Studies on Phospholipids

1. THE DETERMINATION OF AMINO NITROGEN IN UNHYDROLYSED PHOSPHOLIPIDS

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The kephalin content of a fat or tissue has, until recently, been estimated from the difference between the amounts of total and choline-containing phospholipids. In contrast to this indirect procedure, several methods for the direct determination have recently been described. Those due to Artom (1945), Levine & Chargaff (1951) and Axelrod, Reichenthal & Brodie (1953) require a preliminary hydrolysis of the fat for 3–6 hr. This hydrolysis can be dispensed with if a reagent is used which will react with the amino groups of the intact fat.

Lea & Rhodes (1954) have shown that the Van Slyke method cannot be applied to intact fats and have introduced a sensitive and rapid method based on the reaction of ninhydrin with the amino groups. This method, however, has some disadvantages and it occurred to the present authors that the use of 1-fluoro-2:4-dinitrobenzene (FDNB) might result in the formation of dinitrophenyl derivatives which could be used for the study of phospholipids. The use of this reagent has been described in connexion with the isolation of dinitrophenyl (DNP) bases (Ellman & Mitchell, 1954), but the actual DNP-lipids were not isolated. The method to be described provides a means for the direct determination of amino nitrogen in phospholipids. It depends on the quantitative preparation of DNP-lipids and the subsequent measurement of their light absorption.

EXPERIMENTAL

Reagents

Diethyl ether and dioxan were dried over sodium before use in the presence of FDNB. Benzene was A.R. grade and the other solvents were laboratory reagents. 1-Fluoro-2:4-dinitrobenzene was distilled to remove traces of non-volatile materials. *N*-Ethylmorpholine and triethylamine were fractionally distilled before use.

Materials

Dimyristoyl-L- α -kephalin. This compound (85% pure, based on P) was kindly supplied by Dr E. Baer (Found: P, 4.1; N, 2.2. Calc. for $C_{38}H_{74}O_8NP$: P, 4.9; N, 2.2%).

DNP-ethanolamine. This material was prepared by treating 610 mg. (10 m-moles) of ethanolamine, dissolved in

10 ml. of anhydrous diethyl ether, with 2 g. of FDNB (11 m-moles) and 2 g. (19 m-moles) of triethylamine. The mixture was left at room temperature overnight and the excess of FDNB and base were then removed *in vacuo* (0.1 mm. Hg) at 80°. This procedure also removed some DNP-ethanolamine, which was volatile under these conditions. The DNP-ethanolamine was recrystallized from a benzene-diethyl ether mixture (1:1, v/v). The m.p. was 90° (uncorrected; cf. Axelrod *et al.* 1953). The yield was 1.21 g. (53% of theory).

DNP-serine. This compound (m.p. 195° uncorrected, cf. Porter & Sanger, 1948) was prepared by the procedure of Sanger (1945).

Octadecyl 2-aminoethyl hydrogen phosphate. The synthesis of this compound was carried out by the method of Baer, Maurukas & Russell (1952), octadecanol being substituted for a 1:2-diacyl glyceride. The product, obtained in poor yields (9–10% based on the starting material), was used as an analogue of a kephalin (Found: C, 58.9; H, 11.0; N, 2.47; P, 7.07. $C_{30}H_{44}O_4NP$ requires C, 61.2; H, 11.2; N, 3.57; P, 7.90%). The procedure described by Christensen (1940) was also used and gave similar results (see p. 437).

Rat-liver lipids. Fresh rat liver was extracted at room temperature with $CHCl_3$ -methanol (2:1, v/v), 10 ml./g. being used with a Waring Blendor to disintegrate the tissue. The mixture was filtered with suction through a sintered-glass funnel and the residue re-extracted with a similar solvent mixture (5 ml./g.). The suspension was again filtered, the residue re-extracted as before and filtered. The combined filtrates were taken to dryness *in vacuo* at a temperature not exceeding 30° and the residue was dissolved in diethyl ether. The ethereal solution was shaken in a separating funnel with about one-fifth of its volume of 0.1 *N*-HCl and sufficient ethanol to break any emulsion formed. Altogether the ethereal solution was washed twice with 0.1 *N*-HCl and once with water. This treatment with 0.1 *N*-HCl removed amino N without altering the P content; the atomic ratio N:P of the acid-washed fat was close to unity (1.04 ± 0.04). Plasmalogens are known to be sensitive to acid (Fairbairn, 1945) but under the conditions used there was no detectable formation of free aldehydes in the acid-washed fat. The ethereal solution of fat was taken to dryness *in vacuo*, care being taken not to heat the solution above 30°. The fat was then dissolved in light petroleum (b.p. 40–60°) and stored at -10° .

Egg-yolk lipids. These were prepared in a similar manner to the rat-liver lipids.

Ox-brain kephalin. The procedure of Folch (1942), $CHCl_3$ -methanol being used to fractionate the kephalin, was used, and three main fractions and two intermediate ones were obtained. The three principal fractions were stated

by Folch (1942) to be predominantly diphosphoinositide (fraction I), phosphatidylserine (fraction III) and phosphatidylethanolamine (fraction V). All preparations were dialysed for 4 days against distilled water as described by Folch (1942). Dialysis against water did not, however, remove all the amino acids from ox-brain kephalin; only after acid washing of an ethereal solution was it free of amino acids other than serine, as shown by paper chromatography of a hydrolysate. After washing with acid, fraction I contained serine but no ethanolamine, fraction III had mostly serine but some ethanolamine, whereas fraction V had mostly ethanolamine but also some serine.

Analytical methods

Amino nitrogen. (Ninhydrin method.) The procedure of Cocking & Yemm (1954) was modified for use with phospholipids, serine being used as a standard. The sample of the lipid, usually in light petroleum (b.p. 40–60°), was transferred to a 10 ml. graduated tube; the solvent was removed by evaporation and 1 ml. of 2-ethoxyethanol was added. Additional tubes containing 1 ml. of serine (0.5 μ mole/ml.) in 2-ethoxyethanol were taken, together with two tubes each containing 1 ml. of 2-ethoxyethanol as blank. To each tube was added 0.5 ml. of citrate buffer (0.2M, pH 5.0), 0.2 ml. of 5% (w/v) ninhydrin in 2-ethoxyethanol and 1 ml. of KCN solution (1.5 ml. of 0.01M-KCN diluted to 50 ml. with 2-ethoxyethanol). The solutions were mixed and the tubes covered with glass bulbs to prevent evaporation. They were then heated for 30 min. in a boiling-water bath, cooled and diluted to 10 ml. with 80% (v/v) aqueous ethanol. The extinction was measured at 570 $m\mu$ in a spectrophotometer.

Phosphorus. Total phosphorus was determined on about 1 mg. of lipid by the method of Berenblum & Chain (1938) after digestion with 1 ml. of 70% HClO_4 .

Nitrogen. The lipid was ashed with the digestion mixture of Campbell & Hanna (1937) and the NH_3 distilled in the apparatus of Markham (1942). The distillate was collected in the boric acid buffer used by Conway (1942) and titrated with 0.01N-HCl.

Plasmals (fatty aldehydes). The method described by Feulgen & Grünberg (1938) was used. An estimate of the free aldehyde present in a lipid sample was obtained by the use of a 10 min. reaction time in the absence of the HgCl_2 catalyst.

Choline. The lipid (5–20 mg.) was hydrolysed with 5 ml. of a solution containing equal volumes of 6N-HCl and ethanol for 18 hr. at 100°. The fatty acids were removed by extraction with diethyl ether and the aqueous phase was taken to dryness to remove excess of HCl. The residue was dissolved in water and the concentration adjusted so that 0.5 ml. contained between 0.2 and 2 μ moles of choline. The choline was determined by a procedure which depends upon the precipitation of choline phosphomolybdate and which will be described in detail in a subsequent paper.

Ethanolamine and serine. For the determination of the total amino bases, a sample of the lipid (containing 5–10 μ g.-atoms of amino N) was hydrolysed at 100° in 5 ml. of 5N-ethanolic HCl for 1–3 hr. The fatty acids were extracted with diethyl ether and the aqueous phase was taken to dryness with a stream of air. Water (2 ml.) was added and a 0.5 ml. sample treated with FDNB as described below.

RESULTS

Studies on the reaction between FDNB and lipids

Sanger (1945) treated proteins with FDNB in the presence of 5% (w/v) aqueous NaHCO_3 and sufficient ethanol to dissolve the FDNB. When applied to lipids this procedure caused extensive hydrolysis; thus only 29% of the DNP-lipid was intact after treatment for 18 hr. at 20°, the remainder of the DNP compounds being present as DNP-serine, DNP-ethanolamine, DNP-ethanolamine-*O*-phosphoric acid and DNP-serine-*O*-phosphoric acid.

It was thus clear that an aqueous system was unsuitable. Sodium methoxide in ethanol or ethanol-dioxan mixtures was effective in that the reaction with the lipid was complete, but the amount of by-products not derived from the fat was large and difficult to remove. A satisfactory system was one in which the lipid, dissolved in diethylether or benzene, was treated with FDNB in the presence of a tertiary base such as triethylamine or *N*-ethylmorpholine.

The reaction between FDNB and lipid was followed in a spectrophotometer at a wavelength where the absorption due to the FDNB and non-lipid by-products was low compared with that due to the DNP-lipids. Either 420 $m\mu$ or 440 $m\mu$ was suitable, depending on the concentration of lipid. The experimental cell contained 3 ml. of a benzene solution of rat-liver lipid (7.2×10^{-5} M with respect to aminophospholipids), FDNB and triethylamine, and the compensating cell contained the same components except for the lipid. When the concentration of FDNB was 0.023M the time for the completion of the reaction varied from 20 min. in 1.4M-triethylamine to 90 min. in 0.04M-triethylamine. When the concentration of FDNB was increased to 0.07M (triethylamine remaining at 1.4M) the reaction was completed in 10 min. In the absence of triethylamine the reaction was extremely slow.

FDNB and triethylamine reacted to give a product which absorbed strongly in the near ultraviolet but which could be removed by distillation on to a cold finger (see below). In order to facilitate the procedure the concentration of triethylamine was kept to about 0.1M to avoid excessive amounts of this by-product. In the procedure finally adopted (see below), there was at least a threefold excess of FDNB and a 20-fold excess of triethylamine. Under these conditions the reaction was completed within 2 hr. Lipid treated with FDNB as described above yielded no purple colour when further treated with ninhydrin, thus demonstrating the completeness of the reaction between the amino groups and FDNB.

Removal of the reagent blank

FDNB and the by-products of the reaction absorb strongly in the near ultraviolet and unless removed interfere with the determination of DNP-

lipids. In acid ethanol the absorption spectra of FDNB and the by-products are displaced towards shorter wavelengths, and although they still absorb strongly at $345\text{ m}\mu$ (the absorption maximum of DNP-lipids), the absorption due to the reagent blank is low enough at $420\text{ m}\mu$ to permit an accurate measurement of the absorption due to the DNP-lipid. This procedure has been used but the preferred method involves the complete removal of the reagent blank; it has the great advantage that the sample of dinitrophenyl lipid can be used for further investigations, including the determination of ethanolamine and serine (to be described in a subsequent paper).

The FDNB and by-products can be separated from the DNP-lipids by chromatography on Hyflo Super-Cel. This method has been used as a check on the results obtained by distillation on to a cold finger, which was the method used unless otherwise stated.

Absorption spectra of dinitrophenyl-lipids

The determination of DNP-lipids depends on an accurate knowledge of the molecular extinction coefficient at specified wavelengths. This would be best measured on DNP derivatives of pure phospholipids, but such materials were not available. DNP-ethanolamine and DNP-serine in ethanol had values of ϵ ($345\text{ m}\mu$) equal to 16 000 and 16 300 respectively, but both these compounds lack the phosphate group which will influence the value of ϵ . Octadecyl 2-aminoethyl hydrogen phosphate was synthesized by two different procedures (Baer *et al.* 1952; Christensen, 1940) which ensured the presence of a 2-aminoethyl-1-phosphoryl group, although pure compounds were not obtained. These synthetic materials were treated with FDNB and the products chromatographed on Hyflo Super-Cel in order to obtain fractions in which the ratio of the extinction at λ_{max} to the P content was constant (for example, see Table 1). The mean value of the molecular extinction coefficient (calculated in terms of the

atomic concentration of P) at $345\text{ m}\mu$ in light petroleum was $16\,600 \pm 300$ (fiducial limits for $P=0.95$ and the seven samples). Rat-liver DNP-lipids could not be readily separated from other phospholipids by chromatography, and it was not possible to measure the molecular extinction coefficient on natural materials with the same degree of accuracy as on synthetic materials. However, one sample of chromatographed DNP-lipid gave a value of ϵ (computed in terms of the atomic concentration of P) of 15 500 at $345\text{ m}\mu$ and 6100 at $400\text{ m}\mu$.

Measurements at $400\text{ m}\mu$ were sometimes necessary and the value of the molecular extinction coefficient of the synthetic materials in terms of their P content was 6200 ± 130 (light petroleum) compared with 6400 (ethanol) for DNP-ethanolamine and 6500 (ethanol) for DNP-serine.

Determination of amino nitrogen with dinitrophenyl derivatives

The following procedure for the preparation of DNP-lipids and their estimation is based on the foregoing results.

The sample of lipid (5–30 mg.) is incubated with about $100\text{ }\mu\text{moles}$ (1 drop) of FDNB and 0.1 ml. of triethylamine in 5–10 ml. of benzene for 2 hr. at 30° . The solvent and tertiary base are then removed by heating the mixture *in vacuo* (30 mm. Hg) at 80° . The excess of FDNB and by-products are distilled on to a cold finger at a pressure of less than 0.1 mm. Hg and at about 80° . The DNP-lipids are dissolved in a known volume of light petroleum and the absorption at $345\text{ m}\mu$ ($\epsilon=16\,600$) or $400\text{ m}\mu$ ($\epsilon=6200$) is measured.

For larger quantities of lipid (30–500 mg.) the amounts of the reagents are increased proportionately, and it then becomes necessary to dissolve the lipid in light petroleum, which is then distilled off, thus exposing a fresh surface of lipid. This process is repeated several times during the distillation of the FDNB and by-products. The yield of DNP groups

Table 1. *Chromatography of dinitrophenyl derivative of octadecyl 2-aminoethyl hydrogen phosphate*

Octadecyl 2-aminoethyl hydrogen phosphate (509 mg.; 69% pure, based on N content) was treated with FDNB and the resulting DNP material was chromatographed on 200 g. of Hyflo Super-Cel. The value of ϵ ($345\text{ m}\mu$, light petroleum) was calculated in terms of the atomic concentration of P. Of the total N, 96% could be accounted for in fractions 2–5, which possessed well-defined absorption maxima at $345\text{ m}\mu$.

Fraction no.	Eluting solvent	Wt. (mg.)	P ($\mu\text{g. atoms}$)	ϵ	P (%)
1	Benzene-chloroform (1:1, v/v)	35	4	153 000	0.3
2	Ethanol-chloroform (1:9, v/v)	192	233	16 000	3.8
3	Ethanol-chloroform (1:9, v/v)	77	108	16 500	4.4
4	Ethanol-chloroform (1:9, v/v)	257	396	16 700	4.9
5	Ethanol-chloroform (1:9, v/v)	96	111	17 200	3.6
	Total eluted	657	852	—	—
	Amount taken for chromatography	676	997		

per unit weight is independent of the amount of lipid treated with FDNB.

If the lipid contains materials absorbing in the region 340–440 $m\mu$ special precautions are necessary. For example, rat-liver lipids contain vitamin A (λ_{max} 328 $m\mu$) but as this compound does not absorb at 400 $m\mu$ this wavelength can be used for the determination. On the other hand, rat-brain lipids are free from materials which absorb near 345 $m\mu$ so that measurement at this wavelength is satisfactory. In other cases it is sufficient to subtract the absorption at 345 $m\mu$, in the native lipid, from that of the dinitrophenylated lipid; this correction is usually negligible.

If the content of amino N is low (less than 5% of the total lipid P) the absorption spectrum should be measured and compared with that shown in Fig. 1. If the curve is less well defined it may be assumed that absorbing materials other than DNP-lipids are present. In this case the material should be chromatographed on Hyflo Super-Cel, 1 g. of adsorbent to every 2 mg. of lipid being used. The DNP-lipid dissolved in light petroleum is added to the column and the column is washed with $CHCl_3$ (10 ml./g. of adsorbent). The DNP-lipids form a yellow band on the column and are eluted with ethanol- $CHCl_3$ (1:1, v/v) and the absorption spectrum is measured. In some cases the absorption curve of the DNP-lipids possessed neither a maximum nor an inflexion at 345 $m\mu$, and clearly the amount of amino groups in these was negligible.

Comparison of the dinitrophenyl method with other procedures

The method described above was compared with the ninhydrin method and the results are shown in Table 2. In the DNP-method duplicates agree to

Table 2. *Comparison of the ninhydrin and dinitrophenyl methods for the determination of amino nitrogen*

Procedures used are as described in the text.

Sample	Ninhydrin method (Serine as standard)	DNP method [based on ϵ (400 $m\mu$) = 6200]
	(μg.atoms/g. of fresh tissue)	
Rat-liver lipid 1	8.9	10.9
2	10.6	10.9
3	14.2	15.0
4	7.2	7.8
5	11.6	9.4
6	8.9	8.9
7	10.5	10.5
8	16.7	13.2
9	11.4	10.4
10	12.0	11.3
	[based on ϵ (345 $m\mu$) = 16 600]	
	(% by wt.)	
Octadecyl 2-aminoethyl hydrogen phosphate*	2.55	2.48
Dimyristoyl-L-α-kephalin†	1.33	1.62
* Total N = 2.47 %.		† Total N = 2.2 %.

Fig. 1. Absorption spectrum of DNP-kephalin in light petroleum. A sample (3.56 mg.) of ox-brain kephalin was treated with FDNB, the excess of reagents removed and the dinitrophenylated material dissolved in 50 ml. of light petroleum. The lower curve shows the absorption of the blank, indicating that nearly all had been removed on the cold finger.

within 2.4 %, whereas in the ninhydrin method the agreement is to within 3.0 %. Therefore the variability (+ 26 % to - 18 %) in the results cannot be due to poor replication; furthermore, as the differences are random a constant error can be ruled out. In the ninhydrin procedure serine was used as the standard, and, as the ratio of the colour yield of serine to that of ethanolamine varied from 1.05 to 1.24, it is possible that the ratio of the colour yield of serine to that of kephalin might vary by as much. It thus seems likely that the variability in the figures in Table 2 is due in part to the lack of a suitable standard in the ninhydrin method.

The amount of non-choline phospholipids has long been used as an estimate of the kephalin content of lipids. In a sample of rat-liver lipids the non-choline phospholipid (determined as the difference between the total P and the choline contents) was 9.1 μ moles/g. of wet tissue. The direct determination of amino N by reaction with FDNB indicated 9.4 μ moles/g. of wet tissue.

In addition to these comparisons the amino N determined directly on the intact lipid and after hydrolysis was investigated. The reaction with FDNB was modified so that the free bases in the hydrolysate reacted readily. To 0.5 ml. of a hydrolysate of a lipid (obtained as described in the Experimental section) was added 1 ml. of 0.1 M-triethylamine in ethanol and about 100 μ moles (1 drop) of FDNB; the mixture was heated at 50° for 10 min. and taken to dryness *in vacuo*. The residue was acidified with 0.1 ml. of N-HCl and again taken to dryness. The residue was made up to volume in ethanol and the absorption measured at 420 m μ , where the molecular extinction coefficient was 5000. The value of the reagent blank was subtracted from this reading. Thirteen samples of egg-yolk phospholipid fractions in which the ratio of amino N/P varied from 0.79 to 0.024 were analysed by this procedure; the ratio of the amount of amino N in the hydrolysate to the amount in the unhydrolysed fat was 1.02 ± 0.04 (fiducial limits for $P = 0.95$). The excellent agreement obtained demonstrates that the DNP method for amino N can be applied to either intact lipid or hydrolysed material.

Interfering substances

Interference with the DNP method for amino N can arise either through the formation of a compound with the amino group which would not then react with FDNB or through reaction of FDNB with some other functional group where the product absorbed at 345 m μ or at 400 m μ . Aldehydes condense with free amino groups to form Schiff's bases, and thus could interfere. Fatty aldehydes (plasmals) occur in lipids combined as plasmalogens, which are sensitive to acid (Fairbairn, 1945), yielding the free aldehydes. Such free aldehydes

might combine with some of the amino groups and thus partially block the formation of DNP-lipids with a concomitant fall in the amino N content. Free plasmals could not be detected in the lipids studied, and furthermore there was no difference in the plasmal content of a lipid before and after treatment with FDNB.

Another possible reaction would be the combination of amino groups and acid groups; this cannot be excluded but there was no evidence to suggest this. Still another means by which the free amino groups might be masked is the chelation of a bivalent cation with amino groups in the α position relative to a free carboxyl group such as occurs in phosphatidylserine. Although treatment with acid can be expected to remove cations it was of interest to determine whether bivalent cations could inhibit the reaction. In a sample of ox-brain kephalin (fraction III) which had been dialysed against water, but not washed with acid, the value of the atomic ratio DNP groups : P was 0.28. After washing with acid this ratio became 0.61, although no P was removed. Folch (1942) found that fraction III of his ox-brain kephalin contained 12.8 % of ash. It has been found that the addition of bivalent cations to acid-washed lipids depressed the yield of DNP-lipids. (1) Copper acetate (15 μ moles dissolved in ethanol) was added to some ox-brain kephalin (fraction III) containing 1.5 μ moles of lipid P; the yield of DNP-lipid in the presence of copper acetate was only 27 % of a similar sample not treated with copper acetate. (2) The addition of calcium chloride (10 μ moles) to a sample of liver lipid containing 0.23 μ g.atoms of amino N caused the yield of DNP nitrogen to fall to 83 % of the total. These results further indicate the desirability of treating the lipid with acid.

FDNB is known to react with hydroxy compounds to yield products with an absorption maximum at 280 m μ ; on a molecular basis the extinction at 345 m μ is about 5 % of that of a DNP-lipid at the same wavelength. Octadecanol reacted readily with FDNB, but free primary alcohols do not occur in lipids except in traces (e.g. vitamin A alcohol and lysophospholipids). With cholesterol, a secondary alcohol, there was no reaction. Whalley (1950) investigated the reaction between alcohols and FDNB with triethylamine as a catalyst, and stated that primary alcohols react readily whereas secondary and tertiary alcohols react less readily and frequently require warming. In view of the failure to form a DNP derivative of cholesterol it is unlikely that inositol, a secondary alcohol, would react; this could not be tested as inositol is insoluble in organic solvents, and a sample of diphosphoinositide was not available. It can be concluded that alcohols will not interfere, but in any case the DNP derivatives do not possess a maximum

in the spectral region where the DNP-lipids exhibit maximum absorption.

Water reacts with FDNB to form 2:4-dinitrophenol, which has an absorption maximum at 260 $m\mu$ in light petroleum and at 355 $m\mu$ in ethanol. In the presence of acid the maximum in ethanol is at 260 $m\mu$. These properties provide a means for the detection of 2:4-dinitrophenol, and the precautions given in the description of the method suffice to eliminate any interference.

Lecithin did not react with FDNB. In samples prepared by chromatography on magnesium oxide or on alumina there was some absorption at 345 $m\mu$ after reaction with FDNB, but in all cases the absorption spectrum indicated either the absence of amino N or else amounts of less than 1 %.

DISCUSSION

The determination of amino N in intact lipids results in a greatly simplified procedure eliminating the need for 3–6 hr. hydrolysis. The ninhydrin method, modified for use with lipids as described in the present paper and by Lea & Rhodes (1954, 1955), is simple and rapid to use but suffers from several disadvantages. These defects have been recognized in the determination of amino N in amino acids and have resulted in modifications of the procedure of Moore & Stein (1948) being proposed. The purpose of these modifications has been not only to obtain a more stable reagent and lower blanks but also to obtain a higher yield of colour. It is always necessary to use a standard, as the yield of dioxohydrindamine–dioxohydrindylidene is variable. In the present investigation the reagent (based on that of Cocking & Yemm, 1954) is stable for 2 weeks but the blank is always fairly high ($E=0.1-0.25$). It was found that the colour yield varied not only with the material (ethanolamine, serine or phospholipid) but also with the same material over a period of time. Furthermore, the ratio of the colour yield of serine to that of ethanolamine is not constant and was found to vary from 1.05 to 1.24 (see also Levine & Chargaff, 1951), and thus it is possible that the ratio of the colour yield of serine to that of natural kephalin may also vary. It is not practicable to use a synthetic kephalin or a purified natural kephalin as a standard as these materials are difficult to prepare and are not stable (Lea & Rhodes, 1955).

The reaction of FDNB with free amino groups has been much studied since its introduction by Sanger (1945) and the reaction with amino acids is quantitative (Levy, 1954; Isherwood & Cruickshank, 1954). FDNB reacts only with uncharged amino groups and in aqueous solutions the pH has to be about 8–9, usually by the use of a sodium bicarbonate or borate buffer, and under these conditions the

amino-phospholipids react readily. Treatment of phospholipids with 0.2N-alkali at 37° (Dawson, 1954) results in the splitting off of the fatty acids and a high yield of glycerylphosphorylethanolamine, glycerylphosphorylserine and of glycerylphosphorylcholine. In contrast with this, treatment of DNP-lipids with a sodium bicarbonate buffer (pH 8) at 20° for 18 hr. gave a high yield of DNP-ethanolamine and DNP-serine together with smaller amounts of the corresponding phosphorylated compounds. This unexpected alkali lability of the bond between the base and the phosphorus necessitated an anhydrous system such as had been used by Chapman & Parker (1951), who obtained quantitative yields of DNP derivatives of aromatic amines with triethylamine as a proton acceptor. The reaction of lipids with FDNB as described in this paper can reasonably be assumed to be quantitative in view of the results obtained with amino acids and amines quoted above. In support of this, the present results have shown that the DNP-lipid gave no reaction with ninhydrin, indicating the completeness of the reaction with FDNB. The yield of DNP groups was the same on the unhydrolysed as on the hydrolysed fat, showing that the aminophospholipids react as readily as the free bases.

The reaction with FDNB and the determination of the quantity of DNP-lipids formed gives a direct estimate of the free amino N. The correctness of the result so obtained can be assessed from the absorption spectrum and, if necessary, by chromatography. The absorption maximum at 345 $m\mu$ in light petroleum is characteristic of the DNP derivatives of a phospholipid. DNP-ethanolamine and DNP-amino acids which, although insoluble in light petroleum may dissolve to a small extent in this solvent in the presence of fatty materials, have $\lambda_{\max}$ at 332 $m\mu$ (diethyl ether–light petroleum, 1:1, v/v). On chromatography on Hyflo Super-Cel DNP-ethanolamine and DNP-amino acids are eluted by chloroform, whereas the DNP-lipids require ethanol–chloroform for elution. Thus DNP-lipids can be readily distinguished from non-lipid DNP materials.

Although there is some reaction with primary alcohols this is not likely to be important, as free primary alcohols exist only in traces in lipids. Neither lecithin nor cholesterol interferes, but traces of water do, especially with samples with a low kephalin content, and the lipid must therefore be dry.

This method for the determination of amino N on intact lipids is sensitive, and as little as 0.02 μ mole can be estimated. The same sample of DNP-lipid can also be used for other measurements, as for example phosphorus, fatty acids and the determination of ethanolamine and serine (Collins & Wheeldon, unpublished work). The reagents are stable and a standard is not necessary.

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 μ mole can be estimated, and 0.1–2.5 μ moles 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, counter-current 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.