylphosphorylethanolamine in extracts of fresh liver: separation from citrulline

In order to ensure that glycerylphosphorylethanolamine was not an artifact produced as a breakdown product of some other substance, the following experiments were carried out.

Pig liver (220 g.) was placed on ice directly after its removal from the animal and homogenized in a Waring blender. The pH was adjusted to 5 and the homogenate (400 ml.) was poured into 2 l. of boiling distilled water. The

Fig. 1. Chromatogram of pig-liver extract. Y indicates the position of a yellow-coloured spot after development with ninhydrin which does not show on the photograph. 'Basic' refers to the presence of an unidentified basic amino-acid.

precipitated protein was filtered off and the filtrate concentrated to 670 ml. at 40–50°. The latter was dialysed against distilled water and the diffusate concentrated. A mercury precipitation was then carried out as previously described. After decomposition of the mercury salts a two-way chromatogram of the solution was run in phenol/ NH_3 and collidine, and a satisfactory chromatogram obtained (Fig. 1). Similar chromatograms were obtained by treating the concentrated filtrate with ethanol and CHCl_3 as described by Awapara (1948). In the chromatograms produced by

both methods there was a large ninhydrin-positive spot in the position of citrulline on the diagram of spots shown by Dent (1948). This spot could be due to the presence of either citrulline or glycerylphosphorylethanolamine. Since citrulline forms a copper complex with copper carbonate, a decision may be reached as to the identity of the spot by re-running the chromatograms in phenol and collidine with copper carbonate on the chromatograms as described by Crumpler & Dent (1949). In this case only three spots developed after spraying with ninhydrin. These three spots (Fig. 2) were in the positions of taurine, glycerylphosphorylethanolamine, and ethanolamine phosphate (trace). There

Fig. 2. Chromatogram of pig-liver extract treated with $\text{CuCO}_3 \cdot \text{Cu(OH)}_2$. X indicates the approximate position that the amino-acids shown in Fig. 1 would have occupied had the paper not been treated with $\text{CuCO}_3 \cdot \text{Cu(OH)}_2$. 'GPE' refers to glycerylphosphorylethanolamine, and 'Basic' is as in Fig. 1.

is no doubt then that glycerylphosphorylethanolamine is present in fresh liver extracts. Without the use of copper carbonate it is very easy to confuse citrulline and glycerylphosphorylethanolamine as they fail to separate in pyridine/amy alcohol, collidine, or phenol/ NH_3 . The two substances can, however, be separated in butanol/acetic acid in which citrulline runs faster than glycerylphosphorylethanolamine.

In another experiment a different fractionation procedure was used in which the initial heat treatment of the homogenate was avoided. The liver (20 g.) from a 5-week-old rabbit weighing 600 g. was homogenized in distilled water at 0° as previously described. The proteins were precipitated as described by Neuberg & Strauss (1945) by the addition of an equal volume of 21.2% (w/v) perchloric acid to the homogenate (100 ml.). The precipitate was filtered and the perchloric acid removed from the filtrate by the addition of 46 ml. of 50% (w/v) potassium acetate solution. The precipitate of potassium perchlorate was removed by filtration and 50 ml. of the filtrate concentrated to 4 ml. by freeze drying. The concentrate was added to 40 ml. of ethanol which was extracted with 120 ml. of CHCl_3 (Awapara, 1948). The aqueous layer was then treated with mercury as previously described. The decomposed mercury precipitate when run on two-dimensional chromatograms with and without copper carbonate as previously described, indicated the presence of glycerylphosphorylethanolamine. On the copper carbonate treated paper the spot due to taurine was very faint compared with that due to glycerylphosphorylethanolamine and of about the same intensity as that due to ethanolamine phosphoric acid. This confirms previous results with the usual fractionation procedure with Zeo-Karb 225, which showed that the amount of taurine in livers of young rabbits is very small.

Alternative method for isolation of taurine

As has been described, taurine may be isolated by the use of paper columns. However, a simpler method has been utilized which may be employed on a larger scale than is possible with paper columns.

The effluent from the Zeo-Karb 225 column (fraction D) was evaporated to dryness and then repeatedly extracted with hot pyridine to remove sugars (Malpress & Morrison, 1949). Very little ninhydrin-positive material is soluble in pyridine. The residue from the pyridine extraction was then dissolved in water and the solution was treated with phosphotungstic acid as described by Chibnall, Rees & Williams (1943). This phosphotungstic acid precipitate, after decomposition, was shown to have very little ninhydrin material, most of the latter remaining in the supernatant. The supernatant was then hydrolysed with 5N-HCl in a sealed tube overnight, evaporated to dryness twice, and then passed through a column of the anion-exchange resin, Deacidite E (10 g.), to remove the remainder of the HCl. The effluent was then added to a column containing 0.4 g. of the cation-exchange resin, Dowex 50 (The Dow Chemical Co.), which had been activated with 5N-HCl as described for the Zeo-Karb 225. The effluent from this column contained only one ninhydrin-positive substance, which appeared to be taurine. When the solution was concentrated and 4 vol. of ethanol were added to the hot solution, crystals appeared. The crystalline material, transferred to filter paper and chromatographed in amy alcohol/pyridine, could not be distinguished from taurine. A sample of the crystalline material analysed satisfactorily as taurine. (Found: C, 20.0; H, 5.8; N, 10.9. Calc. for $\text{C}_2\text{H}_7\text{O}_3\text{NS}$: C, 19.2; H, 5.6; N, 11.2%.) The melting point of the crystals was 328° . The nitroprusside test was positive.

Altogether 22 mg. of crystalline taurine was obtained and it was calculated that from 1 kg. pig liver about 100 mg. could thus be obtained.

RESULTS AND DISCUSSION

The ninhydrin-positive water-soluble constituents of liver have been separated into two major fractions, those that are adsorbed on a cation-exchange resin, and those that are not. In pig-liver homogenates 57% of the water-soluble nitrogen was dialysable, of which 48% was adsorbed on the cation-exchange resin, Zeo-Karb 225. Zeo-Karb 225, rather than 215, was used in the present work. The former is a unifunctional sulphonated polystyrene resin which is lightly cross linked with divinylbenzene, whereas the latter is a sulphonated phenol-formaldehyde resin. The polystyrene resins have a greater molecular permeability than the phenol resins. Thus, Zeo-Karb 215 tends to adsorb irreversibly the aromatic amino-acids (Partridge, 1949), whereas the polystyrene resins (Zeo-Karb 225 and Dowex 50) do not. Since it was hoped in the present work to avoid irreversible adsorption of peptides present in liver extracts, polystyrene resins were obviously preferred.

This paper is chiefly concerned with the acidic

substances that are not adsorbed on a cation-exchange resin, but in pig liver the following substances have also been identified by chromatography: glutathione, aspartic acid, glutamic acid, ethanolamine, serine, glycine, glutamine, alanine, β -alanine, lysine, proline, valine, leucine, phenylalanine, and tyrosine (cf. Awapara, Landua & Fuerst, 1950a). We have not detected citrulline, although the methods used do not exclude its presence, and the same considerations apply to methionine. Ethanolamine is sometimes, but not always, present in the extracts and it is suspected that free ethanolamine may be a breakdown product of glycerylphosphorylethanolamine.

Only three ninhydrin-positive substances were consistently identified as being present in the effluent from the ion-exchange column and these were all precipitated as their mercury salts. Of these substances, glycerylphosphorylethanolamine and taurine represented the major part of the nitrogen from adult pig livers, whereas ethanolaminephosphoric acid only constituted a small proportion. In young rabbit livers, in which there is very little taurine, glycerylphosphorylethanolamine predominated.

Awapara, Landua & Fuerst (1950b) have shown that ethanolaminephosphoric acid occurs in many organs of the normal rat, and the results now obtained with pig liver confirm that it is not necessarily associated with tumour tissue as was at first thought by Outhouse (1937).

Awapara *et al.* (1950a) have reported the presence of taurine in rat liver, but did not isolate it. Our results suggest that in the particular pig liver concerned there was about 100 mg. of taurine/kg. so that the amount of taurine in the liver is quite considerable.

Although the method described here for the isolation of taurine from pig liver was fairly satisfactory, it is not regarded as the best possible method, and preliminary experiments with the strongly basic anion-exchange resin, Deacidite F (The Permutit Co. Ltd.) indicated that a simpler method for the isolation of taurine from fraction A of our present procedure could be devised.

In the isolation of glycerylphosphorylethanolamine from liver extracts the chief difficulty is in its separation from taurine. The two substances in many ways have very similar properties and the only way of separating the two which has so far been worked out is by the use of paper columns. This method, however, suffers from the fact that it can deal only with comparatively small quantities. In our isolation of glycerylphosphorylethanolamine, electrodialysis was used, but although this procedure removed a certain amount of interfering material, and most of the ethanolaminephosphoric acid, the loss of nitrogen (only 25 % remained in the centre compart-

ment) was considerable and there is little doubt that there was a loss of glycerylphosphorylethanolamine in this step.

Glycerylphosphorylethanolamine has been found not only in pig liver but also in the liver of rabbits and in a foetal sheep liver by us, and in low concentration in rat liver (Walker, 1951). It has also been identified in this Institute in an extract of brewer's yeast. Dr Walker has also detected it in ox spleen, kidney, brain, and liver. The possibility that glycerylphosphorylethanolamine is split from some larger labile molecule, e.g. phospholipin, during the course of isolation, cannot be completely excluded. However, glycerylphosphorylethanolamine was detected in liver extracts when the protein was removed by cold perchloric acid (Neuberg & Strauss, 1945) and also when acid was excluded and the protein precipitated by heat. It is very probable, therefore, that glycerylphosphorylethanolamine occurs as such in the cell.

The identification of glycerylphosphorylethanolamine in the tissues of various animals is, so far as is known, the first report of the occurrence of this substance free in cells. Feulgen & Bersin (1939) obtained it in crystalline form as the hydrolysis product of muscle plasmalogens, and recently Thannhauser, Boncoddio & Schmidt (1951) obtained it from a brain plasmalogen. In the latter case the α isomer exclusively was obtained. Since the sample of glycerylphosphorylethanolamine obtained in the present work reacted with periodate under conditions in which α - but not β -glycerophosphates react, it is certain that some of the α isomer was present. However, chromatography in propanol-ammonia, in which solvent it is possible to separate the α - and β -glycerophosphates, indicated that on alkaline hydrolysis both glycerophosphates were produced. Baer & Kates (1948b) have already shown that on hydrolysis of glycerylphosphorylcholine an equilibrium mixture of the α - and β -glycerophosphates is produced. Thus the production of β -glycerophosphate on hydrolysis of glycerylphosphorylethanolamine does not necessarily indicate the presence of β -glycerylphosphorylethanolamine in the sample.

That glycerylphosphorylethanolamine has not previously been identified in tissue extracts would seem to be due to its instability and its association with citrulline in two-dimensional chromatograms. The two substances run identically in the solvents normally used for two-dimensional chromatography, namely phenol/ NH_3 and collidine, and also in amyl alcohol/pyridine. However, the two substances are separated by a fourth solvent, namely butanol/acetic acid. The chromatographic similarity between two substances with such different structures is rather surprising and suggests a note of caution in too ready an acceptance of chromato-

graphic evidence alone in the identification of unknown substances.

No experiments have yet been carried out to determine the biological significance of glycerylphosphorylethanolamine. It would be surprising if this phosphate ester were not of biological significance since it is present in comparatively high concentrations in the livers of a variety of animals and apparently also in other tissues. Fig. 2 is typical of the chromatograms obtained from the liver extracts of various animals.

SUMMARY

1. The nitrogenous water-soluble constituents of liver have been separated into two groups by use of an ion-exchange resin, Zeo-Karb 225.

2. The group of compounds not adsorbed on Zeo-Karb contained three substances giving coloured derivatives with ninhydrin.

3. Two of these substances were identified as taurine and ethanolaminephosphoric acid.

4. Two methods were devised for the isolation of crystalline taurine from pig liver.

5. The third 'ninhydrin-positive' substance was isolated from pig liver and, by degradative methods and by comparison with a synthetic specimen, it was shown to be glycerylphosphorylethanolamine.

6. Evidence was obtained that glycerylphosphorylethanolamine is a normal constituent of liver and not an artifact produced from phospholipins.

7. Glycerylphosphorylethanolamine was found in rabbit liver and foetal sheep liver as well as in pig liver. It is also found in other types of tissue.

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