Phospholipins

1. PARTITION CHROMATOGRAPHY OF EGG-YOLK PHOSPHOLIPINS ON CELLULOSE

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Bevan, Gregory, Malkin & Poole (1951) have claimed that choline-containing phospholipins (phosphatidylcholine and sphingomyelin) can readily be separated from those containing ethanolamine and serine (phosphatidylethanolamine and phosphatidylserine) by partition chromatography on filter paper or on cellulose columns. Phospholipins containing amino groups were detected by reaction with ninhydrin and choline-containing phospholipins by reaction with phosphomolybdic acid followed by reduction with stannous chloride to molybdenum blue.

'Lecithin' obtained from egg yolk was found to give four slow-moving fractions (R_F 0·02, 0·05, 0·17 and 0·28) reacting with ninhydrin but not with phosphomolybdic acid, and a well separated fraction (R_F 0·90) reacting with phosphomolybdic acid. Synthetic saturated $\alpha\alpha'$- and $\alpha\beta$-phosphatidyl ethanolamines reacted with ninhydrin and had low R_F values, while $\alpha\beta$-dipalmitoyl phosphatidylcholine and hydrogenated lecithin of groundnut reacted with phosphomolybdic acid and gave R_F 0·97.

Similar results on a larger scale were obtained with cellulose columns, and it was concluded that this procedure provides an efficient means of isolating small samples of uncontaminated choline-containing phospholipins.

Applying these methods to egg-yolk phospholipins we have been unable to achieve any useful separation of the amino- and choline-containing phospholipins, but have identified the slow-moving ninhydrin-reacting substances as free amino-acids.

EXPERIMENTAL

Preparation of egg phospholipin. The yolks of forty-eight hen eggs were separated, freeze-dried and extracted with boiling methanol for 1·5 hr. After removal of the solvent and re-solution in a small volume of ether the phospholipins were precipitated with acetone at 4°. Solution in ether and precipitation with acetone were repeated four times, traces of insoluble material which separated when the ether solution was allowed to stand at 4° being discarded. The final residue was taken up in chloroform and held at $-30°$ *in vacuo.* (Found: N, 2·00; P, 3·95 %. Atomic ratio N/P = 1·12.)

Chromatographic procedures. Chromatography on paper was carried out as described by Bevan *et al.* (1951) except that a temperature of 20° was used in place of 18° and that the papers were sprayed with the reagents rather than dipped. This latter modification seemed advisable in view of the fact that the spots were appreciably soluble in the reagents. Butanol was generally employed as solvent for the ninhydrin reagent in place of ethanol and was more satisfactory for this purpose.

Cellulose columns were prepared from Whatman ashless tablets powdered to pass an 80 mesh/in. sieve. Fractions were collected by means of an automatic fraction cutter (Snow, 1952).

Analytical methods. Total N was determined by micro-Kjeldahl and P by the method of Allen (1940) after digestion with perchloric acid. Filter paper alone gave no blank P value, but initial values in P recovery experiments were determined in the presence of filter paper. Choline was determined by the method of Entenman, Taurog & Chaikoff (1944) and free amino N by the Van Slyke manometric method after hydrolysis of the phospholipin with acid and removal of the fatty acids.

Data using paper chromatography. When egg phospholipin in 10 % (w/v) solution in $CHCl_3$ was applied to paper and the chromatograms were developed, a marked ninhydrin reaction was obtained in the region of R_F 0·0–0·1, but fast-moving ninhydrin-reacting material was also present giving a strong spot at R_F 0·85 (0·8–0·9) with a faint trail extending from R_F 0·5. A strong phosphomolybdic reaction was obtained at R_F 0·85.

The five-times precipitated phospholipin used was then replaced by a crude total-lipid preparation from yolk or by a phospholipin precipitated once in the presence of $MgCl_2$. On running the solvent front off the paper, four slow-moving ninhydrin-reacting spots (R_F 0·01, 0·02, 0·08, 0·13) similar to those reported by Bevan *et al.* (1951) appeared, the remainder of the picture being as before.

Determination of the distribution of P on the chromatograms showed that there was no peak corresponding to the slow-moving ninhydrin-reacting substances, which therefore contained no P. A total of 97–99 % of the P applied to the paper could be recovered between R_F 0·4 and 1·0, with most of it between R_F 0·8 and 0·9 (Fig. 1*a*). Synthetic α-palmitoyl-α'-linoleoyl phosphatidylethanolamine behaved similarly (Fig. 1*b*), although in this case the small quantity of material available did not permit accurate determination of the total recovery in terms of the P applied to the paper. The presence of ethanolamine as well as of choline in the egg phospholipin recovered from the region of the peak at R_F 0·85 was confirmed by paper chromatography of the water-

soluble fraction of an acid hydrolysate (Levine & Chargaff, 1951).

Passage of the egg phospholipin in a water-ethanol-ether solvent through either an acidic or a basic ion-exchange column reduced the atomic N/P ratio from 1·12 to 1·00 and completely eliminated the slow-moving ninhydrin-reacting materials from the chromatogram without otherwise affecting the picture.

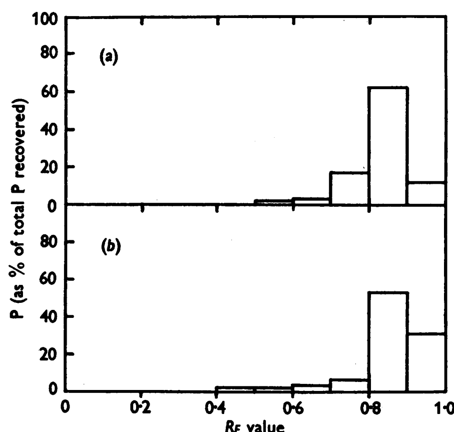

Fig. 1. Distribution of phosphorus on paper chromatograms. For conditions see text. *a*, Egg phospholipin; *b*, synthetic α-palmitoyl-α′-linoleoyl phosphatidylethanolamine.

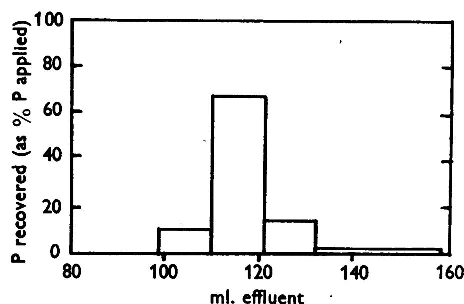

Fig. 2. Recovery of phosphorus of egg phospholipin from a cellulose column (size *B*). For conditions see text. Total recovery = 97·5 %.

Attempts to improve the performance of the method by using paper impregnated with quinol, ethylenediaminetetraacetic acid or phosphate-citrate buffers between pH 3 and 8 were unsuccessful.

Results using cellulose columns. Two columns of length 15 cm. and diam. 3·7 cm. (*A*) and length 50 cm. and diam. 2·2 cm. (*B*) were used, with collection of fractions of 10 and 12 ml., respectively. P determinations on these fractions showed that practically the whole of the phospholipin applied to the columns (92–98 %) appeared as a single band close behind the solvent front. Analysis of the material present in the peak from column *B* (Fig. 2) showed that after hydrolysis 21·5 % of its total N was present as amino-N, as compared with 26·1 % in the egg phospholipin applied to the column. Paper chromatography of the eluate showed that the slow-moving ninhydrin-reacting substances had been retained quantitatively by the column. When an approximate correction for the amino-N content of these substances (identified below as amino-acids) was applied to the initial value a figure of 21 % was obtained, indicating that all of the phospholipin amino N had, in fact, passed down the column. Confirmation of this absence of fractionation was obtained by separate analysis of the front and rear portions of the peak from column *B* which were found to possess similar amino-N contents.

Identification of the slow-moving ninhydrin-reacting substances. When the acidic or basic ion-exchange columns which had been used to absorb the substances responsible for the ninhydrin reactions at low R_F values were treated with alkali or acid respectively, fractions giving a strong ninhydrin reaction were obtained. These were washed with ether to remove contaminating phospholipins. Emulsification prevented quantitative recovery of the non-phospholipin N from the aqueous phase, but recoveries were about half of that required to bring the original N/P ratio of the crude phospholipin down from 1·12 to 1·00, and of this approximately 60 % was free amino N.

Two-dimensional paper chromatography in *n*-butanol-acetic acid-water and phenol-ammonia-water at 20° gave correct R_F values and no separation from admixed authentic samples in the cases of serine, glycine, threonine, alanine, lysine, arginine, proline, tyrosine, valine, leucine and phenylalanine. A further substance running at R_F 0·09 in *n*-butanol-acetic acid-water (40:10:50 by vol.) and at R_F 0·37 in phenol (saturated aqueous solution + 1 %, w/v, NH_3) and giving a clear blue coloration with ninhydrin was not identified.

Presence of the amino-acids in fresh egg yolk. The presence of free amino-acids in fresh egg yolk was confirmed by agitating the yolk with ether-ethanol-water (20:30:50 by vol.), centrifuging down the insoluble material and passing the supernatant fluid through a column of sulphonated polystyrene. After washing and displacing with NaOH a solution was obtained in which the amino-acids listed above were again identified. No attempt was made to search exhaustively for other amino-acids which may have been present.

DISCUSSION

The results obtained in the present work differ from those of Bevan *et al.* (1951) in several respects. In no case has slow-moving ninhydrin-reacting material (R_F < 0·5) been obtained from paper chromatograms of phospholipin preparations which have been freed from contaminating amino-acids. On the other hand, fast-moving ninhydrin-reacting material believed to be mainly phosphatidylethanolamine has been found to travel with the phosphatidylcholine and to resist separation from it on both paper and cellulose columns. The failure of Bevan *et al.* to detect this material may well have been due to their practice of dipping the papers in an ethanol solution of ninhydrin. We find that a spot, whether of egg phospholipin or of a synthetic phosphatidylethanolamine, which gives a strong mauve reaction on spraying with ninhydrin in butanol gives a weaker brownish spot on spraying with ninhydrin in

ethanol, while after immersion for a few seconds in the ethanol solution the colour is very faint or absent. Since completion of this work we have found that the ninhydrin reagent used for the estimation of amino-acids (Moore & Stein, 1948), which contains a buffer in methyl cellosolve solution, is much more satisfactory than the butanol solution, and its use is at present being explored as the basis of a method for the estimation of unhydrolysed amino-containing phospholipins. With this reagent the synthetic α-palmitoyl-α′-linoleoyl phosphatidylethanolamine gave a well defined spot at R_F 0·92 (0·87–0·96). The synthetic kephalin also reacted with the phosphomolybdic reagent used by Bevan *et al.* (1951) for the detection of choline-containing phospholipins, showing that the reagent is not specific for these substances. Lack of response to the phosphomolybdic test and, above all, the absence of any detectable P content shows that the slow-moving ninhydrin-reacting materials are not amino-containing phospholipins.

The presence of water-soluble lipid-insoluble nitrogenous substances, particularly urea or amino-acids, in phospholipins which give a clear solution in petroleum ether, chloroform or ether has been reported previously (Wittcoff, 1951). It is probable that the atomic N/P ratios greater than 1·00 which are commonly reported for phospholipin preparations are often due to this. Even the highly purified egg lecithins used by Pangborn (1941, 1951) gave ratios of 1·05, 1·01 and 1·06, respectively. The use of ion-exchange resins in combination with a suitable solvent or passage through a cellulose column (preferable if recovery of the amino-acids is not required) will remove these substances and offers an alternative to procedures involving washing with water or dialysis which are liable to be troublesome with phospholipins.

Very little information relating to the presence of free amino-acids in egg yolk appears to have been published. Ninhydrin-reacting substances presumed to be amino-acids have been detected by chromatography (La Cour & Drew, 1947), and tyrosine (Bracaloni, 1940) and glutamine (Archibald, 1944) have been identified. Independent investigations by Hawthorne in this laboratory, however, have also established the presence of at least fourteen different free amino-acids in egg yolk (Hawthorne, 1952).

SUMMARY

1. The separation reported by Bevan *et al.* (1951) of phospholipins containing ethanolamine and choline by partition chromatography on paper or on cellulose columns could not be confirmed. These methods effectively separated contaminating amino-acids which had been carried by the phospholipins into clear ether or chloroform solution.

2. Removal of these amino-acids by cellulose or by ion-exchange resins reduced the atomic N/P ratio of crude egg phospholipins from 1·12 to 1·00.

3. Among the free amino-acids present in the crude phospholipins and in the fresh yolk, serine, glycine, threonine, alanine, lysine, arginine, proline, tyrosine, valine, leucine and phenylalanine have been identified.

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