SUMMARY

1. 1-Fluoro-2:4-dinitrobenzene was shown to react in the presence of triethylamine with the free amino groups of phospholipids dissolved in benzene.

2. This reaction serves as the basis of a method for the quantitative estimation of the free amino groups in the phospholipids. The method is considered to be more reliable than the ninhydrin method in that the reagent is stable, the blank negligible and the need for a standard is eliminated. As little as 0·02 μmole can be estimated, and 0·1–2·5 μmoles represents the usual range.

3. Lecithin and other lipids do not interfere but with low concentrations of kephalin special precautions are necessary.

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Studies on Phospholipids

2. 2:4-DINITROPHENYLKEPHALINS

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The separation of kephalin* from lecithin can be accomplished by solvent fractionation, countercurrent distribution or by chromatography, but clear-cut separations are not easily obtained and the procedures are not suitable for use as analytical methods. Recently, Lea, Rhodes & Stoll (1955) have had some success with a chromatographic procedure applied to egg-yolk phospholipids.

Methods for separating kephalin and lecithin depend on differences in solubility or on the firmness with which the phospholipids are adsorbed on to certain materials. These differences in properties in turn depend upon the ionizable groups present in the phospholipids, and it occurred to the present authors that if it were possible to convert kephalin into a non-ionizable molecule, without altering the lecithin, the separation of the two should be readily effected.

* Throughout this paper kephalin is taken to mean a phospholipid containing a free amino group.

The reaction of 1-fluoro-2:4-dinitrobenzene (FDNB) with the free amino groups (Wheeldon & Collins, 1957) to form the non-ionized dinitrophenyl (DNP) groups is suitable, and these products possess the further advantage of being coloured, thus facilitating chromatography. Lipids treated with FDNB are still acidic substances and methylation with diazomethane to form the methyl esters will convert the kephalins into neutral compounds, and in addition Baer & Maurukas (1955) have shown that diazomethane is without action on lecithin. Therefore a mixture of lecithin and of kephalin (phosphatidylethanolamine, phosphatidylserine and the corresponding acetal phospholipids), treated successively with FDNB and diazomethane, should be readily separated, as the methylated DNP-kephalin would be a neutral non-ionizable substance while lecithin would still be fully ionized. The present paper describes the development of this idea.

EXPERIMENTAL

Materials

Rat-liver lipids and ox-brain kephalin fractions were obtained as described by Wheeldon & Collins (1957).

Hen egg-yolk phospholipids. The yolks of 10 eggs were disintegrated in a Waring Blendor in 2 l. of acetone and the mixture was filtered. The filtrate was discarded and the residue, after washing with acetone, was extracted with 500 ml. of $CHCl_3$–ethanol (1:9, v/v) and then three times with 200 ml. of ethanol. The extracts were combined, concentrated *in vacuo* to about 200 ml., filtered and made to 1 l. with acetone. The acetone-insoluble material (fraction *A*) was filtered off, suspended in a small volume of ethanol and reprecipitated by the further addition of acetone. The acetone-soluble material (fraction *B*) in the supernatant after the precipitation of fraction *A* was concentrated *in vacuo* and more acetone added to give a precipitate. Fraction *A* was sparingly soluble and fraction *B* was freely soluble in ethanol.

To remove the lecithin from these preparations, treatment with cadmium chloride was carried out: fraction *A* was dissolved in diethyl ether–ethanol (1:1, v/v) and fraction *B* was dissolved in ethanol; to each solution was added a saturated solution of cadmium chloride in methanol until no further precipitate was formed. The precipitate from fraction *A* was discarded, as it formed a colloidal solution with diethyl ether, but the precipitate from fraction *B* was washed once with diethyl ether and the washings were added to the original supernatant. The two solutions were concentrated *in vacuo*, more diethyl ether was added and the solutions were washed twice with 0·1 N-HCl and once with water, to remove cadmium chloride. The solvent was distilled off and the lipid was weighed and diluted to volume in light petroleum. Fraction *A* (3·2 g.) contained 3·72% of P, 1·65% of N (atomic ratio N:P = 0·98), 0·18% of choline N and 0·99% of amino N. Fraction *B* (6·2 g.) contained 3·52% of P, 1·56% of N (atomic ratio N:P = 0·98), 0·87% of choline N and 0·37% of amino N.

The lecithin from egg-yolk phospholipids was precipitated with cadmium chloride (as described above) and the cadmium chloride–lecithin complex was washed ten times with diethyl ether. This preparation of lecithin contained 3·51% of P, 1·51% of N and 0·1% of amino N, and the atomic ratio N:P was 0·95.

Rat-liver lipids. These were labelled with ^{32}P and were obtained as follows: 4 g. of rat liver was disintegrated in 0·25 M-sucrose with a Potter–Elvehjem homogenizer and diluted to 50 ml. The final mixture contained 0·25 M-sucrose, 5 mM-$MgCl_2$, 4·5 mM-glycerol, 1·7 mM-2-amino-2-hydroxymethylpropane-1:3-diol buffer (pH 7·4), 33 mM-sodium succinate, 2 mM-adenosine monophosphate and 0·5 mM-sodium phosphate (pH 7·4) containing 200 μC as $NaH_2{}^{32}PO_4$. This mixture was shaken in air at room temperature for 2 hr., dried in the frozen state and the lipid extracted from the dry material by the procedure described by Wheeldon & Collins (1957).

Synthetic materials. *N*-2:4-Dinitrophenyl-1-amino-octadecane was prepared by condensing 1-amino-octadecane and FDNB by the procedure described by Wheeldon & Collins (1957). The product (m.p. 45–46°, uncorr.) was crystallized from light petroleum. The absorption spectrum had maxima at 328 mμ (ϵ = 18 600, light petroleum), 330 mμ (ϵ = 18 600, *cyclo*hexane), 347 mμ (ϵ = 18 600, chloroform) and 347 mμ (ϵ = 17 400, ethanol) (Found: C, 66·3; H, 9·4; O, 14·8; N, 9·8. $C_{24}H_{41}O_4N_3$ requires C, 66·2; H, 9·4; O, 14·7; N, 9·7%).

A sample of dimyristoyl-L-α-kephalin was kindly supplied by Dr E. Baer (Found: P, 4·13; N, 2·2; theory requires P, 4·88; N, 2·2%).

N-2:4-Dinitrophenyl-2-aminoethyl octadecyl hydrogen phosphate (86% pure based on the P content) was prepared and chromatographed on Hyflo Super-Cel as described by Wheeldon & Collins (1957). The fraction used in the present investigation had an absorption maximum at 345 mμ and ϵ = 16 700, light petroleum, where ϵ was computed in terms of the atomic concentration of phosphorus (Found: P, 4·78. $C_{26}H_{46}O_6N_3P$ requires P, 5·55%).

Diazomethane. This was prepared from nitrosomethylurea as described by Arndt (1943). Before use the solution of diazomethane in diethyl ether was dried over KOH pellets for at least 1 hr. The concentration of diazomethane in this solution was determined from the absorption at 410 mμ, where the molecular extinction coefficient is equal to 3 (Brinton & Volman, 1951).

The sample of dinitrophenylated lipid or *N*-2:4-dinitrophenyl-2-aminoethyl octadecyl hydrogen phosphate was dissolved in diethyl ether containing a tenfold excess of diazomethane, calculated as μmoles of diazomethane/μg.atom of P. The mixture was left at 20° for 1 hr. and the solvent and excess of diazomethane were removed *in vacuo*.

Methods

The methods used for the determination of N, total P, plasmalogen and choline and for the preparation of DNP-lipids have been previously described (Wheeldon & Collins, 1957).

Phosphorus and radioactivity measurements. Radioactivities were measured as described by Ennor & Rosenberg (1952), except when the specific radioactivity (counts/min./μg.atom of P) was very low. In such cases the radioactivity (counts/min.) of the total lipid extract was measured (in 10 ml. of solvent) in a liquid counter, the counts being corrected for the difference in counting efficiencies between the solvent used and *iso*butanol.

Washing the lipid with 0·1 N-HCl did not completely remove contaminating radioactivity and it was necessary to show that the specific radioactivities obtained were not influenced by the radioactivity of non-lipid impurities. A solution containing both inorganic ^{32}P and acid-soluble phosphate esters containing ^{32}P, but free of lipid P, was prepared as follows. Rat liver (1 g.) was disintegrated in a Potter–Elvehjem blendor with 0·9% NaCl containing about 25 μC of $NaH_2{}^{32}PO_4$ (approx. 0·01 M, pH 7·4); this mixture was kept for 1 hr. at 20° and the protein and lipid were precipitated by 2 ml. of ice-cold 5 N-$HClO_4$. The precipitate was removed by centrifuging, the supernatant was neutralized with 2 ml. of 5 N-KOH at 0° and the precipitate of $KClO_4$ was removed by centrifuging. The clear supernatant was diluted to 5 ml., and 3 ml., containing $2·3 \times 10^6$ counts/min. of inorganic ^{32}P and $0·7 \times 10^6$ counts/min. of esterified ^{32}P, was emulsified with a solution of rat-liver lipids containing 120 μg.atoms of P in $CHCl_3$–ethanol (3:1, v/v). The mixture was taken to dryness and the residue was suspended in diethyl ether. This solution, which contained $3·1 \times 10^6$ counts/min., was washed with 0·1 N-HCl and yielded a solution containing 4560 counts/min. and 119 μg.-atoms of P. The lipids were then treated successively with

FDNB and diazomethane and chromatographed on Hyflo Super-Cel (as described below). No radioactivity was eluted from the column by light petroleum, $CHCl_3$ or ethanol–$CHCl_3$ (1:9, v/v), corresponding respectively to the neutral lipid, '328 kephalin' fraction and the '345 kephalin' fraction. The ethanol eluate, corresponding to the choline phospholipids, contained 137 counts/min., which was equivalent to 2 counts/min./μg.atom of P.

Ethanolamine and serine. A sample of DNP-lipid (5–20 mg.) was hydrolysed by heating with 2 ml. of saturated $Ba(OH)_2$ and 2 ml. of ethanol at 100° for 6 hr. The mixture was acidified with 10 N-HCl and the fatty acids were extracted with diethyl ether. The aqueous phase was taken to dryness and bases were extracted from the residue with ethanol. The ethanolic solution was removed by decantation and evaporated to dryness; the residue was dissolved in the minimum volume of 0·1 N-HCl and spotted on paper for chromatography. The solvent system used was phenol saturated with water and known amounts of serine and ethanolamine were used as markers in adjacent sections of the paper. The bases were located, after chromatography, by spraying the paper with 0·5 % (w/v) ninhydrin in acetone. This procedure enabled an approximate estimate by visual comparison of the relative proportions of ethanolamine and serine to be made in a small sample of lipid.

Fatty acids. The determination of carboxylic esters by the hydroxamate method (Shapiro, 1953) could not be applied to DNP-lipids and the following procedure has been developed (cf. Popják & Tietz, 1954). The DNP-lipid (about 5 mg.) was hydrolysed by boiling with 5 ml. of 0·1 N-ethanolic KOH for 10 min. The hydrolysate was acidified by the addition of 0·05 ml. of 10 N-HCl and the mixture was taken to dryness. The fatty acids were dissolved in diethyl ether, which was decanted from the solid residue; this extraction was repeated twice.

In order to remove the DNP materials, which interfered with the subsequent titration, the ethereal solution of fatty acids was passed through a column (5 cm. × 1 cm.) of silica gel, which had previously been washed with ethanol and then with diethyl ether. The eluate containing the fatty acids was taken to dryness and the residue was dissolved in 5 ml. of 90 % (v/v) aqueous ethanol. A 1 ml. sample was then titrated with 0·1 N-KOH in a slow stream of nitrogen to exclude carbon dioxide and to facilitate mixing. Bromothymol blue was used as the indicator. The procedure was standardized for each batch of samples with known amounts of oleic acid.

RESULTS

Reaction of dinitrophenyl-lipids with diazomethane

When a sample of DNP-2-aminoethyl octadecyl hydrogen phosphate (an analogue of DNP-phosphatidylethanolamine), which was sparingly soluble in light petroleum, was treated with diazomethane the absorption maximum changed from 345 to 328 mμ (both measured in light petroleum, see Fig. 1) and the product was freely soluble in light petroleum. *N*-2:4-Dinitrophenyl-1-amino-octadecane with no acidic group has $\lambda_{max.}$ at 328 mμ (light petroleum) and therefore the change in the absorption maximum from 345 to 328 mμ was presumably due to the methylation of the acidic group. When a DNP-lipid was methylated the change was not so marked (see Table 1 and Fig. 1) and further treatment with diazomethane had no effect: the implications of this are discussed below.

Fractionation of methylated dinitrophenyl-lipids

It was expected that chromatography or countercurrent distribution would readily separate the un-ionized methyl esters of DNP-kephalin from the ionized lecithin and that the absorption maximum of the methylated DNP-kephalin would be at 328 mμ in light petroleum. In fact, three DNP derivatives were obtained and these will be referred to as the 328, 345 and 355 kephalins, where the figures refer to the value of the absorption maxima in light petroleum.

Chromatography on Hyflo Super-Cel. When phospholipids were chromatographed on Hyflo Super-Cel no phosphorus-containing material was eluted until ethanol was applied to the column, and therefore it was expected that the methylated DNP-kephalins would be eluted by a less polar solvent.

For each milligram of methylated DNP-lipid (rat liver or ox brain) 0·5–1 g. of adsorbent was used; the sample (usually between 1 and 100 mg.) was dissolved in light petroleum and applied to the column. Elution with this solvent was continued until the yellow band of DNP-kephalin ceased to move; the neutral lipid and much of the unsaponifiable material had been removed at this stage. Elution with benzene (or benzene–$CHCl_3$, 1:1 v/v) removed a yellow band from the column, which was expected to account for all of the DNP-kephalin and which in fact contained only the 328 kephalin. Prolonged elution with $CHCl_3$ failed to remove any more material from the column, but ethanol–$CHCl_3$ (1:9, v/v) eluted a second yellow band, which contained the 345 kephalin. Compared with the 328 kephalin fraction this band was more diffuse, with considerable trailing, and quantitative elution was difficult without also eluting the lecithin. Ethanol or aqueous ethanol (90 %, v/v) eluted the remainder of the lipids. When egg-yolk lipids, after treatment with FDNB and diazomethane, were chromatographed in the same way three yellow bands were formed on the column. The two lower ones contained the 328 and 345 kephalins but the third one, which was eluted with ethanol–$CHCl_3$ (2:8, v/v), contained the 355 kephalin, which came off the column just in front of the lecithin.

The recovery of DNP groups on chromatography could be tested by comparing the sum of the absorptions of the fractions with that of the unchromatographed material at a given wavelength. While the recoveries could be as high as 98 %, they were sometimes lower; in one experiment the recovery was 94 % at 400 mμ, 89 % at 350 mμ and 87 % at 330 mμ. With the egg-yolk phospholipids,

where vitamin A was absent, the recovery could be more easily investigated. The sum of the absorption curves of the 328 and 345 kephalin fractions was subtracted from the absorption spectrum of the unchromatographed material: the resulting 'difference' spectrum agreed in position of $\lambda_{max.}$ and general shape with the absorption spectrum of the 355 kephalin fraction. The amount of the 355 kephalin isolated by chromatography was always less than the amount computed from the 'difference' spectrum obtained as described above (see Table 4).

The absorption spectra of the various fractions obtained by chromatography of the methylated DNP-phospholipids prepared from egg-yolk lipid are shown in Fig. 2. These results showed that even when the kephalin content of a sample was less than 7 %, the 328, 345 and 355 fractions could be readily separated from one another. This was indicated both by the fact that on chromatography distinct yellow bands were formed on the column which

Table 1. *Change in the absorption spectra of dinitrophenyl-lipids on treatment with diazomethane*

The absorption spectrum of a DNP-amine is included for comparison. The value of $\epsilon_{max.}$ for the synthetic products is computed in terms of the atomic concentration of P. The solvent was light petroleum.

	$\lambda_{max.}$	$\epsilon_{max.}$	$\frac{E \text{ at } 350\ m\mu}{E \text{ at } 330\ m\mu}$
N-2:4-Dinitrophenyl-1-amino-octadecane	328	18 600	0·44
DNP-2-aminoethyl octadecyl hydrogen phosphate	345	16 700	1·13
Methyl ester of DNP-2-aminoethyl octadecyl hydrogen phosphate	328	17 700	0·42
DNP-lipid (rat liver)	345	—	1·21
Methylated DNP-lipid (rat liver)	332	—	0·75

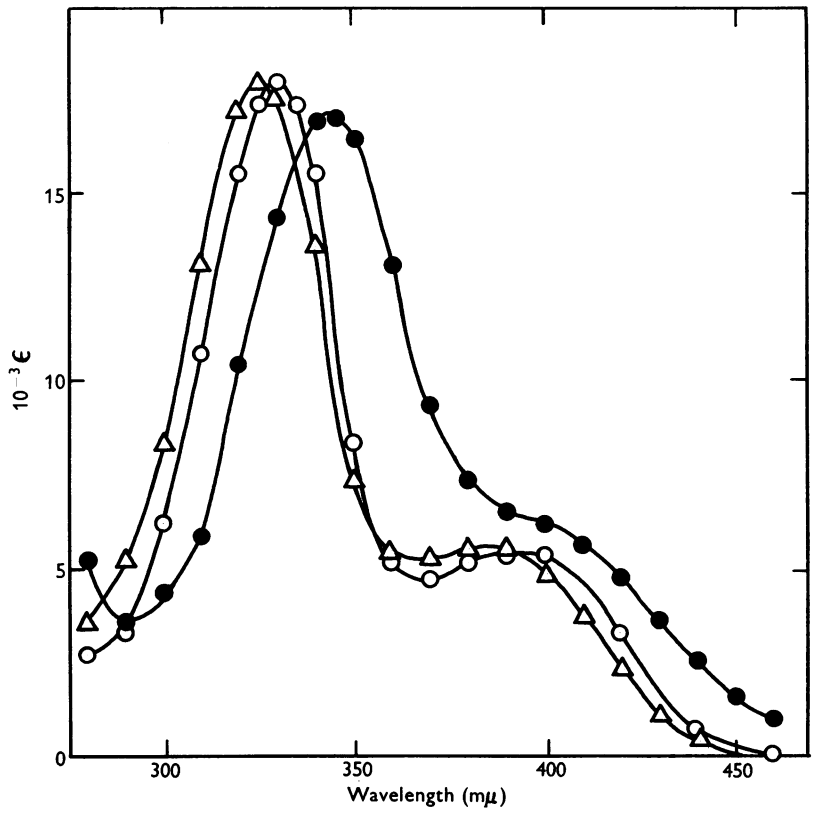

Fig. 1. Absorption spectra of DNP compounds. △, DNP-aminoethyl octadecyl methyl phosphate (86% pure; ϵ was computed in terms of the concentration of P); ●, DNP-aminoethyl octadecyl hydrogen phosphate (86% pure; ϵ was computed in terms of the concentration of P); ○, methylated DNP-lipid from rat-liver lipids insoluble in acetone, with the extinction at the maximum put equal to 17 500 to facilitate comparison with the other spectra. Solvent used was light petroleum.

were eluted by characteristic solvents, and by the fact that the absorption spectra of these fractions were clearly differentiated from one another. With 328 kephalin isolated after chromatography the ratio DNP groups:P was unity even when the lipid sample contained only 1·3 % of this material.

Countercurrent distribution. As an alternative method of fractionation, countercurrent distribution was investigated. The solvent system used was obtained by equilibrating 100 vol. of light petroleum (b.p. 40–60°), 85 vol. of ethanol and 15 vol. of water. The partition coefficients of the 328 and 345 kephalins between the two phases were respectively 0·5 and 8, and on the basis of these figures a simplified system of countercurrent distribution was developed. The whole process described below could be completed in 30 min. and required no special apparatus other than four separating funnels.

The sample of methylated DNP-lipid was dissolved in the light-petroleum-rich phase; the concentration of lipid should not exceed 2 % (w/v). An equal volume of the other phase was added and after three distributions in which the lower phase was transferred, the upper (non-polar) phase in funnel 1 contained 328 material free of the 345 kephalin. A fourth funnel was introduced at the end of the line to receive the bottom phase from funnel 3; the upper phase from funnel 1 was removed and stored, and funnel 1, now empty, was removed. Funnel 2 now became effectively funnel 1 for a second distribution, from which another fraction of the 328 kephalin was obtained. A third distribution was performed in the same way, yielding a further fraction of the 328 kephalin, and in addition the bottom phase of the end funnel contained 345 material free of 328, which was removed and stored. The complete procedure was

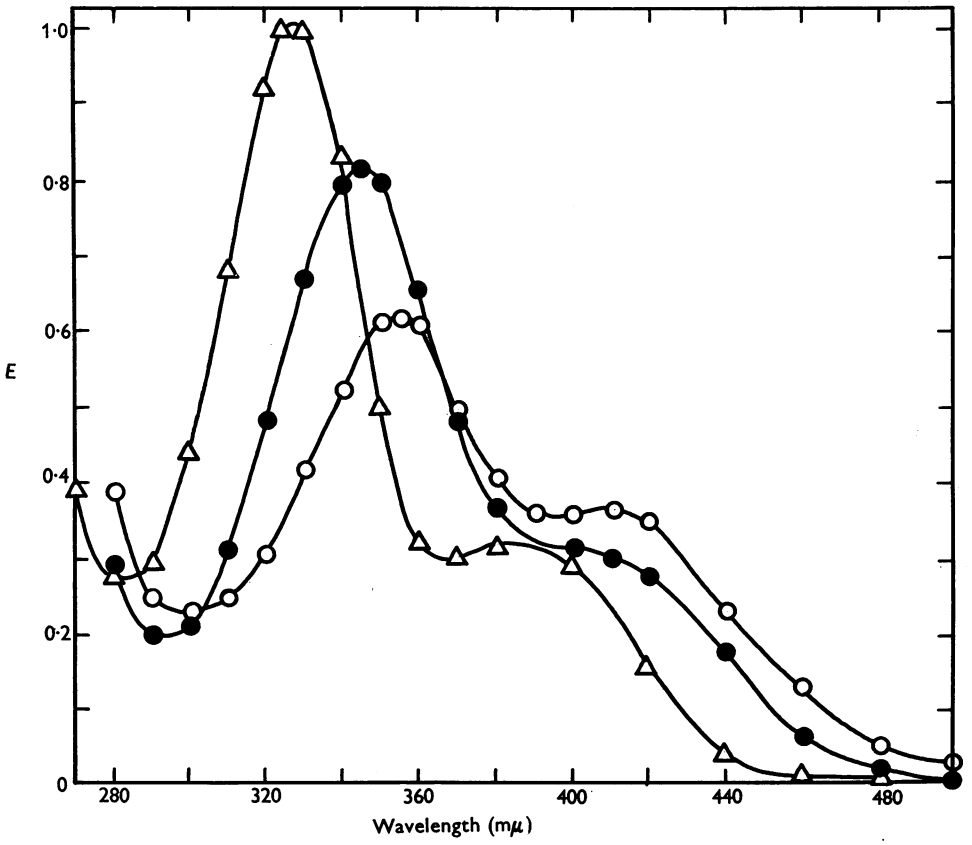

Fig. 2. Ethanol-soluble phospholipids from egg yolk. Ethanol-soluble phospholipids (63 mg.), after treatment with FDNB and diazomethane, were chromatographed on 30 g. of Hyflo Super-Cel. The 328 kephalin (△) was eluted with benzene and had the atomic ratio DNP N:P = 0·95. 345 Kephalin (●) was eluted with benzene–chloroform and chloroform, and had the atomic ratio DNP N:P = 0·55. 355 Kephalin (○) was eluted with ethanol–chloroform (2:8, v/v). In the last case correction has been made for loss of extinction on chromatography. All absorption curves are shown as if measured in the same volume of light petroleum.

repeated on the material remaining, yielding a further three 328 fractions and one 345 fraction. The resultant six 328 fractions were combined, as also were the two 345 fractions. The countercurrent distribution had thus divided the fat into two fractions, namely, a non-polar fraction containing the 328 kephalin together with the neutral lipid and a small amount of unidentified phospholipid, and a polar fraction containing the 345 kephalin and lecithin. With egg-yolk phospholipids the polar fraction also contained the DNP-kephalin with absorption maximum at 355 mμ. In the separation obtained by countercurrent distribution the recovery of extinction was 100 ± 3 %, and this method of analysis gave slightly higher values for the 345 kephalin compared with chromatography. Table 2 compares the results with the two methods on the same sample of rat-liver lipid. The chromatographic procedure was indispensable if purified fractions were to be obtained, in contrast with the other method which gave two fractions only, but with quantitative recoveries.

328 *Kephalin*

Countercurrent distribution separated the 328 kephalin completely from the 345 kephalin and the choline-containing phospholipids, yet the ratio DNP groups: atoms of P was always less than unity. However, chromatography removed fractions containing P with no associated DNP groups (see Table 5); the 328 kephalin was thus rechromatographed until the ratio of the extinction at 328 mμ to the P content was constant. The mean value of ϵ (328 mμ) in light petroleum, computed in terms of the atomic concentration of P, was $17\,800 \pm 360$ (32 samples, fiducial limits for $P = 0{\cdot}95$). The methylated DNP derivatives of octadecyl 2-aminoethyl hydrogen phosphate and of dimyristoyl-L-α-kephalin were chromatographed and the fractions isolated, with $\lambda_{max.}$ at 328 mμ (see Fig. 1), which comprised over 94 % of the DNP groups, had ϵ (328 mμ) equal to 17 700 and 17 900 respectively, these figures again being calculated in terms of the P content. Finally, as the value of ϵ (328 mμ) for *N*-2:4-dinitrophenyl-1-amino-octadecane was 18 600, a value of ϵ (328 mμ) near to 18 000 is clearly correct for this chromophore. Thus it can be concluded that in the 328 kephalin, where ϵ (328 mμ, based on P content) is 18 000, the atomic ratio of DNP groups: P is unity.

It was expected that the 328 kephalin would correspond to phosphatidylethanolamine and phosphatidylserine and would thus contain fatty acids, amino N (as ethanolamine and serine) and P in the molar proportions 2:1:1. In agreement with this expectation the only bases present were ethanolamine and serine, except with egg-yolk phospholipids, in which serine was absent. Fatty acids and fatty aldehydes (1–10 moles %) were also present. As a rule the chromatographic separations were on too small a scale to permit the fraction to be weighed, but in one experiment with egg-yolk phospholipids the main 328 kephalin fraction had the following composition: 34 μg.atoms of P, 72 μmoles of fatty acids and 38 μmoles of DNP groups in 33 mg. The methyl ester of *N*-2:4-dinitrophenyldioleylphosphatidylethanolamine would contain, in 33 mg., 36 μg.atoms of P, 72 μmoles of fatty acids and 36 μmoles of DNP groups. Less than 1 μmole of plasmalogen was present. Thus it can be concluded that the 328 kephalin consists of the methyl esters of DNP-phosphatidylethanolamine and DNP-phosphatidylserine together with small amounts of the corresponding acetal compounds.

345 *Kephalin*

The polar kephalin fraction obtained from rat-liver lipids by countercurrent distribution contained approximately 5·5 moles of choline-containing phospholipids to 1 mole of DNP-kephalin with $\lambda_{max.}$ at 345 mμ. The 345 kephalin was not completely stable on chromatography, as indicated both by the extensive 'tailing' of the band and by the low recovery of DNP groups. If all the yellow material on the column was collected some choline-containing phospholipids were included in the final eluates of 345 kephalin. Such fractions containing all the 345 kephalin might contain up to 20 moles % of choline. If the material was rechromatographed or if only the main portion of the 345 kephalin was collected then no choline was present. Ethanolamine and serine (except in the egg-yolk phospholipids, which lack serine) were both present, as also were fatty acids and fatty aldehydes. The ratio DNP groups: atoms of P can be assessed from the value of the molecular extinction coefficient (based on the P content) of 8920 ± 330 (measured at 345 mμ in light petroleum; mean of nine preparations). If the ratio DNP groups: atoms of P were 0·5 then the value of $\epsilon_{max.}$ for the chromophore would be $17\,840 \pm 660$. This compares with 16 600

Table 2. *Analysis of methylated dinitrophenyl-lipids from a rat liver*

A comparison of the results obtained by using the chromatographic procedure and by using the countercurrent distribution method. All figures are in μmoles of kephalin/g. of wet tissue.

DNP-lipid fraction with $\lambda_{max.}$ at	Chromatography (1)	Chromatography (2)	Countercurrent distribution
328 mμ	6·2	6·3	6·0
345 mμ	3·2	3·3	4·1
Total	9·4	9·6	10·1

for DNP-lipids and 17 800 for the 328 kephalin. Thus it can be concluded that within the experimental error the ratio DNP groups:P is 0·5.

355 *Kephalin*

Material from the egg-yolk phospholipids with $\lambda_{max.}$ at 355 mμ (see Fig. 2) after methylation has not been found elsewhere and was not present in hen-liver lipids. The spectrum resembles that of dinitrophenol but differs from it in that the ratio of the extinctions at 400 to 355 mμ is 0·8 for dinitrophenol and 0·6 for the DNP-kephalin fraction, and that it is unaffected by treatment with acid. This material is found chiefly in the ethanol-soluble fraction of the kephalin and is a minor component. The value of the molecular extinction coefficient (based on the P content) varied from 2150 to 8070.

Kephalin content of rat-liver lipids

Table 3 shows the proportion of 328 and 345 kephalins in rat-liver lipids.

Further examination of the dinitrophenylkephalins

Although the 328 kephalin was easily separated from lecithin, the usefulness of this separation would be limited if the remainder of the DNP-kephalin was either the result of incomplete methylation or an artifact. The following evidence indicates quite clearly that neither of these alternatives is correct.

Reaction with diazomethane. The DNP derivatives of dimyristoyl-L-α-kephalin and of 2-aminoethyl octadecyl hydrogen phosphate when treated with diazomethane yielded 94 and 100 % respectively of their DNP groups in the form of 328 kephalin. Furthermore, analysis of the 328 kephalin showed it to contain fatty acids, base and phosphorus in the proportions expected for kephalin; thus it followed that it was the 345 and 355 kephalins which required further investigation.

The synthetic compounds gave an almost quantitative yield of the 328 kephalin, showing that methylation was complete and making it unlikely that samples of DNP-lipids were incompletely methylated owing to insufficient excess of diazomethane. This possibility was none the less tested in experiments with DNP-lipids from rat liver, in which the excess of diazomethane was increased from 10 to 100-fold and in which the reaction time was increased to 12 hr., but there was no alteration in the yield of the 345 kephalin. Further, the 345 kephalin, isolated after countercurrent distribution, was unaffected by further treatment with diazomethane.

If cations or basic groups were present then some of the acid groups might be unable to react with diazomethane (cf. lecithin). As the lipid had been washed with acid before the reaction with FDNB, cations would be absent, and excess of triethylamine did not alter the yield of the 345 kephalin.

Diazomethane is an extremely reactive substance, and the possibility existed that in addition to esterifying acid groups side reactions might be occurring. However, the yield of 345 kephalin was unaltered, even when the methylation was carried out at $-60°$.

Fractionation of lipids with ethanol. Three forms of methylated DNP-kephalin can be distinguished, and one of these, the 328 kephalin, corresponds to a mixture of phosphatidylethanolamine and phosphatidylserine. The other forms of DNP-kephalin, if not artifacts, must correspond to hitherto unrecognized compounds. It has long been accepted that preparations of kephalin seldom analyse correctly for a mixture of phosphatidylethanolamine and phosphatidylserine, and the present authors thought it possible that this might be due to the presence of materials from which the 345 and 355 kephalins were derived.

Kephalin is usually regarded as ethanol-insoluble, but it has long been known that part of the kephalin is in fact soluble (Maclean, 1915; Rudy & Page, 1930). With Folch's (1942) discovery of phosphatidylserine it was generally assumed that this was the ethanol-insoluble material, and that phosphatidylethanolamine represented the ethanol-soluble fraction. However, egg-yolk phospholipids, in which serine is absent (Chargaff, Ziff & Rittenberg, 1942), still contain kephalin fractions which are sparingly soluble in ethanol. The present authors thought it possible that these differences in solubility in ethanol might be due to the presence of

Table 3. *Phospholipid composition of rat liver*

From results presented in the present paper and from other workers. All figures are as % of the total lipid P.

		Present result	Artom (1943)	Taylor & McKibbin (1953)
Choline-containing phospholipids	Lecithin	66±6	52	—
	Sphingomyelin		8	—
Kephalin	328 fraction	22±4	40	—
	345 fraction	12±3		
Inositol phospholipids (dog liver)		—	—	7

several kephalins corresponding to the three methylated DNP-kephalins.

Fraction III of the ox-brain kephalin (Wheeldon & Collins, 1957) represented material which was less soluble in ethanol than fraction V, and the proportions of the 345 kephalin were respectively 10 and 20% of the total DNP-kephalin. This difference, while not large, was an indication that the kephalins did differ in solubility in ethanol. Egg-yolk phospholipids, which were free of phosphatidylserine, were used in a further investigation; the fractionation with ethanol and cadmium chloride is described in the Experimental section. Table 4 summarizes the distribution of the three components of methylated DNP-kephalin found in egg-yolk phospholipids, and the absorption spectra of the fractions obtained by chromatography are shown in Fig. 2. It will be seen that the 328 kephalin corresponds to material which was relatively insoluble in ethanol, and the other two DNP-kephalins correspond to materials which made up the major portion of the kephalin which was soluble in ethanol and also of the small amount of kephalin remaining in the lecithin preparation.

These results show that the 328 kephalin corresponds to material which has a characteristic property of kephalin, namely ethanol insolubility, whereas the other types of DNP-kephalin represent anomalous forms.

Incorporation of ^{32}P *into the dinitrophenylkephalins.* If it can be shown that the different forms of DNP-kephalins incorporate ^{32}P at different rates then this would be further evidence that they are distinct forms of kephalin and that the 345 kephalin is not an artifact.

In experiments in which ^{32}P was incorporated *in vitro* into rat-liver lipids, the following precautions were taken to ensure that the three fractions obtained by chromatography were free of contaminating compounds containing ^{32}P. The values of the specific radioactivities of the 328 kephalin shown in Table 6 were measured on fractions in which the value of ϵ (328 mμ, calculated in terms of the gram-atomic concentration of P) was within 5% of 18 000, thus indicating that the atomic ratio N:P was equal to 1·00 within the experimental error. If several fractions of the 328 kephalin (with $N:P = 1{\cdot}00 \pm 0{\cdot}05$) were obtained on chromatography then the weighted mean (in respect to P) of the specific radioactivities was calculated. These considerations are illustrated in Table 5.

Table 4. *Analysis of phospholipid fractions from egg yolks*

Egg-yolk phospholipids, fractionated as described in the text into an ethanol-insoluble, an ethanol-soluble and a lecithin fraction, were treated with FDNB and subsequently with diazomethane. These materials were chromatographed on Hyflo Super-Cel. The figures represent μmoles of DNP groups as a percentage either of the total N or (in parentheses) of the total DNP groups in the sample, assuming $\epsilon_{max.}$ values of the three forms to be equal.

DNP-lipid fraction with $\lambda_{max.}$ at	Ethanol-insoluble (fraction *A*)	Ethanol-soluble (fraction *B*)	Lecithin
328 mμ	52 (87)	10 (42)	1·3 (19)
345 mμ	5 (8)	8 (33)	0·8 (11)
355 mμ (as isolated)	0 (0)	4 (17)	3·6 (52)
355 mμ (estimated by difference)	3 (5)	6 (25)	4·8 (72)
Total amino N	60 (100)	24 (100)	6·9 (100)

Table 5. *Rechromatography of a dinitrophenylkephalin fraction with* $\lambda_{max.}$ *at* 328 mμ

A dinitrophenylkephalin fraction into which ^{32}P had been incorporated was rechromatographed and the fractions were arbitrarily divided into a number of successive fractions. On each fraction the absorption spectrum, P content and radioactivity were measured. The specific radioactivity of the total lipid P was put equal to 100 and the relative specific radioactivity of each fraction computed. The weighted mean (in terms of P) of these fractions (3–7 inclusive), where ϵ (328 mμ calculated in terms of the P content) was not less than 17000, was equal to 14.

Fraction	P (μg.atoms)	$\lambda_{max.}$ (mμ)	ϵ (328 mμ)	Relative specific radioactivity
1	1·23	—	—	0
2	0·06	—	—	29
3	0·44	328	18 600	37
4	1·08	328	19 300	26
5	0·85	328	17 300	9
6	1·95	328	17 700	8
7	1·05	328	17 100	6
8	1·75	328	16 800	19
9	0·31	Inflexion at 328	10 000	60
10	0·10	—	—	36

Table 6. *Comparison of the incorporation of* ^{32}P *into the two forms of kephalin and into lecithin*

Homogenates of rat liver were incubated with $NaH_2{}^{32}PO_4$ (see text for details), the lipid was extracted and treated successively with FDNB and diazomethane. The material was then chromatographed and the specific radioactivities of the fractions were measured. The specific radioactivities of the total lipid P have been put equal to 100.

		Expt. no. 1	2	3	4
Methylated DNP-kephalin	With $\lambda_{max.}$ 328 mμ	14	2	2	60
	With $\lambda_{max.}$ 345 mμ	636	890	380	197
Choline phospholipids	Ethanol eluate	10	33	68	33
	By chromatography on MgO	<0·2	<2	—	—

In a series of 345 kephalin fractions obtained by chromatography there was a good correlation between the specific radioactivity and the atomic ratio of DNP groups to P. The specific radioactivity of the 345 kephalin was taken as the highest obtained in a chromatography (or rechromatography) in which ϵ (345 mμ) was not less than 6000.

The specific radioactivity of the phospholipids eluted by ethanol was taken as equivalent to that of the choline phospholipids, but a more accurate estimate was obtained by chromatography on magnesium oxide (Taurog, Entenman, Fries & Chaikoff, 1944), which serves to isolate the choline phospholipids.

Experiments in which a rat-liver homogenate was incubated *in vitro* with $NaH_2{}^{32}PO_4$ indicated that the uptake of ^{32}P into the two kephalin fractions was very dissimilar. The specific radioactivity of the 345 kephalin was from 3·3 to 450 times that of the 328 kephalin and from 5·6 to 280 times that of the ethanol eluate, which was itself always greater than that of the choline phospholipids (see Table 6).

DISCUSSION

Diazomethane, which has long been used to form methyl esters of acids, has recently been shown by Baer & Maurukas (1955) to cleave kephalin, with the formation of the dimethyl ester of phosphatidic acid. Lecithin, however, was unaffected and these authors concluded that a free amino group was necessary for 'diazomethol ysis'. The fraction of methylated DNP-kephalin with $\lambda_{max.}$ at 328 mμ obtained after chromatography contained fatty acids, phosphorus and DNP groups in the expected proportions and thus there was no cleavage. The DNP derivatives of the samples of dimyristoyl-L-α-kephalin and of 2-aminoethyl octadecyl hydrogen phosphate, which were insoluble in light petroleum, became freely soluble on treatment with diazomethane. On chromatography the DNP groups remained attached to phosphorus, as shown by analysis of the fractions. Thus it can be concluded that diazomethol ysis does not occur with the DNP-kephalins.

In non-polar solvents such as light petroleum the absorption maximum of *N*-2:4-dinitrophenyl-1-amino-octadecane is at 328 mμ. DNP-serine and DNP-ethanolamine in a mixture of diethyl ether and light petroleum had $\lambda_{max.}$ at 332 mμ. On the other hand, DNP-lipids and DNP-2-aminoethyl octadecyl hydrogen phosphate have $\lambda_{max.}$ at 345 mμ in light petroleum. In ethanol the absorption maxima in all these cases is at 345 mμ. The above facts can be harmonized if it is assumed that in the absence of hydrogen bonding the absorption maximum of the DNP chromophore is at 328 mμ and with hydrogen bonding it is at 345 mμ. In ethanol the hydrogen bonding would be with the solvent, whereas with the DNP-lipids and DNP-2-aminoethyl octadecyl hydrogen phosphate in light petroleum there would be intramolecular hydrogen bonding with the acid groups. This view is supported by the fact that after methylation of DNP-2-aminoethyl octadecyl hydrogen phosphate the absorption maximum is at 328 mμ in light petroleum. In the 345 kephalin it is thus possible that a group is present in the molecule which forms a hydrogen bond with the DNP group and which does not react with diazomethane.

The experiments with the egg-yolk phospholipid fractions showed that relatively more of the material giving rise to the 345 and 355 kephalins was soluble in ethanol than was the case with the remainder of the kephalin. This was an indication that the DNP-kephalin fractions corresponded to materials pre-existing in the native lipid.

The *in vitro* experiments in which ^{32}P was incorporated into the lipid resulted in very little uptake into the fractions which correspond to phosphatidylethanolamine, phosphatidylserine and lecithin, and ^{32}P was incorporated almost entirely into the phosphorus associated with the 345 kephalin. However, as the ratio DNP groups:P was 0·5, it could not be assumed that the radioactivity was exclusively on P atoms adjacent to DNP groups. Chromatography, however, yielded no evidence that the phosphorus with high specific radioactivity could be separated from the DNP group with $\lambda_{max.}$ at 345 mμ.

From the evidence discussed above, the 345 kephalin contains a group which interacts with the DNP group but which does not react with diazomethane. The native phospholipid which is the precursor of the 345 kephalin is more soluble in ethanol than is phosphatidylethanolamine and, in an *in vitro* system, it exchanges with inorganic ^{32}P more rapidly than phosphatidylethanolamine. The 355 kephalin, like the 345 kephalin, is more soluble in ethanol than phosphatidylethanolamine but the nature of the interaction with the DNP group is presumably more complex.

The results described in this paper provide the means for the quantitative determination of the kephalin fractions and also for their isolation. In the absence of the 355 kephalin, countercurrent distribution in 85% (v/v) aqueous ethanol–light petroleum resolves the kephalin into two fractions, and the kephalin content of each can be determined from their light absorption. If ethanolamine and serine are estimated on both fractions then the proportions of phosphatidylethanolamine, phosphatidylserine and the ethanolamine and serine forms of the 345 kephalin can be determined. If the 355 kephalin is present, the polar fraction obtained on countercurrent distribution will contain a mixture of 345 and 355 kephalins, and an estimate of their proportions can be obtained from the absorption spectrum by comparison with the absorption curves of the purified fractions obtained by chromatography.

If the objective is the isolation of the kephalin fractions then chromatography is essential. It is possible to isolate the 328 kephalin (phosphatidylethanolamine and phosphatidylserine) even when present only to the extent of 1·3% and in which the atomic ratio of N:P is unity. Whereas the isolation of the 328 kephalin by chromatography is nearly quantitative, the 345 kephalin cannot be isolated quantitatively and recoveries are about 80%. The 355 kephalin is more labile and it is doubtful whether a pure preparation of this material has been obtained.

The material eluted by ethanol consists of the choline-containing phospholipids, but with rat-liver lipids this fraction also contains the inositol phospholipids (F. D. Collins, unpublished observations).

The isolation of the DNP derivatives of the kephalins instead of the native forms is no disadvantage if the object is to study the distribution of isotopic tracers, and for this purpose is in fact an advantage as the kephalin is spectroscopically labelled.

SUMMARY

1. Diazomethane reacts with dinitrophenylkephalins to form the corresponding methyl esters, while lecithin is unaffected.

2. By chromatography or countercurrent distribution the following methylated dinitrophenylkephalin fractions could be distinguished: (i) material with $\lambda_{max.}$ at 328 mμ, which consisted of a mixture of the methyl esters of dinitrophenylphosphatidylethanolamine, dinitrophenylphosphatidylserine and of the corresponding dinitrophenylacetal phospholipids; (ii) material with $\lambda_{max.}$ at 345 mμ in which the atomic ratio dinitrophenyl groups:P was 0·5; (iii) material with $\lambda_{max.}$ at 355 mμ, which was obtained only from egg-yolk phospholipids and which was a minor component.

3. Evidence is presented showing that the fraction of the methylated dinitrophenylkephalin with $\lambda_{max.}$ at 345 mμ was unaffected by further treatment with diazomethane.

4. More material giving rise to the dinitrophenylkephalin fraction with $\lambda_{max.}$ at 345 mμ was present in the ethanol-soluble fraction of egg-yolk phospholipids compared with the ethanol-insoluble portion.

5. After the *in vitro* incorporation of ^{32}P, the relative specific radioactivity of the phosphorus associated with the dinitrophenylkephalin with $\lambda_{max.}$ at 345 mμ was much greater than that of either the dinitrophenylkephalin with $\lambda_{max.}$ at 328 mμ or the choline phospholipids.

6. It is concluded that the methylated dinitrophenylkephalins with $\lambda_{max.}$ at 345 mμ and at 355 mμ represent hitherto unrecognized forms of kephalin.

7. Procedures are described both for the determination of these forms of kephalin and for their separation from each other and from choline-containing phospholipids.

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