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## Phosphorylcholine in Rat Tissues

By R. M. C. DAWSON\*

*Department of Biochemistry, University of Oxford*

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During a recent investigation on the role of glyceryl-phosphorylethanolamine and glycerylphosphorylcholine in rat-liver lipid metabolism, a large phosphorus-containing spot was observed on two-dimensional chromatograms of the water-soluble phosphorus-containing compounds of liver, in a position which was different from that of the known major components of this fraction. This substance has been isolated and identified as phosphorylcholine. It is present in rat liver at a fairly high concentration and accounts for approximately 9% of the water-soluble phosphorus in the tissue. Evidence has also been obtained that phosphorylcholine is present in the testes, spleen, intestines, kidney and brain of the animal, while only trace amounts occur in muscle, heart and blood.

Inukai & Nakahara (1935) have isolated the picrate of phosphorylcholine from ox liver, but it has been suggested that because of the minute yield obtained ( $1 \times 10^{-4}$ %) this might have been formed from lipid material during the drastic procedures used in the isolation (Lundquist, 1947). The latter author has found a high concentration of the ester in fresh human semen, where it is thought to provide a substrate for seminal phosphatase.

\* Betty Brookes Fellow.

## EXPERIMENTAL

*Isolation of phosphorylcholine from rat liver.* The livers from ten rats were quickly removed, and homogenized in 500 ml. of 10% (w/v) trichloroacetic acid solution. The precipitate was centrifuged down and washed with a further 100 ml. of trichloroacetic acid solution. The combined supernatants were extracted 3 times with equal volumes of ether, and ethanol was added until the mixture contained 70% (v/v). After standing 1 hr. at 0° the precipitate of glycogen was filtered off under reduced pressure using a filter aid. The filtrate was concentrated under reduced pressure to 15 ml., and ethanol again added to 70% (v/v). After standing, a further precipitate of glycogen was removed by filtration and the filtrate concentrated to 10 ml. The solution was then passed through a column of Amberlite IR-100 H<sup>+</sup> resin (10 cm.  $\times$  0.8 cm.<sup>2</sup>) and washed through with a further 25 ml. of water. The effluent was concentrated to a small volume and applied as starting strips on two Whatman no. 3 filter-paper chromatograms (40 cm. wide). The chromatograms were developed for 18 hr. with a phenol-NH<sub>3</sub> solvent, the solvent removed in a drying cabinet and marker strips sprayed with acid molybdate (Hanes & Isherwood, 1949) and irradiated with ultraviolet light. The phosphorus-containing substances moving near the edge of the solvent front ( $R_f$  approximately 0.8-1.0) were eluted from the paper by extracting it 3 times with 100 ml. of water and the extract was filtered and concentrated to a small volume. It was then applied as the starting

strip on two Whatman no. 1 filter-paper chromatograms (40 cm. wide) which were developed for 16 hr. with a propanol-NH<sub>3</sub> solvent (Hanes & Isherwood, 1949). After drying off the solvent, marker strips on either side were sprayed to detect phosphorus-containing compounds, and the most prominent band at approximately  $R_F$  0.23 (phosphorylethanolamine reference marker,  $R_F$  0.20) was eluted from the paper by successive extractions with water (50 ml.). The extract was filtered, concentrated to 20 ml. and boiled for 5 min. with successive small portions of charcoal (extracted with HCl and HF) until decolorized. The clear solution was then again passed through a column of Amberlite IR-100 H<sup>+</sup> (10 cm. × 0.8 cm.<sup>2</sup>) and to the effluent (30 ml.) was added 1 ml. of 6N-HCl. It was then taken to dryness under reduced pressure and the residue taken up in 3 ml. of water and neutralized to pH 7 with lime water. The solution was concentrated to 0.4 ml. and 4 ml. of ethanol added. After standing for 30 min. at 0° the precipitate was centrifuged off and dried. The solid was taken up in

with 70% (v/v) ethanol. After hydrolysis in 2N-HCl, the solution was centrifuged and made alkaline with 5N-NaOH (pH 11-13) any precipitate which formed being removed before the addition of ammonium reineckate.

**Phosphorylcholine.** Calcium phosphorylcholine chloride was prepared by the method of Plimmer & Burch (1937) and gave a satisfactory analysis for C, H, N and P. It contained no acid-labile P impurity (cf. Baer & McArthur, 1944) and was free of diester (Jackson, 1935). It showed chromatographic behaviour in phenol-water and *tert.*-butanol-picric acid solvents identical with that of an authentic sample of phosphorylcholine kindly supplied by Professor E. Baer (Toronto). Phosphorylethanolamine was synthesized by the method of Outhouse (1937).

**Tissue samples.** These were removed from the rats as quickly as possible after death (<6 min.). Samples of small intestine were dissected free from mesentery and the contents removed by washing through with water. Blood was removed by cardiac puncture performed under ether anaesthesia.

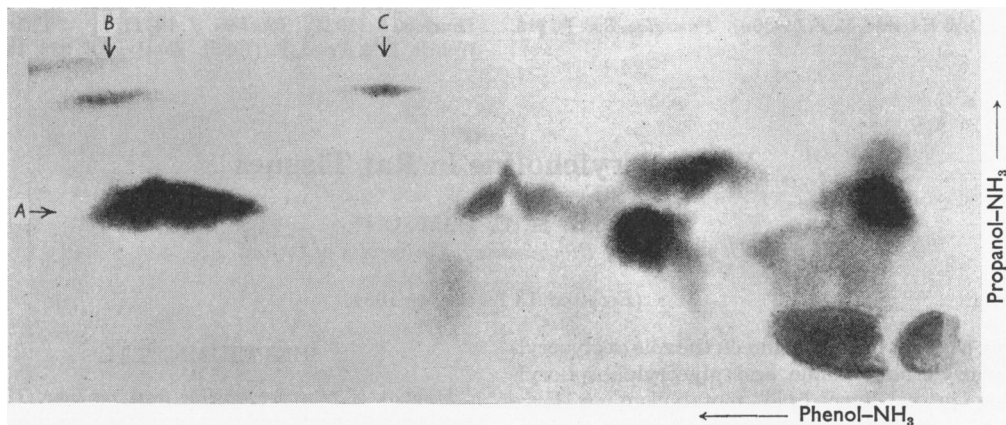


Fig. 1. Two-dimensional paper chromatogram of the water-soluble P-containing compounds in rat liver. A, Phosphorylcholine; B, glycerylphosphorylcholine; C, glycerylphosphorylethanolamine. The haze near the origin is due to decomposition of a labile P-ester in the strongly alkaline propanol-NH<sub>3</sub>.

0.4 ml. of water, any residue removed by centrifuging and the clear solution treated with 2 ml. of ethanol. The calcium salt was centrifuged off and dried at 100°. Yield 3-5 mg. colourless needles or irregular crystals.

**Chromatography, detection and estimation of P.** The procedures used have been described in a previous paper (Dawson, 1955). The phosphorus in chromatographic spots was assayed by cutting out the paper spots and also an equivalent area of paper blank, completely oxidizing the pieces of paper with HClO<sub>4</sub> and estimating inorganic P in the solutions by the method of Fiske & Subbarow (1925).

**Choline.** This was estimated by the method of Entenman, Taurog & Chaikoff (1944), and in hydrolysed tissue extracts by a slight modification of the procedure of Engel, Salmon & Ackerman (1954). In this modified method tissue extracts for hydrolysis were prepared by extraction of the tissues with 10% (w/v) trichloroacetic acid solution. The extract was then washed 3 times with ether to remove trichloroacetic acid and any glycogen was removed by precipitation

## RESULTS

Fig. 1 shows a photograph of a typical two-dimensional chromatogram of the water-soluble P-containing substances in rat liver. It can be seen that phosphorylcholine (A) like glycerylphosphorylcholine (B) is characterized by its fast running in the phenol-NH<sub>3</sub> solvent, a property which can be used to separate it from the bulk of the other P-containing compounds occurring in the fraction.

The phosphate ester constituting spot A was found to be extremely stable to hydrolysis for 2 hr. at 100° both in N-HCl (<4% hydrolysis) and N-NaOH (<2% hydrolysis), which is in agreement with the acid and alkaline stability of phosphorylcholine (Plimmer & Burch, 1937; Baer & McArthur, 1944). The calcium salt of the phosphate ester whose

isolation is described in the Experimental section was identified as phosphorylcholine from the following observations:

(1) When heated with 2N-HCl in sealed tubes at 123° for 24 hr., the compound was almost quantitatively hydrolysed and choline and inorganic P were liberated in equimolar proportions. (Choline/P, molar ratio, prepn. 1; 1.02 prepn. 2; 0.98.)

(2) The compound was converted into the aurochloride by the method of Jackson (1935). The product on recrystallizing from 2N-HCl consisted of long slender yellow needles (m.p. corr. 176.5°). Jackson records that phosphorylcholine aurochloride crystallizes in yellow needles m.p. (corr.) 174–176°.

(3) The hydrolysis curve of the substance in 2N-HCl at 123° closely followed a similar curve prepared for synthetic phosphorylcholine.

(4) The substance was inseparable from synthetic phosphorylcholine on paper chromatography in four solvent systems widely used in phosphate ester chromatography; *tert.*-amyl alcohol-formic acid (Hanes & Isherwood, 1949),  $R_F$ , 0.19; butanol-pyric acid (Caldwell, 1953),  $R_F$ , 0.49; phenol-NH<sub>3</sub>,  $R_F$ , 0.93; propanol-NH<sub>3</sub> (Hanes & Isherwood, 1949),  $R_F$ , 0.23. Phosphorylethanolamine reference markers run on the chromatograms had  $R_F$  values of 0.17, 0.54, 0.39, 0.20 in these solvents respectively.

(5) A radioactive sample of the substance was prepared by isolating it from a rat liver 4 hr. after the animal had been injected with 1 mc of labelled phosphate. To an aqueous solution of the sample was added a large excess (400 mg.) of the synthetic calcium salt of phosphorylcholine. The salt was precipitated out by the addition of ethanol (80% v/v) and the specific radioactivity of the phosphorus contained in the compound measured. The phosphorylcholine was then purified by crystallizing it 5 times from 80% ethanol-water (v/v) and the final product was converted into phosphorylcholine aurochloride by the method of Jackson (1935). The yellow needles of the aurochloride were finally recrystallized from 2N-HCl (m.p. 177°). During the process of purification and after the conversion into the aurochloride the specific radioactivity of the P contained in the compounds remained constant, showing that no separation of the radioactive phosphate ester and phosphorylcholine had occurred.

*Concentration of phosphorylcholine in liver.* Phosphorylcholine has been estimated chromatographically in six liver samples which were removed from female rats (185–188 g.) under deep thiopentone anaesthesia, and immediately frozen in liquid oxygen. The mean level was  $38.1 \pm 4.3$  (S.E.) mg./100 g. wet tissue with individual values varying between 27.9 and 58.0. Good recovery of synthetic phosphorylcholine was obtained when this was

added to a liver sample. No evidence could be obtained of any rapid disappearance of phosphorylcholine from post-mortem liver, 97% being recovered after 7 min. and more than 84% 18 min. after death.

*Distribution of phosphorylcholine in other tissues of the rat.* A number of other tissues from the rat were examined for the presence of phosphorylcholine. The tissues were removed as quickly as possible from the rats and frozen in liquid oxygen until analysed. Extracts from 1.0 g. of tissue were prepared by a method previously described (Dawson, 1955). Two-dimensional paper chromatograms (phenol-NH<sub>3</sub>; propanol-NH<sub>3</sub>) of the extracts were developed with and without a synthetic phosphorylcholine marker. These indicated that phosphorylcholine was also present in testes, kidney, spleen, small intestine and brain, and in trace amounts in the heart, rectus femoris muscle and blood (Table 1).

Table 1. *The distribution of phosphorylcholine in rat tissues*

Values are expressed as mg. per 100 g. wet wt.

Tissue	Chromatographic estimation; female rat, (198 g.)	Hydrolytic estimation; combined tissues 3 female rats (ca. 160 g. each)
Testis	76.1*	85.8†
Spleen	28.0	30.3
Intestine	27.8	22.6
Kidney	9.7	7.1
Brain	8.1	6.6
Muscle (rectus femoris)	1.4	Trace
Heart	1.4	Trace
Blood	1.1	None detected

\* Combined tissue from 3 male rats, 74–96 g.

† Combined tissue from 7 male rats, 73–96 g.

Further evidence for the presence of phosphorylcholine in these tissues was obtained by comparing the choline present in mildly hydrolysed extracts of the tissues (30 min., 2N-HCl, 100°) with strongly hydrolysed extracts (24 hr., 2N-HCl, 123°). The mild hydrolysis is sufficient to liberate choline from known acid-soluble choline-containing compounds (glycerylphosphorylcholine, acetylcholine, propionylcholine) but not phosphorylcholine, while the strong hydrolysis liberates practically all the choline contained in synthetic phosphorylcholine. The results confirmed the conclusions on phosphorylcholine distribution obtained from the chromatographic studies (Table 1). It was found necessary to combine the tissues from several animals for the purpose of the estimation, as although the alkaline precipitation of choline as the reineckate is more

specific than other methods of choline estimation it is relatively insensitive. The reineckate precipitates were examined by paper chromatography in water-saturated butanol (Engel *et al.* 1954), which showed that the extra reineckate precipitation occurring after strong acid hydrolysis of the tissue extracts consisted of choline reineckate. The quantitative results of these experiments are recorded in Table 1.

### DISCUSSION

The relatively mild conditions used for preparing aqueous extracts of the rat tissues leave little doubt that free phosphorylcholine occurs as an important constituent in the testes, liver, spleen, small intestine, kidney and brain of the rat. On the other hand, muscular tissue (heart and rectus femoris) and blood contain relatively little of this phosphate ester. The level found in rat liver is over 500 times greater than the yield of the substance isolated from ox liver by Inukai & Nakahara (1935).

Two ways are known by which phosphorylcholine can be formed enzymically; the action of lecithinase *C* on lecithin, and the direct phosphorylation of choline by adenosine triphosphate catalysed by choline phosphokinase. Lecithinase *C* has so far only been demonstrated in certain bacteria (Macfarlane & Knight, 1941), foetal pig cartilage (Levine & Follis, 1949) and recently in plant mitochondria (Goodwin & Waygood, 1954). On the other hand, choline phosphokinase occurs in mammalian liver, brain, intestinal mucosa and kidney (Wittenberg & Kornberg, 1953). It seems likely, therefore, that the appreciable quantities of phosphorylcholine occurring in rat tissues are formed through the activity of the latter enzyme.

The metabolic significance of phosphorylcholine in tissues is being investigated. Two possibilities suggest themselves: it may act as a store of acid-soluble choline, and it may also play a part in phospholipid synthesis (Kornberg & Pricer, 1952), although the work of Kennedy (1954) has shown that at least one pathway of lecithin synthesis occurring in liver does not require phosphorylcholine as an intermediate; in brain tissue phosphorylcholine can act to a limited extent as a pre-

cursor of acetylcholine (Kometiani, 1952) by a synthetic mechanism which is independent of adenosine triphosphate (Berry & Stotz, 1953).

### SUMMARY

1. Phosphorylcholine has been identified in aqueous extracts of rat liver, where it accounts for 9% of the water-soluble P.

2. Chromatographic and hydrolytic evidence indicates that the substance also occurs in appreciable quantities in rat testes, spleen, kidney, small intestine and brain, but only in trace amounts in heart, muscle and blood.

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