Ethanolamine *O*-Phosphoric Acid in Rat Brain

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The presence of ethanolamine phosphoric acid in brain tissue was first suggested by the work of Stone (1943), who found that a small fraction of the acid-soluble phosphorus compounds in dog brain possessed properties similar to those of this substance. Using chromatographic methods, Awapara, Landua & Fuerst (1950) have reported the presence of the compound in rat brain. The biochemical interest of ethanolamine phosphoric acid in tissues lies in its possible role as an intermediary in the metabolism of the phospholipins. From the results of experiments with ethanolamine phosphoric acid labelled with radioactive phosphorus, ^{32}P, Chargaff & Keston (1940) concluded that the compound is not an intermediary in the synthesis of phospholipins, and suggested that it might arise in the tissues by the breakdown of phosphatidyl ethanolamine. In this connexion, Tyrrell (1950) has recently described a 'cephalinase' which is present in nervous tissue.

In the present investigation the presence of free ethanolamine phosphoric acid in rat brain has been confirmed, and the uptake of radioactive phosphorus into this compound has been measured. The results suggest that the major portion of the ethanolamine phosphoric acid present in rat brain is not produced by the breakdown of phosphatidyl ethanolamine. Experiments have also shown that synthesis of ethanolamine phosphoric acid can take place in isolated brain tissue by a process which appears to be independent of the breakdown of phosphatidyl ethanolamine.

EXPERIMENTAL

Young rats (30–40 g.) were used throughout the investigation. For experiments *in vivo* these were injected intraperitoneally with a solution of KH_2PO_4 containing 40–280 μc.^{32}P. After the experimental period they were stunned and immediately immersed in liquid oxygen. The frozen whole brain was removed, crushed, weighed on a torsion balance and transferred to a centrifuge tube containing 5 ml. of water at 100°. To the hot aqueous suspension was added the requisite amount of tungstic acid for protein precipitation, as recommended by Schurr, Thompson, Henderson & Elvehjem (1950) (i.e. 0·6 ml./g. of brain, of a solution prepared by mixing 5 vol. of 10% sodium tungstate and 7 vol. of 0·6 N-H_2SO_4). The suspension was kept at 100° for 2 min. and stood for at least 50 min. at room temperature before centrifuging.

Chromatographic isolation of ethanolamine phosphoric acid phosphorus. The suspension of brain tissue was centrifuged, and approximately 4 ml. of the supernatant desalted in an apparatus similar to that used by Consden, Gordon & Martin (1947). Distilled water was used in the anode compartment and was changed several times during the course of the desalting. A large portion of the radioactivity of the extract was removed by the process. The percentage of the radioactivity remaining after the desalting increased with the length of time allowed for ^{32}P exchange in the animal.

Samples of the desalted extract equivalent to 0·05 g. brain were subjected to two-dimensional chromatography. Whatman no. 4 paper was used because it had a lower P content than Whatman no. 1 paper. It was irrigated first with water-saturated phenol and then with water-saturated 'collidine/lutidine' (Dent, 1948), but ammonia and diethylamine were not added to the chromatography cabinet. The chromatograms were dried below 70° after each irrigation and then sprayed with 0·1% ninhydrin in water-saturated butanol. After development overnight at room temperature the ethanolamine phosphoric acid spot (*X* in Fig. 1) was cut out, together with an equivalent area around the spot which served as a control. (This equivalent area was measured by weighing on a torsion balance.) The spots and associated paper from two chromatograms were combined and oxidized by heating with 2 ml. of 60% (w/w) perchloric acid; the reaction was completed by the addition of 0·2 ml. 30% (w/v) H_2O_2 and 0·05 ml. HNO_3 when necessary. (Precautions were taken to prevent personal injury in the event of an explosion.) The residual solution was evaporated to approximately 0·6 ml.; made up to 10 ml. with distilled water and determinations made of the radioactivity and phosphorus content. The control area of paper was assayed in a similar manner.

Ether-soluble phospholipin phosphorus. The precipitate obtained on centrifuging the brain suspension was treated with 30 ml. of 5% trichloroacetic acid solution and, after re-centrifuging, the residue was stirred with 30 ml. water. After standing overnight at 0° the residue was rewashed with 30 ml. water, then twice with 15 ml. volumes of acetone at 0°. The residue was slowly dried at 37° and the phospholipins extracted by refluxing for 30 min. with 25 ml. of a 50% (v/v) $CHCl_3$-ethanol mixture. The solution of phospholipins was evaporated to dryness under reduced pressure and the residue treated with 15 ml. of cold diethyl ether. After centrifuging, the ether was removed from the supernatant by evaporation. The ether-soluble phospholipin residue was oxidized with perchloric acid and H_2O_2, and determinations made of the radioactivity and P content.

The supernatant from a tungstic acid precipitation of brain suspension contained a negligible amount of ether-soluble phospholipins, indicating that the precipitation was complete.

Inorganic + acid-labile phosphorus. A sample (1 ml.) of the supernatant from the tungstic acid precipitation was hydrolysed with N-HCl for 10 min. at 100°. After cooling, 1 ml. of 0·8 M-$CaCl_2$ was added and the pH adjusted to 8·5 with NaOH solution. The suspension was kept at 0° for

15 min., centrifuged and the residue suspended in 7 ml. of distilled water. Perchloric acid (0·6 ml. of 60%, w/w) was added and then 2·5 ml. of 5% (w/v) ammonium molybdate solution. The phosphomolybdic acid was extracted into 10 ml. of *iso*butanol. The extract was then washed with an equal volume of N-H_2SO_4 and made up to 10 ml. with *iso*butanol. The ethanolic solution was assayed for radioactivity and the phosphomolybdic acid then reduced with $SnCl_2$ to measure the phosphate colorimetrically.

In some experiments the hydrolysis with N-HCl was omitted, but the high value of the 'inorganic P' figure showed that very considerable hydrolysis of the labile phosphoric esters had occurred during the experimental procedure. It is known that there is an extremely rapid turnover of the P in these labile phosphoric esters of the brain (Linberg & Ernster, 1950), and there would probably be little difference between the specific activities determined with and without preliminary hydrolysis with N-HCl.

Determination of the radioactivity. The procedure and corrections used have been described in a previous paper (Dawson & Richter, 1950). As the inorganic and acid-labile P were assayed in *iso*butanol, a small correction was applied to allow for the different absorption of β-radiation by this solvent. All counting rate measurements were referred to a solution containing 5% (w/w) perchloric acid. For convenience, the specific activity used was calculated as the counting rate/mg. P.

Phosphorus determinations. The method of Berenblum & Chain (1938) was used, except in experiments *in vitro* when the inorganic and ether-soluble phospholipin P was estimated by the procedure of Fiske & Subbarow (1925). Both methods have the advantage that the colour development is not influenced by large variations in the concentration of perchloric acid used.

Experiments in vitro. Experiments demonstrating ethanolamine phosphoric acid synthesis by isolated brain tissue were carried out in Warburg vessels. The rats were killed by decapitation, the whole brains finely minced with a razor and suspended in Krebs bicarbonate-Ringer solution (200 mg. wet weight brain tissue/ml.). After incubation in the presence of labelled KH_2PO_4 the contents of each Warburg vessel were treated with 0·22 ml. of tungstic acid solution and heated at 100° for 5 min.

The suspension was centrifuged and the inorganic and labile P estimated in a 0·4 ml. sample of the supernatant. The rest of the supernatant was treated with enough solid $Ca(OH)_2$ to make a saturated solution, and the precipitated calcium phosphate removed by centrifuging. Excess $Ca(OH)_2$ was removed as $CaCO_3$ by saturating with CO_2 and boiling. The solution was desalted and subjected to chromatography to isolate the ethanolamine phosphoric acid. Ether-soluble phospholipins were isolated by a procedure similar to that described previously, except that the tungstic acid precipitate was given additional washes with 5% trichloroacetic acid solution and water.

Synthesis of ethanolamine phosphoric acid. Ethanolamine phosphoric acid was prepared by the method of Outhouse (1937), m.p. 241° (uncorr.).

RESULTS

Chromatographic separation of ethanolamine phosphoric acid. Two-dimensional chromatograms of trichloroacetic acid and tungstic acid extracts of whole rat brain frozen *in situ* with liquid oxygen, showed a relatively large spot (*X*) under the glutamic acid spot (Fig. 1). The intensity of this spot was reduced in chromatograms of extracts prepared from autolysed brain. With extracts from human brain, several days post-mortem, only a very faint spot was observed. The spot was not seen on chromatograms of acid hydrolysates of rat brain proteins.

The ninhydrin-reacting material which formed spot *X* was judged to contain ethanolamine phosphoric acid from the following observations:

(1) The material showed identical chromatographic behaviour to that of synthetic ethanolamine phosphoric acid.

(2) It was highly stable to acid hydrolysis. After 24 hr. hydrolysis of brain extracts at 100° in 5·5N-hydrochloric acid a considerable portion of spot *X* survived. On prolonging the hydrolysis for 72 hr. a faint spot was still visible, but a new spot appeared on the chromatogram. This new spot occupied the same position as free ethanolamine and was still present when the chromatogram was treated initially with basic copper carbonate to eliminate α-amino-acids (Crumpler & Dent, 1949).

(3) The spot was still present after the brain extract had been hydrolysed for 67 hr. at 100° in N-sodium hydroxide. All the spots on this chromatogram were of reduced intensity due possibly to the removal of the amino-acids by the silica gel produced on neutralization of the sodium silicate formed by the action of the alkali on the glass container. Plimmer & Burch (1937) have reported that synthetic ethanolamine phosphoric acid is very stable to hydrolysis with N-sodium hydroxide.

(4) On preliminary treatment of the chromatogram with basic copper carbonate, according to the procedure of Crumpler & Dent (1949), the spot was still visible, together with those of taurine and γ-aminobutyric acid.

(5) Analysis of the spot cut from the chromatogram showed that it contained an amount of phosphorus significantly higher than that in an equal area of paper surrounding the spot.

(6) In further experiments measurements were made of the intensity of the ninhydrin colour produced by extracting the spot with water according to the method of Roberts & Frankel (1950). The aqueous extract and the filter-paper pulp were then combined and analysed for phosphorus. It was found that the ratio between the intensity of the ninhydrin colour and the phosphorus content was identical, within the limits of experimental error, with that obtained when synthetic ethanolamine phosphoric acid was subjected to the same procedure.

Identification of the unknown spot by an isotopic procedure. To confirm the presence of ethanolamine

phosphoric acid in rat brain, a rat under ether anaesthesia was injected intracisternally with 100 μc. of ^{32}P. After 4 hr. the animal was decapitated, the head immediately immersed in liquid oxygen, and a tungstic acid extract of the brain prepared. After desalting, the solution was subjected to two-dimensional paper chromatography and the unknown spot *X* located with acid. This evidence confirmed that the unknown spot *X* on chromatograms of rat-brain extracts consisted of ethanolamine phosphoric acid.

The uptake of ^{32}P into the ethanolamine phosphoric acid of the brain in vivo. In experiments in which the uptake of ^{32}P into the ethanolamine phosphoric acid of the brain *in vivo* was determined, it was found necessary to inject the rats with phosphate of a high

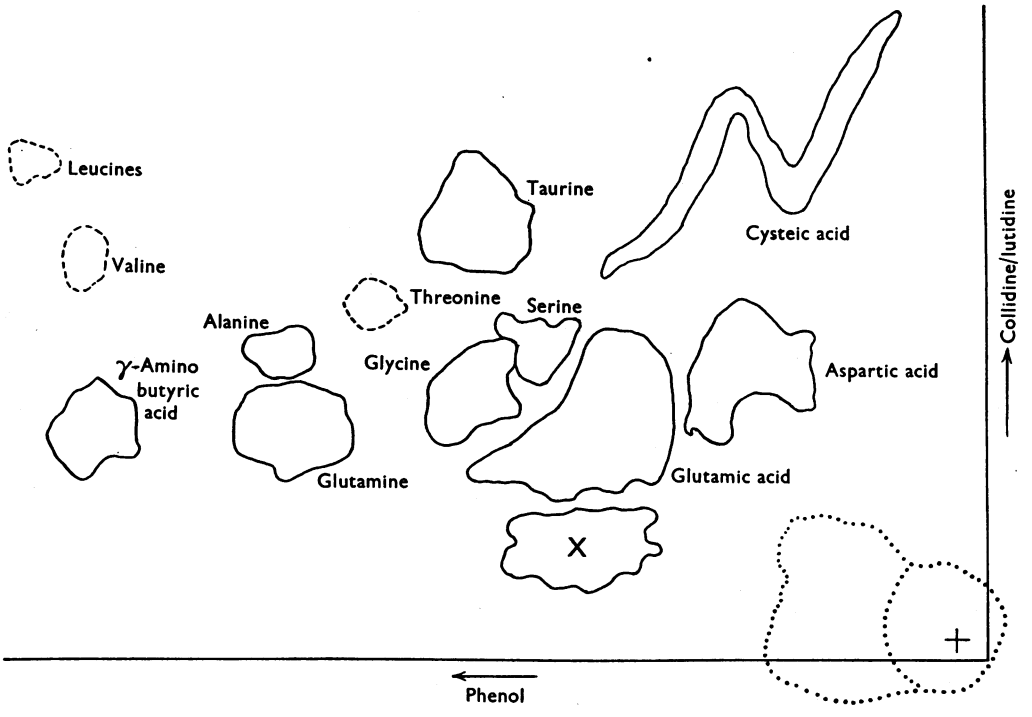

Fig. 1. Typical pattern of ninhydrin-reacting substances produced by the two-dimensional chromatography of rat-brain extract (equivalent to 0·04 g. wet tissue) which had previously been oxidized with H_2O_2. Interrupted lines indicate faint spots. Dotted line indicates the limit of the spread of inorganic phosphate and acid-labile phosphate when the extract had not been desalted to remove these substances.

ultraviolet light (Woiwod, 1949; Fowden, 1951). A solution of a small quantity of the unknown substance (equivalent to 0·1 g. wet brain tissue) was obtained by elution of the chromatographic spots with boiling water. After evaporation to 2 ml., 150 mg. synthetic ethanolamine phosphoric acid was dissolved in the solution, and then crystallized out by the addition of ethanol to 80 %. The specific activity of the product was determined. This process of recrystallization from 80 % ethanol was repeated a further five times during which the specific activity remained absolutely constant. The final product was then converted into the flavianic acid derivative (Outhouse, 1936) and crystallized from aqueous *n*-butanol, m.p. 225°. The specific activity of the phosphorus in this compound was identical with that in the original ethanolamine phosphoric activity in order to ensure a measurable counting rate from the ethanolamine phosphoric acid spot. As the blood-brain barrier is not very permeable to injected phosphate it is unlikely that the phosphorus metabolism of the brain would be appreciably affected by the radiation.

Experience showed that tungstic acid extracts of whole brain tended to give better defined ethanolamine phosphoric acid spots than trichloroacetic acid extracts. The area of paper around the ethanolamine phosphoric acid spot possessed no detectable radioactivity and its phosphorus content approximated closely to that of the paper blank.

Chromatograms of brain extracts, which had not been desalted, were examined for inorganic phosphate and phosphoric esters by the method of Hanes & Isherwood (1949). Part of these substances

remained at the point of application of the extract and part trailed in the phenol run to a position half way between the ethanolamine phosphoric acid spot and the origin (Fig. 1). Movement in the collidine/lutidine run was negligible. No inorganic phosphate was detected by this method at the position of the ethanolamine phosphoric acid spot, presumably because of its high stability to acid hydrolysis. No inorganic phosphate or phosphate esters could be detected on the chromatograms of desalted brain extracts. When chromatograms of a mixture of synthetic ethanolamine phosphoric acid and radioactive inorganic phosphate were run, the ethanolamine phosphoric acid spot showed no radioactivity.

The results presented in Table 1 show that, after ^{32}P had been allowed to 'exchange' within the animal for 3 hr., the ethanolamine phosphoric acid in the brain had a specific activity approximately 50–70% of that of the inorganic + acid-labile P fraction and fifteen to nineteen times greater than the specific activity of the corresponding ether-soluble phospholipin fraction. In two experiments in which shorter times (45 and 60 min.) were allowed for phosphorus 'exchange', the ratios of the specific activities of the ethanolamine phosphoric acid and the ether-soluble phospholipins were even greater (Table 1).

The synthesis of ethanolamine phosphoric acid by minced rat-brain tissue. In preliminary experiments designed to show the synthesis of ethanolamine phosphoric acid *in vitro*, minced rat-brain tissue was suspended in glucose-phosphate-Ringer solution and incubated under conditions which ensured a high rate of respiration. The solution obtained after protein removal with tungstic acid was desalted and subjected to two-dimensional chromatography. It was found that the intensity of the ethanolamine phosphoric acid spot did not increase above that of a control when 0·03 M-ethanolamine hydrochloride was added to the brain suspension prior to the incubation. Similar results were obtained when the experiments were carried out in the presence of 0·016 M-sodium fluoride. However, the possibility existed that the detection of an increased ethanolamine phosphoric acid production by this method would be prevented by the breakdown of the compound by phosphatase activity. Consequently, experiments were made in which the minced brain tissue was suspended in glucose-bicarbonate-Ringer

Table 1. *Specific activities of the ethanolamine phosphoric acid, ether-soluble phospholipins and inorganic + acid-labile phosphate in rat brain after the injection of* ^{32}P

Rat no.	Wt. of rat (g.)	Duration of experiment (min.)	Dose ^{32}P (μc./g.)	Specific activity of: Ethanolamine phosphoric acid P (counts/min./mg. P)	Specific activity of: Ether-soluble phospholipin P (counts/min./mg. P)	Specific activity of: Inorganic + acid-labile P (counts/min./mg. P)	Ratio of specific activities: Ethanolamine phosphoric acid P/ether-soluble phospholipin P	Ratio of specific activities: Ethanolamine phosphoric acid P/inorganic + acid-labile P
1	35·0	180	1·7	11,820	—	15,850	—	0·74
2	37·0	45	1·2	1,785	30	8,130	59	0·22
3	30·6	100	1·3	1,730	109	5,500	16	0·31
4	38·6	185	1·1	2,460	165	4,850	15	0·51
5	37·8	60	7·4	8,650	335	33,400	26	0·26
6	34·3	120	7·3	12,400	732	37,000	17	0·33
7	37·5	180	7·5	22,200	1,172	37,000	19	0·60

Table 2. *Synthesis of ethanolamine phosphoric acid by minced rat brain*

(Each Warburg vessel contained 340 mg. minced rat brain; 3·35 ml. Krebs bicarbonate-Ringer solution; 4 μc. ^{32}P as KH_2PO_4 (0·03 mg. P). Glucose concentration 0·018 M. Temp. 37·5°. Gas phase 95% O_2, 5% CO_2.)

Time of incubation (min.)	Additional substrate	Specific activity of: Ethanolamine phosphoric acid P (counts/min./mg. P)	Specific activity of: Ether-soluble phospholipin P (counts/min./mg. P)	Specific activity of: Inorganic P + acid-labile P (counts/min./mg. P)	Ratio of specific activities of ethanolamine phosphoric acid P/ether-soluble phospholipin P
0	—	0	350	191×10^4	0
60	—	>7,200	1,880	186×10^4	>3·8
180	—	10,700	2,370	186×10^4	4·5
180	0·0035 M-Ethanolamine HCl	29,400	2,270	176×10^4	13·0

solution and incubated aerobically with radioactive potassium dihydrogen phosphate. The ethanolamine phosphoric acid isolated in these experiments was found to be radioactive (Table 2), demonstrating that synthesis had occurred, and its specific activity was higher than that of the ether-soluble phospholipins, isolated from the same sample of tissue. Moreover, when 0·0035 M-ethanolamine hydrochloride was present during the incubation period, the synthesis of ethanolamine phosphoric acid was accelerated so that after 2 hr. its specific activity was thirteen times greater than that of the corresponding ether-soluble phospholipins.

The value for the specific activity of the ether-soluble phospholipin phosphorus at zero incubation time given in Table 2, points to the extreme difficulty of freeing phospholipins from contaminating inorganic phosphate. Fries, Schachner & Chaikoff (1942) found the same difficulty in their experiments on the uptake of ^{32}P into the phospholipins of surviving brain tissue.

Concentration of ethanolamine phosphoric acid in whole rat brain. The recovery of phosphorus from spot *X* corresponded to a mean value (eight brains) of 9 mg. ethanolamine phosphoric acid/100 g. whole brain, though individual values showed a wide variation from 4 to 13 mg./100 g. brain. Awapara *et al.* (1950) record a minimal content of 11 mg./100 g. for adult rat brain obtained by estimation of the spot on chromatograms with ninhydrin.

When 10 μg. ethanolamine phosphoric acid standards were run on a two-dimensional chromatogram, the recovery of phosphorus from the spot was only about 60 %. The recovery was little improved by drying off the solvents from the chromatogram at room temperature. Fowden (1951) has recently shown that when the amino-acids are estimated colorimetrically with ninhydrin the recoveries are greatly improved by low temperature drying of the chromatograms. Further experiments showed that although ethanolamine phosphoric acid hardly moved in the collidine/lutidine run, some loss of phosphorus occurred; a washing-out process may account for this observation. Visible tailing of the ethanolamine phosphoric acid spot occurred in the phenol run. These results indicate that estimation of the phosphorus content of the ethanolamine phosphoric acid spot on a two-dimensional chromatogram irrigated first with phenol and then collidine/lutidine is not a satisfactory method for the quantitative estimation of the substance in tissues and the results only indicate minimal values.

DISCUSSION

There is now good evidence that the ninhydrin-reacting substance occurring below the glutamic acid spot on two-dimensional chromatograms of brain extracts consists of ethanolamine phosphoric acid. As the spot is present on chromatograms of extracts prepared after the freezing of the brain *in situ* with liquid oxygen, the substance is not likely to have been formed by post-mortem enzymic activity. It has been suggested by Chargaff & Keston (1940) that ethanolamine phosphoric acid arises in tissues by the enzymic breakdown of phosphatidyl ethanolamine. The present experiments showed that when rats were injected with potassium dihydrogen phosphate containing radioactive phosphorus and killed after a few hours, the specific activity of the brain ethanolamine phosphoric acid phosphorus was many times greater than the specific activity of the corresponding ether-soluble phospholipin phosphorus. This strongly suggests that the whole or a large portion of the ethanolamine phosphoric acid in the brain does not arise by the catabolism of brain phosphatidyl ethanolamine. It is interesting in this connexion that Sloane Stanley (1951) obtained no evidence of the release of nitrogenous compounds in his studies on the breakdown of kephalin by brain homogenates.

The ether-soluble phospholipin fraction isolated in the present investigation was undoubtedly complex and contained lecithin as well as phospholipins containing serine, ethanolamine and inositol (Folch, 1942). The results of Artom (1945) indicate that the ether-soluble phospholipins isolated from rat brain contain about 40 % of phosphatidyl ethanolamine. Therefore, in the present experiments, even if the entire ^{32}P turnover of this ether-soluble phospholipin fraction was located in the phosphatidyl ethanolamine portion, the resulting specific activity of this compound after 3 hr. exchange would still be six times less than that of the corresponding ethanolamine phosphoric acid. It should not be assumed that the turnover of the phosphatidyl ethanolamine itself is homogeneous. The phospholipins probably exist in the cell in different chemical forms (α and β; lipoproteins) each with its own turnover rate. Furthermore, the turnover rate of the compound in different anatomical and cytological regions may not be constant. However, to account for the results obtained, ethanolamine phosphoric acid could only be produced by phosphatidyl ethanolamine breakdown if it were selectively formed from a portion of the phosphatidyl ethanolamine with a turnover rate very much higher than that of the rest of the fraction.

The question arises whether ethanolamine phosphoric acid is carried to the brain in the blood or synthesized, wholly or partly, in the brain. Radioactive ethanolamine phosphoric acid was isolated from a minced brain suspension respiring in the presence of labelled potassium dihydrogen phosphate and as the specific activity of the substance was

many times higher than that of the corresponding ether-soluble phospholipin fraction, it would seen that it had not arisen by phosphatidyl ethanolamine breakdown. If the radioactive ethanolamine phosphoric acid was produced by a simple phosphorylation, it would appear that the rate of synthesis *in vitro* must be extremely slow. This assumes that the labile phosphate groups of adenosinetriphosphate are as rapidly exchanged *in vitro* as they have been shown to do in the intact brain (Linberg & Ernster, 1950). However, the possibility that ethanolamine phosphoric acid arises from the breakdown of other phosphorus-containing compounds, such as acetal phospholipin (Thannhauser, Boncoddo & Schmidt, 1951), cannot be excluded.

The role of ethanolamine phosphoric acid in brain metabolism is not yet clear. As the formation of the compound in brain tissue appears to be independent of phosphatidyl ethanolamine breakdown and as it has a relatively high phosphorus turnover in the intact brain, the acid cannot be excluded as a precursor of phosphatidyl ethanolamine on the basis of the present results. From the work of Chargaff & Keston (1940) it would appear that ethanolamine phosphoric acid is not an intermediary of phosphatidyl ethanolamine synthesis in the liver or intestinal tract. Their results, however, are not completely conclusive, as our new knowledge of the solubility of the phospholipins (Folch, 1942) creates uncertainty as to the composition of the 'kephalin' fraction isolated by these workers.

SUMMARY

1. Ethanolamine phosphoric acid has been identified in extracts of rat brain frozen *in situ* with liquid oxygen.

2. The recovery of phosphorus from a sample of synthetic ethanolamine phosphoric acid run on a two-dimensional chromatogram irrigated with phenol and collidine/lutidine was not quantitative. Therefore the recoveries from tissue extracts by this chromatographic procedure represent minimal values only.

3. After 3 hr., the specific activity of the ethanolamine phosphoric acid of the brain in the intact rat injected with ^{32}P was approximately 50–70 % of the specific activity of the inorganic + acid-labile phosphate fraction and many times greater than the specific activity of the ether-soluble phospholipin fraction.

4. The results suggest that the major portion of the acid had not originated by the breakdown of the phosphatidyl ethanolamine in the brain.

5. The synthesis of ethanolamine phosphoric acid by isolated rat-brain tissue has been demonstrated.

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