bated for 16–18 hr. with borate buffer, pH 10, in the presence of n-butanol; purification by calcium phosphate gel at alkaline and acid pH; repeated precipitations of the enzyme with half-saturated ammonium sulphate; and separation from inactive proteins by dialysis.

2. From 1 kg. ox kidneys it is possible to obtain 620 mg. of a preparation with a Q_{O_2} value not less than 700.

3. Uricase prepared according to the present method was free from catalase at an early stage of purification.

4. The suitability of this enzyme preparation for the purpose of uric acid determination is discussed.

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A Method for Measuring the Deposition of 32P in Phosphatidylethanolamine and its Application to Rat-brain Tissue

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There is at present no method for determining the radioactivity of phosphatidylethanolamine in small samples of animal tissues labelled with radioactive phosphorus (32P). Such a determination requires the isolation of a pure sample of phosphatidylethanolamine.

It was shown by Folch (1942) that phosphatidylethanolamine is appreciably soluble in ethanol, so that the serine- and inositol-containing phospholipins are selectively precipitated on adding ethanol to an ethereal solution of the phospholipins. Therefore, former methods for determining the specific activity of 'kephalin' phosphorus which were based on ethanolic precipitation do not give a true indication of the phosphorus turnover in phosphatidylethanolamine. Solvent fractionation methods for the isolation of phosphatidylethanolamine require large quantities of tissue and careful analytical

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control. Moreover, for the fractionation to be successful, it is necessary to avoid the preliminary treatment of the tissue with trichloroacetic acid solution. Precipitation of the phospholipins with trichloroacetic acid helps to free them from troublesome contamination with acid-soluble phosphorus of high specific activity.

The method to be described for determining the specific activity of phosphatidylethanolamine phosphorus is based on the finding that the phospholipin can be catalytically hydrolysed by dilute mercuric chloride solution to a water-soluble derivative of ethanolamine whose behaviour on chromatography and hydrolysis corresponds to glycerylphosphorylethanolamine (GPE). This compound can be separated by paper chromatography and its radioactivity measured. The specific activity of the phosphatidylethanolamine phosphorus can then be calculated.

METHODS

Determination of the specific activity of ^{32}P in phosphatidylethanolamine

A sample of brain tissue (0·25–1·0 g.) was quickly homogenized in 20 ml. of 20% (w/v) trichloroacetic acid solution and allowed to stand for 2 hr. at room temperature to destroy acetal phospholipins. The precipitate was well washed with more acid and finally with water, and after dehydration with acetone was extracted for 1 hr. with a boiling mixture of equal volumes of $CHCl_3$ and methanol. The solution of phospholipins was shaken with 2 vol. 0·25 M-$MgCl_2$ and the $CHCl_3$ layer which separated was evaporated to dryness *in vacuo*. The residue was taken up in boiling ether, and after 1 hr. at 5° the precipitate was removed by centrifuging. The ether was evaporated and the ether-soluble phospholipins hydrolysed in a sealed tube (or under reflux) with 1–2 ml. 0·02 M-$HgCl_2$ for 3 hr. at 100°. The hydrolysate was centrifuged and the residue washed with 4 ml. water. The combined supernatant and washings were extracted with ether (five–six extractions) to remove unchanged lipid material. If necessary, any emulsion formed was broken by centrifuging. The aqueous solution was freed from dissolved ether with a stream of N_2, and then electrolytically desalted to remove $HgCl_2$. Complete removal of the $HgCl_2$ was found to be essential as it interfered with chromatography. The solution, after concentration *in vacuo*, was applied to a filter-paper sheet and a one-dimensional chromatogram prepared using a water-saturated phenol solvent. The chromatogram was dusted near the origin with basic copper carbonate since this treatment was found to reduce the P content of the GPE spot very slightly, and thus help to prevent contamination. After drying the chromatogram at 50° the GPE spot was located with ninhydrin and the specific activity of its P determined in a similar manner to that previously described for ethanolamine phosphoric acid spots on chromatograms (Ansell & Dawson, 1951).

Metabolism experiments

Isolated rat-brain preparations were suspended in Krebs-bicarbonate-Ringer and incubated aerobically at 37°.

To ensure a measurable deposition of ^{32}P in the brain phospholipins during short term *in vivo* experiments, the isotope was injected intracisternally. Cisternal punctures for this purpose were made by the technique of Jeffers & Griffith (1942).

RESULTS

The initial experiments were made with the following synthetic saturated kephalins: DL-α-dimyristyl, DL-α-dipalmityl, DL-β-dimyristyl. These were hydrolysed with mercuric chloride solution and after removal of unchanged kephalin and mercuric chloride were subjected to two-dimensional paper chromatography using phenol/ammonia and 'collidine' solvents (Dent, 1948). Ninhydrin spraying showed that each hydrolysate contained an ethanolamine derivative moving to a similar position to that of true α-GPE. Although the synthetic α-kephalins gave a single spot on the chromatogram, the spot obtained from the β-kephalin tended to

'separate' in the collidine run, so that its intensity was concentrated in two regions of the spot. If the spot is actually GPE, this may indicate that some migration of the phosphoryl group can occur during the hydrolysis, the α- and β-isomers of GPE separating very slightly in the collidine run. On the other hand, a similar behaviour is often shown on two-dimensional chromatograms by compounds such as aspartic or glutamic acids in which the possibility of such isomers does not exist (Dent, 1948). The phenol/ammonia run readily distinguished our ethanolamine derivative (R_F, 0·65) from ethanolamine (R_F, 0·90), ethanolamine phosphoric acid (R_F, 0·34) and unchanged kephalin (R_F, 0·84 +). When the chromatograms were sprayed with reagents for detecting phosphorus (Hanes & Isherwood, 1949; Bandurski & Axelrod, 1951) a spot appeared which exactly coincided with the position of the ninhydrin-reacting spot.

When the ether-soluble phospholipins of brain tissue were freed from amino-acid and protein impurities and hydrolysed with mercuric chloride solution, the only ninhydrin-reacting spot visible on a two-dimensional chromatogram of the hydrolysate was in the position of GPE. The spot contained phosphorus, the ratio of the ninhydrin colour and phosphorus (Ansell & Dawson, 1951) being very slightly higher than in the GPE-like derivative prepared from synthetic kephalin. This result can probably be explained by the contamination of the spot with traces of nitrogenous impurities. The spot was still detected with ninhydrin when the chromatogram was initially dusted with basic copper carbonate to eliminate substances with an α-amino-carboxylic acid grouping (Crumpler & Dent, 1949). When the mercuric chloride hydrolysate was hydrolysed with 5 N-hydrochloric acid (16 hr.) the GPE spot was no longer visible on the chromatogram but a new one appeared in the position of ethanolamine. In further experiments the GPE-like substance was located on a phenol chromatogram by running it together with a parallel marker, eluted with water and hydrolysed with 0·4 N-barium hydroxide at 100° for 75 min. Chromatography of the product on paper showed that the original substance had been completely hydrolysed with the production of a new spot in the ethanolamine position.

A specimen of phosphatidylethanolamine isolated from ox brain by the procedure of Folch (1942) and hydrolysed with mercuric chloride solution gave, when subjected to paper chromatography in phenol, a distinct ninhydrin reacting and phosphorus-containing spot in the GPE position. It was not found possible to separate chromatographically synthetic L-α-GPE from the GPE-like substance prepared from either a crude lipid extract of brain or ox-brain phosphatidylethanolamine. The solvent

systems used were phenol saturated with 0·1 % ammonia, R_F 0·65; n-butanol:ethylene glycol: water (4:1:1, v/v), R_F 0·22; n-butanol:pyridine: water (4:1:5, v/v) upper layer, R_F 0·04; n-butanol: acetic acid:water (4:1:5, v/v) upper layer, R_F 0·13.

Specificity. It has been shown that GPE is formed when acetal phospholipin is hydrolysed with mercuric chloride solution (Feulgen & Bersin, 1939). Although acetal phospholipin is known to occur in brain tissue (Thannhauser, Boncoddo & Schmidt, 1951) the procedure used in the present investigation ensured that the tissue stood in the presence of acid for a sufficient length of time to destroy this phospholipin (Feulgen & Bersin, 1939; Hack, 1947). This was confirmed by a preliminary hydrolysis of the ether-soluble phospholipins with N-potassium hydroxide for 24 hr. at 37° which destroys phosphatidylethanolamine but not acetal phospholipin (Feulgen & Bersin, 1939; Klenk, 1944; Hack, 1947). After the alkaline hydrolysis the unchanged phospholipins were precipitated with acetic acid at pH 6 and extracted with ether or chloroform. After mercuric chloride hydrolysis on these alkali-stable phospholipins no GPE spot was observed on the subsequent chromatogram, and when the position was located by citrulline markers (Campbell & Work, 1952) analysis showed that the phosphorus content of the paper in this area was negligible.

Several experiments were carried out subjecting 3–4 mg. of synthetic DL-α-dipalmitoyllecithin to the experimental procedure, and no interference with the method could be detected. It proved difficult to free natural phosphatidylcholine from traces of phosphatidylethanolamine, and consequently on

hydrolysing 10 mg. samples with mercuric chloride solution a faint ninhydrin-reacting and phosphorus-containing spot was observed on the chromatogram at the GPE position. No choline could be seen in the GPE position on spraying with reagents for detecting this base (Bevan, Gregory, Malkin & Poole, 1951). A 7 mg. sample of phosphatidylserine, isolated from ox brain by the procedure of Folch (1942) and containing a trace of phosphatidylethanolamine, gave after hydrolysis a very faint ninhydrin-reacting GPE spot. The intensity of this spot was completely independent of the presence of copper carbonate on the chromatogram which indicated that phosphatidylserine does not interfere with the method and that the faint GPE spot had arisen from the phosphatidylethanolamine impurity. Any traces of unchanged phospholipins not removed by the ether extractions appeared to move to the end of the phenol run, where they could be located by examination in ultraviolet light or by spraying to detect phosphorus. It is not known to what extent the fatty acid composition of phosphatidylethanolamine affects the rate of hydrolysis by mercuric chloride solution, but yields of GPE equivalent to 50 % of the phosphatidylethanolamine content of the tissue have been obtained. Yields from synthetic saturated kephalins tended to be less than this, possibly because they emulsify far less readily with the mercuric chloride solution during the hydrolysis. When 150 μg. samples of synthetic L-α-GPE were hydrolysed with mercuric chloride solution for half the normal hydrolysis time, desalted and subjected to chromatography, the average recovery of phosphorus from the GPE spot was approximately 70 %.

Table 1. *A comparison of the specific activities of phosphatidylethanolamine phosphorus and ether-soluble phospholipin phosphorus in rat brain after* ^{32}P *exchange*

System used	Wt. rat (g.)	Activity ^{32}P (μc.)	Time allowed for ^{32}P exchange (hr.)	Ratio of specific activities of: phosphatidyl-ethanolamine P (GPE) / ether-soluble phospholipin P*
Suspension, whole brain, cell-free	{150 —	{8 μc./400 mg. brain	{ 3 2	<0·11 <0·13
Minced whole brain	—		2	<0·09
Sliced whole brain	164	15 μc./400 mg. brain	{ 2 2	0·08 0·05
Intact animal, intraperitoneal injection	{ 50 {108 {108	40 50 50	28 24·5 24·5	0·77 0·90 0·65
Intact animal, intracisternal injection	{ 90 {347	20 30	3 3	<0·35 0·39

* Ethereal solution of phospholipins washed twice with N-HCl.

< indicates that the counting rate of the 'phosphatidylethanolamine' was too low for accurate measurements, and consequently a maximum value was used to evaluate the ratio.

The deposition of ^{32}P in brain phosphatidylethanolamine

Some results obtained by using the method to determine the specific activity of phosphorus in phosphatidylethanolamine in rat brain after ^{32}P exchange are given in Table 1. It is seen that when suspensions obtained in a 'homogenizer', minces or slices of rat-brain tissue, are incubated in a glucose-Ringer solution containing labelled phosphate, the specific activity of the phosphatidyl-ethanolamine phosphorus is considerably less than the specific activity of the phosphorus from the whole ether-soluble phospholipin fraction. Thus, after 2–3 hr. incubation the specific activity of the phosphatidylethanolamine phosphorus is only about a tenth of that of the ether-soluble phospholipin phosphorus, even though this latter fraction contains some 40 % phosphatidylethanolamine.

Similarly, a few hours after intracisternal injection of labelled phosphate the phosphatidyl-ethanolamine phosphorus of rat brain has a specific activity which is much less than that of the ether-soluble phospholipin phosphorus. Some 24 hr. after the intraperitoneal injection of ^{32}P, the specific activity of the phosphatidylethanolamine phosphorus is beginning to approach that of the whole phospholipin fraction.

DISCUSSION

The present experiments leave little doubt that a water-soluble and phosphorus-containing deriva-tive of ethanolamine is split off from phosphatidyl ethanolamine on hydrolysis with mercuric chloride solution. While efforts to crystallize the compound have not yet met with success (cf. Campbell & Work, 1952) there is strong evidence that the sub-stance is glycerylphosphorylethanolamine. How-ever, it must be emphasized that for the purpose of the present method an absolute identification of the substance is not essential so long as it is assured that the compound is derived from phosphatidyl-ethanolamine.

The results show that incorporation of ^{32}P into the phosphatidylethanolamine of isolated rat-brain tissue represents only a very small part of the total phospholipin-phosphorus turnover. This could be explained by assuming that the incubation condi-tions chosen were not favourable for the synthesis of phosphatidylethanolamine. However, in short term in vivo experiments, the specific activity ratio between phosphatidylethanolamine phosphorus and the total ether-soluble phospholipin phosphorus is also low. This suggests that the rate of renewal of the phosphatidylethanolamine phosphorus in rat brain is much lower than that of phosphorus in other components of the phospholipin fraction.

It was shown by Ansell & Dawson (1951) that the specific activity of ethanolamine phosphoric acid phosphorus in rat brain was many times higher than that of the ether-soluble phospholipin phosphorus. This was interpreted to mean that the acid was not formed by phosphatidylethanolamine breakdown. If the present results are related with these it can be calculated that a short time after ^{32}P injection, the ethanolamine phosphoric acid in rat brain has a specific activity several hundred times that of the phosphatidylethanolamine. Unless the turnover of the phosphatidylethanolamine fraction is extremely heterogeneous, this confirms that ethanolamine phosphoric acid is formed by a pathway which is independent of phosphatidylethanolamine cata-bolism.

SUMMARY

1. A method has been devised for measuring the incorporation of ^{32}P into the phosphatidyl-ethanolamine fraction of tissues.

2. The phosphatidylethanolamine in a lipid extract is catalytically hydrolysed with mercuric chloride solution, and a phosphorus-containing derivative of ethanolamine which behaves like glycerylphosphorylethanolamine is separated by paper chromatography.

3. The method has been used to measure the deposition of ^{32}P in the phosphatidylethanolamine of rat brain.

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