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"THE COMPOSITION OF CEPHALIN"

A THESIS

submitted for the degree of

DOCTOR OF PHILOSOPHY

at the

AUSTRALIAN NATIONAL UNIVERSITY

by

L.W. WHEELDON.
The research described in this thesis was carried out in Canberra between July, 1953 and January, 1956 during the tenure of an Australian National University Scholarship, for which I am indebted to the Council of the University.

The isolation of cephalin on a small scale suited to metabolic experiments was suggested to me as a project by my supervisor, Dr. F.D. Collins, who introduced me to the field of lipid analysis and gave me every encouragement and support to pursue my own ideas. For this I am very grateful and in particular for the services of his assistant, Mrs. M. Labutis, who generously carried out all the phosphorus analyses reported in the thesis.

The novel approach to the problem of isolating cephalin, that is by chemical modification of polar substituents, arose from discussion with Dr. Collins and was our mutual interest throughout. Thus, the promise of early experiments in the preparation of 2,4-dinitrophenyl cephalin (Chapter 5) and the methyl ester of the latter (Chapter 7) opened up many avenues in which we participated equally. However, acknowledgement has been made wherever Dr. Collins' results have been included to support the thesis.
I also wish to record my indebtedness to Dr. D.M.F. Phillips, who informed me of the possibilities of the Janovskv reaction, which were developed as described in Chapter 4. The preparation of the manuscript was greatly facilitated by the services of Mr. Gordon Arthur, who reproduced the figures and Mr. Val Paral, who photographed them.

L.W. Wheeldon

L.W. Wheeldon.

October, 1956.
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CHAPTER 1.

GENERAL INTRODUCTION

(a) GENERAL PROPERTIES AND NOMENCLATURE OF PHOSPHOLIPIDS.

Lipids as a class are much less uniform in their structural make-up than other classes of biologically important substances such as proteins, nucleotides and carbohydrates. This is particularly true of the phospholipids which have structural affinities with proteins and carbohydrates on the one hand and more characteristic lipids such as neutral fats and waxes on the other. Fats are often described as being insoluble in water and soluble in "fat solvents", for example, diethyl ether, petroleum ether, chloroform, acetone; this criterion breaks down with the phospholipids, of which sphingomyelin is insoluble in ether and petroleum ether and cephalin and phosphatidic acid will form clear solutions in water at suitable pH.

All naturally occurring phospholipids contain a long-chain
2. Alkyl residue which may be in ester, acetal or amide linkage with the rest of the molecule. Orthophosphoric acid and either fatty acids or fatty aldehydes are obtained upon hydrolysis.

1. Phosphoglycerides.

Following the suggestion of Folch and Sperry (1948) the generic term "phosphoglycerides" and the prefix "phosphatidyl" have come into use for the naming of lipids which contain acyl glyceryl phospho as a building stone. The earlier term was "monoaminophosphatides", emphasising the atomic ratio of nitrogen to phosphorus of one. The members of this class are lecithin (phosphatidyl choline, I), cephalin (phosphatidyl-ethanolamine or -serine, II), diphosphoinositide (phosphatidyl inositol phosphate, III), phosphatidic acid (IV) and others as yet incompletely characterised. This class includes lysophospholipids which contain only one fatty acid molecule per phosphorus atom. Lysolecithin and lysocephalin are known for their powerful haemolytic action.

2. Plasmalogens (phosphoglyceracetals).

Related to the above class are the plasmalogens which are frequently given the prefix "phosphatidal-" since glycerol is in combination with a fatty aldehyde (plasmal) through an acetal linkage. The structure shown for phosphatidal ethanolamine (V) is based upon the composition of the material first isolated
I
\[ CH_2-O-CO-R \]
\[ CH-O-CO-R \]
\[ CH_2-O-P-O-CH_2-CH_2-N-CH_3 \]
\[ O-O \]
\[ \text{CH}_3 \]

II
\[ CH_2-O-CO-R \]
\[ CH-O-CO-R \]
\[ CH_2-O-P-O-CH_2-CH_2-NH_3 \]
\[ O-O \]

III
\[ CH_2-O-CO-R \]
\[ CH-O-CO-R \]
\[ CH_2-O-P-O-CH_2-\text{CH(OH)CH(OH)CH(OH)} \]
\[ \text{CH}_2-\text{CH}-O-P-OH \]
\[ \text{OH} \]

IV
\[ CH_2-O-CO-R \]
\[ CH-O-CO-R \]
\[ CH_2-O-P-O-\text{CH(OH)} \]
\[ \text{OH} \]
\[ \text{CH(OH)CH(OH)} \]
\[ \text{OH} \]
(Feulgen and Voit, 1924) which has been crystallised (Thannhauser, Boncoddio and Schmidt, 1951) but this is now believed to be an artefact (Baer and Stancer, 1953). Elenk and Debuch (1954) have suggested an alternative hemiacetal structure, which will be discussed in the concluding chapter.

3. **Sphingolipids.**

Carter, Haines, Ledyard and Norris (1947) suggested the name "sphingolipides" for the group of lipids which contain the fatty amine sphingosine. Of the two best known representatives of this class, only sphingomyelin (VI) is in accord with the definition of a phospholipid given above. Cerebroside (VII) contains neither glycerol nor phosphoric acid but is characterised by a sugar in combination with a long chain 2-amino, 1,3-diol. Carter and Greenwood (1952) have recently shown that the sugar is attached at the terminal carbon atom of sphingosine. Fischer (1954) has proposed the generic terms "phosphoglyceratid" for phosphoglyceride, "phosphosphingamide" for sphingomyelin and "sphingoglycoside" for cerebroside.

The classical term "cephalin" is used in this thesis synonymously with "aminophospholipid", that is, phospholipids possessing a free amino group.

(b) **The Isolation and Determination of Phospholipids.**

1. **Isolation and Determination of Total Phospholipids.**

For complete extraction of phospholipids from tissues, a
polar solvent such as ethanol must be used in combination with a more general lipid solvent like diethyl ether or chloroform. Such extracts are heavily contaminated with non-lipid material and must in turn be dried and re-extracted with a more selective lipid solvent such as petroleum ether. "Petrol-soluble" phosphorus is an expression commonly used to indicate the phospholipid content of a lipid extract. This value at best only approximates to the true phospholipid content. Additional purification procedures must be carried out to obtain reliable analysis, especially of isotopically labelled phospholipids. These procedures will be described in Chapter 2.

2. **Isolation and Determination of Individual Phospholipids.**

   **Solvent Fractionation.**

   Early investigators attempted to discover the phospholipid composition of tissues by making use of the known solubility properties of the recognised components. Their results bear little significance for two reasons: (1) the difference in solubility within one class of lipids, between those which contain saturated fatty acids and those which contain unsaturated fatty acids - and (2) the remarkable solvent effect of lipids upon each other. While all phospholipids are virtually insoluble in cold acetone, they are appreciably soluble in a cold acetone solution of glycerides. If the solution is cooled too much then saturated glycerides precipitate. In recent time, McKibbin and Taylor (1949)
have described a method for the quantitative precipitation of phospholipids based upon the observation of Bloor (1929) that the addition of magnesium chloride lowers the solubility of phospholipids in acetone.

Further manipulations which may be applied to the acetone-insoluble fraction are shown in the scheme opposite. None of these steps effects a quantitative separation but the general plan has been in use for almost a hundred years for the preparation of phospholipid samples enriched with a particular component. Folch (1942) made use of it in his preparation of phosphatidyl ethanolamine and phosphatidyl serine from brain fat. The long established use of these procedures has led to the belief that the overall composition of the cephalin of tissues, for example, may be inferred from that of the alcohol-insoluble portion. This belief is possibly erroneous since it takes no account of the cephalin in the alcohol-soluble fraction.

Chromatography and Counter-Current Distribution.

A recent review by Holman (1952) shows that the chromatographic separation of fatty acids, fat soluble vitamins, pigments, sterols, etc., is well established.

The chromatography of phospholipids has been found difficult, owing to their instability and the tenacity with which they adhere even to weak adsorbents. No procedure which allows recovery of all the component phospholipids of a total lipid extract has yet been found. Indeed it is doubtful whether this will ever be
achieved, in view of the complexity of native phospholipid mixtures. The most that has been done to recover one species with the loss of the remainder. Trappe (1941) employed alumina for the removal of phospholipids and fatty acids from lipid extracts. The phospholipids were not recovered. Chromatography has been used to remove cerebrosides in the purification of sphingomyelin (Thannhauser, Setz and Benotti, 1938), for the purification of cerebrosides (Klenk, 1942) and of gangliosides (Klenk and Rennkamp, 1942). Chromatography on hydrated magnesium silicate has been applied to the preparation of the component of beef heart lecithin which is serologically active in the diagnosis of syphilis (Rice and Oster, 1951). These procedures have not been widely adopted and must be regarded as merely pointers to the possibilities. Taurog, Entenmann, Fries and Chaikoff (1944) on the other hand, developed a simple and efficient method of the separation of the choline-containing phospholipids which has been adopted by other workers. In this method, the total phospholipids are adsorbed on to magnesium oxide from petroleum ether solution and the choline-containing phospholipids are eluted with methanol. However, the remainder of the phospholipids cannot be eluted. This is the only isolation procedure which has been adopted as a quantitative analytical method.

A pure lecithin with two unsaturated fatty acids in the molecule (dipalmitoleyl-L-α-glyceryl-phosphorylcholine) was
isolated for the first time by chromatography on alumina by
Hanahan and Jayko in 1952 and there is a recent report of the
isolation of egg yolk cephalin from silica (Lea, Rhodes and Stoll,
1955). A combination of solvent fractionation and chromatography
on silica gel enabled McKibbin and Taylor (1952) to isolate a
hitherto unknown phospholipid which they called "polyglycerol
phosphatide".

Paper chromatography has been used successfully in the
separation of phosphatidyl ethanolamine and phosphatidyl serine by
Amelung and Böhm (1954) and of lecithin from lysolecithin by Lea,
Rhodes and Stoll (1955). While this method works satisfactorily
with simple mixtures of purified phospholipids, there is no report
of its successful application to total phospholipid preparations.
Recently, Marinetti and Stotz (1955) who used the method of
Amelung and Böhm (1954), were unable to effect a resolution of
the total phospholipids of rat tissues. This is not surprising in
view of the lack of success of counter-current distribution in
separating the major phospholipids of a total lipid extract.
(Cole, Lathe and Ruthven, 1953; Lovern, 1952). The results of
counter-current distribution emphasise both the complexity of native
phospholipid mixtures and the occurrence of a large number of
unidentified forms (Garcia, Lovern and Olley, 1956).

It may be appreciated from the brief account given above that
attempts at the quantitative isolation of pure phospholipids from a
from a complete extract are still at an exploratory stage. Much of the work quoted requires confirmation and there is considerable scope for further research into methods of isolation.

**Determination of a Characteristic Fragment.**

The phospholipid composition of a lipid extract is usually determined by degradation to the characteristic structural units of the known phospholipid components. For example, lecithin and sphingomyelin are degraded to choline, cephalin to ethanolamine and serine; cerebrosides and sphingomyelin may be determined as sphingosine and the former also as sugar liberated on hydrolysis. Inositol-containing phospholipids are determined as inositol and plasmalogens as aldehyde.

Degradation is invariably effected by acid or alkaline hydrolysis. Extraction of the neutral aqueous hydrolysate with ether separates the products of hydrolysis into ether-soluble (fatty acids, fatty aldehydes, sphingosine, etc.) and water-soluble (glyceryl phosphate, bases, sugar, inositol, etc.) fractions. The conditions of hydrolysis vary according to the fragment to be determined. The acetal structure is very labile to acid and pharmal determination is carried out after hydrolysis of the plasmalogens in glacial acetic acid at room temperature in the presence of mercuric chloride which is a catalyst. The weak bases containing free amino groups are liberated after less than half an hour's refluxing in constant boiling (6 N) hydrochloric acid;
however, the strongly basic choline residue requires at least twelve hours refluxing in constant boiling hydrochloric acid for quantitative liberation. Inositol diphosphate is fairly readily liberated from phospholipids on acid hydrolysis, but quantitative conversion to free inositol in which form it may be determined microbiologically (Beadle, 1944) requires very lengthy hydrolysis (Brante, 1949).

The determination of choline, ethanolamine and serine will be the subject of Chapters 3 and 4; plasmalogen determinations are included in Chapter 6.

Sphingomyelin has been determined by a method which depends upon its resistance to hydrolysis (Schmidt, Benotti, Hershman and Thannhauser, 1946) as compared with glycerolphospholipids. This analysis is best carried out on a methanol eluate from magnesium oxide (method of Taurog and others, 1944, for isolation of choline-containing phospholipids) since unidentified phospholipids which do not contain choline have been found to interfere with the method (Brante, 1949; Dawson, 1954a). A more direct method for the determination of sphingomyelin is based on the formation of the N-succinyl derivative of sphingosine, which can be chromatographed and titrated with alkali as described by Wittenberg (1955).

Cerebrosides are a minor component of most lipid extracts and can be determined reliably only after some preliminary purification. A recent publication described a chromatographic purification by
means of a mixed bed of ion-exchange resins. The purified cerebrosides are then estimated colourimetrically with the anthrone reagent for sugars (Radin, Lavin and Brown, 1955).

(c) **Objects of the Present Investigation.**

Phospholipids are recognised as a structural element of cell membranes and organelles such as mitochondria. In mitochondria phospholipids account for about one fifth of the dry weight (Barnum and Huseby, 1948; Swanson and Artom, 1950; Levine and Chargaff, 1952). It is very probable that they play an important part in the enzymatic activities of mitochondria by specific orientation of the proteins. Slater (1953) has implied such a role for phospholipids in electron transport but there is little direct evidence for such a function apart from the report that succinoxidase is inactivated by a lecithinase (Goodwin and Waygood, 1954).

The main lines of biochemical research on phospholipids have been concerned with pathways of synthesis and breakdown. These problems can be dealt with by the isolation of water-soluble precursors such as the phosphorylated base, glycerylphosphoryl base, etc. Dawson (1954b and 1954c) has made outstanding contributions to this field. However, in order to make an effective study of the role of phospholipids once they are formed in mitochondria, cell membranes, plasma lipoproteins, etc., techniques must be available for the isolation on a micro scale of samples of individual phospholipids of uniform composition (neglecting the fatty acid
portion).

The present work was commenced in 1953 with the ultimate aim of learning something about the role of preformed phospholipids within the cell. Since phospholipids are a universal part of the architecture of cell membranes and inclusions, the problem is a structural one as much as functional. Although degradative methods have provided important information about the synthesis and catabolism of phospholipids (Dawson, 1955), one can only speculate about the part performed by phospholipids in the maintenance of vital processes, such oxidative phosphorylation. For example, one can think of the acidic groups associated with the phosphorus and the terminal carbon of serine being involved in transient esterification reactions in situ. This possibility might have been examined by use of the $^{18}$O technique of Cohn (1953) had time permitted. However, for this and other possible approaches, isolation of the phospholipid in a pure form is desirable as a first step.

Moreover, degradative methods for the analysis of phospholipids take no account of possible complex arrangement of the fragments in the parent molecule. In view of increasing evidence of the complexity of phospholipids (which will be discussed in later chapters), a sounder approach to metabolic studies would be the formation of an identifiable derivative of the native phospholipid under conditions mild enough not to prejudice the stability of labile linkages which may be present. The derivative should have a well-defined absorption
spectrum so that it might be estimated by spectrophotometry and preferably be coloured, so as to facilitate chromatography.

To implement this idea, cephalin was chosen for the following reasons. (a) Previous attempts (Borgström's, 1952) to chromatograph native cephalin were unsuccessful. (b) Cephalin possesses a reactive free amino group which should be readily acylated, thereby abolishing the positive charge on the amino group and perhaps facilitating its recovery from adsorbents. Choline-containing phospholipids on the other hand, could not be essentially modified without disruption of the whole molecule.

Fluoro-2,4-dinitrobenzene the advantages of which are exemplified in protein chemistry, fulfils the above requirements and is a specific reagent for the amino group. The reagent reacts readily with amino groups under mild conditions (Sanger, 1945) and the N-2,4-dinitrophenyl derivatives have a yellow colour and a well defined absorption maximum in the near ultra-violet.

As will be shown, dinitrophenylation provides a convenient means for the direct determination of lipid amino-nitrogen but the ease of elution from adsorbents of the dinitrophenyl derivative as compared with native cephalin was not great enough to fulfil the object of an improved method for the isolation of cephalin. However dinitrophenylcephalin, being in effect a free acid and having a protected amino group, was readily esterified by reaction with diazomethane. Since this reagent does not react with salts of strong bases, choline-
containing phospholipids were again unaffected. Therefore a completely non-ionised derivative of cephalin was obtained which apart from being readily separable from the majority of remaining phospholipids, possessed a characteristic absorption spectrum, identical with that of the same derivative of synthetic cephalin. This latter feature was of great importance since spectroscopic evidence of the existence of complex amino-phospholipids was an additional result of methylation of the dinitrophenyl derivatives.

Since the field of phospholipid analysis was new to me, I started my work with an examination of methods for the determination of the principal phospholipid constituents. Arising from this were new procedures for the determination of the nitrogenous bases, ethanolamine, serine and choline, in phospholipid hydrolysates. These techniques are described in the first chapters and provided a means of appraisal of the acylation reaction as a method for the direct determination of lipid amino nitrogen. The interpretation of analytical results led to the recognition of the complexity of non-choline-containing fraction of native phospholipid mixtures. Therefore the main object of devising a useful procedure for the isolation of the aminophospholipids will be presented as part of a general study of the composition of cephalin.
CHAPTER 2 -

THE PREPARATION AND PURIFICATION OF PHOSPHOLIPIDS.

(a) Extraction.

Phospholipids are polar substances, yet characterised by their solubility in so-called "fat solvents" such as petroleum ether, etc. Owing to their polar nature they are not quantitatively extracted from tissues by these solvents alone. With wet tissues it is essential in the first place to have a dehydrating agent: acetone, ethanol, anhydrous sodium sulphate are examples. However, even tissues which have been dried in the frozen state require a polar solvent for complete extraction of phospholipids. Such treatment results in the denaturation of protein and it is reasonable to infer that phospholipids are bound in the cell in the form of lipoprotein complexes.

In Table 1 are shown the quantities of phospholipid removed from egg yolk by serial extraction, starting with a weakly polar solvent (diethyl ether) at room temperature and concluding with a
TABLE 1

**Total Phospholipid in Serial Extracts of Egg Yolks.**

(shown as wt. in gms. of acetone insoluble/yolk after ether separation and acid-washing).

<table>
<thead>
<tr>
<th></th>
<th>Ether</th>
<th>Ethanol:ether (1:4)</th>
<th>Ethanol:ether (1:3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>at +15°C</td>
<td>0.54</td>
<td>0.35</td>
<td>0.37</td>
</tr>
<tr>
<td>at -10 to 0°C</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>at +60°C</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
much more strongly polar solvent mixture (ethanol:diethyl ether) at an elevated temperature. The experimental details were as follows:

Six egg yolks were rubbed to a dry powder with anhydrous sodium sulphate in a cold mortar. Cold ether was added and decanted at intervals until all the protein had been decanted in the ether in the form of a fine suspension, leaving behind largely hydrated sodium sulphate. The ether extract was clarified by centrifugation and the supernatant taken to dryness below 30°C. The residue after centrifuging was suspended in ethanol:ether (1:4 by vol.) and allowed to stand overnight at -10°C. The filtrate was taken to dryness below 50°C. The residue from this second treatment was finally suspended in equal parts of ethanol and ether at 60°C. for an hour, followed by 24 hours at room temperature. This extract was also taken to dryness in vacuo. All extracts were submitted to acetone separation using redistilled acetone and the phospholipids dissolved in a small quantity of ether and left at -10°C. overnight, after which the insoluble fraction was removed by centrifuging at 0°C. The supernatant ether solutions were acid-washed (to be explained in the next section), dried in vacuo, weighed and made to volume in petroleum ether.

The solvent mixture introduced by Bloor (1915) consisting of three parts of ethanol to one part of diethyl ether by volume, has found general use for the routine extraction of lipids. This solvent has been used in the course of the present work for the extraction of rat liver, in the following way:

Rat liver was rapidly dissected into thin strips with a pair of scissors, the pieces being dropped into sufficient boiling ethanol to dehydrate the tissue. The ethanol extract was poured off into a sintered glass funnel and the dried tissue ground with sand or anhydrous sodium sulphate under ethanol:ether (3:1 by vol.). The disintegrated tissue was finally transferred to the sintered glass funnel and washed with several portions of ethanol:ether.

Chloroform:methanol was first introduced for the extraction of sources rich in sphingolipids (viscera in certain pathological _
conditions; see Haven and Levy, 1941). The results of chloroform:methanol extraction of brain tissue have been compared by Folch, Ascoli, Lees, Meath and LeBaron (1951) with the yields by alternative procedures. Plant tissues may be a special case, since they are often rich in inositol containing lipids. Rewald (1937) has used benzene:ethanol mixtures for the extraction of vegetable phospholipids.

Chloroform:methanol (2:1 by vol.) has been employed in the present work for the extraction of rat tissues in a survey of their phospholipid composition (Chap. 6). In this case the material was suspended in the solvent and disintegrated at room temperature in a Waring blender.

It is not widely realised that complete denaturation of protein is not a necessary condition for the extraction of phospholipids. The use of acetone powders in enzyme chemistry is an example but acetone would not effect complete extraction of phospholipids. However, Morton (1950) who introduced fractionation with butanol at low temperature to obtain in water solution a number of enzymes previously known only in particulate form, claims that solubilisation is accompanied by complete liberation of phospholipids (private communication).

The usefulness of butanol as an extractant for phospholipids was tested as a matter of interest, with the following procedure:

Freshly excised rat liver (1.9 gm.) was homogenised in a Waring blender at 0°C. in 0.9% potassium chloride for about two minutes. The homogenate was freeze-dried and suspended in n-butanol at -10°C. overnight. After centrifugation, the butanol extract was taken to dryness in vacuo, but on account of the low volatility of butanol, the temperature was raised to about 80°C. After
centrifugation, the butanol extract was taken to dryness in vacuo, but on account of the low volatility of butanol, the temperature was raised to about 80°C. After acid-washing the fat extracted weighed 91.2 mgms. The analytical results were as follows (expressed as μ moles / gm. fresh tissue); total phosphorus, 26; choline-nitrogen, 17; amino-nitrogen (ninhydrin method cf. Chap. 6), 4.4.

If these results are compared with those in Table 10 of Chapter 6, it will be seen that dry butanol extraction provides excellent replication of the results of methanol:chloroform extraction of the wet tissue, with regard to phosphorus and choline but amino-nitrogen is low. It therefore seems probable that lengthy heating in butanol has caused some loss of lipid amino groups. This result emphasises the caution necessary in interpreting the results of new extraction procedures.

(b) Purification.

Introduction.

A troublesome feature of the polar nature of phospholipids is their ability to solubilise substances which are not, in the pure state, soluble in fat solvents. This includes both inorganic and organic material. The nature and extent of the contamination depends on the tissue extracted. Frequent contaminants of lipids from animal sources are urea, taurine and purine bases; but amino acids and peptides may be expected in most impure phospholipid preparations. However the distinction between contaminants and what may be genuine, but unrecognised lipids, is not always clear cut. Wynn and Williams (1950) first reported the presence of
petroleum ether-soluble and non-dialysable peptides in blood plasma which had been deproteinised and desalted. This observation has been confirmed (Bliss, Ruhi, Lecomte and Macheboeuf, 1953) and from the latter paper it appears that there is at least as much of this material in blood plasma as there is cephalin which indeed is low (only 5-6% of total phospholipid). Substances of this type have been isolated from brain by Folch and Lees (1951) and termed "proteolipides"; one proteolipide was isolated in crystalline form. "Strandin", also described by Folch, is of a similar nature (Folch, Arsove and Meath, 1951). These proteolipides are only a minor component of brain phospholipid. It is surprising that Folch and Lees (1951) were unable to demonstrate their presence in blood plasma.

It has repeatedly been shown that Bloor's extract contains considerable quantities of non-lipid impurities (e.g., Brante, 1949). The amount of attention devoted to the removal of impurities has varied a great deal. Even some experienced workers (Artom and Fishman, 1943; Levine and Chargaff, 1952) are satisfied to take the initial extract to dryness and re-extract with chloroform or petrol. Such a procedure is without doubt inadequate, as my own results will show.

Some workers have attempted to remove water-soluble material from the tissue prior to lipid extraction: precipitation of plasma lipoproteins with colloidal iron has been employed by Folch and van Slyke (1939) while Dawson (1954 b) washes with trichloroacetic
acid a precipitate which includes proteins and bound lipids.

Phospholipids have most frequently been purified after extraction either by acetone precipitation from an emulsion in water or saline (Chargaff, Ziff and Rittenberg, 1942) or by dialysis (Folch, 1942). Evidence will be presented to show that the latter procedure is inadequate, at least so far as brain cephalin is concerned.

Dialysis was employed by Sinclair (1948) in a study of the atomic ratio of nitrogen to phosphorus in plasma phospholipids. In the petrol:chloroform soluble portion of an ethanol:ether extract which had not been dialysed, the atomic ratio of nitrogen to phosphorus varied in a number of species from 1.45 to 2.66. After dialysis the range was 0.99 to 1.39. For a mixture of lecithin and cephalin this ratio should be 1.00; for sphingomyelin, 2.00. Thus, if one were assured of the purity of an extract, it would be possible to calculate the proportions of these three phospholipids from the atomic ratios of total nitrogen to phosphorus and of choline-nitrogen to phosphorus: that is, if there are no complex lipids present. This was attempted by Sinclair (1948), who found values for sphingomyelin in good agreement with those obtained by the method of Schmidt and others (1946).

Only comparatively recently has it been realised that distribution in a two-phase solvent system containing water provides a logical, simple and as far as present experience allows, effective
means of removing non-lipids.

Hevesy (1948) recommended washing an ether:ethanol solution of phospholipids with dilute hydrochloric acid, solely as a means of removing contamination radioactive phosphorus. McKibbin and Taylor (1949) washed a chloroform solution of phospholipids with an aqueous solution of magnesium chloride, the emulsions formed being broken by freezing, thawing and centrifuging. Folch, Ascoli, Lees, Meath and LeBaron (1951) washed chloroform:methanol solutions of brain lipids by submerging the solution beneath a large volume of water and allowing it to stand overnight. Convection in the lipid solution is maintained by diffusion of methanol into the water, resulting in a temporarily heavier surface layer which sinks to the bottom. The process takes place without emulsification. However, such a tedious device is not necessary to prevent emulsification if the proportion of ethanol to chloroform is carefully adjusted. Subsequently, Folch, Lees and Sloane-Stanley (1954) have adapted the system to a separating funnel. Acid-washing has been employed as a routine purification step throughout this work and was carried out with a two-fold purpose: (a) to remove non-lipids; (b) to leave the polar groups of the phospholipid in a characterised form. Native cephalin is known to be associated with a considerable amount of ash (Folch, 1942) on account of its acid nature. In later chapters the preparation of derivatives of native cephalin, involving alteration of the acid
and basic groups, will be described. It was thought that the reactions concerned would take place less ambiguously if the cephalin was free of cations.

Lea and Rhodes (1953), investigating a claim by Bevan, Gregory, Malkin and Poole (1951) of the chromatography of phospholipids on cellulose columns, could not confirm any separation of phospholipids but found in the process a very efficient means of removing non-lipids. In the present work this procedure has been compared with acid-washing. It is essentially filtration rather than chromatography.

Experimental.

A. Experiments with Total Phospholipid Preparation.

The procedures to be described were applied to egg yolk phospholipids, which were extracted as described in the previous section.

Acid Washing of Fat. This was carried out as follows: 0.1 - 1.0 g of phospholipids dissolved in about 150 ml of ethanol: ether (1:9 by vol.) were shaken in a separating funnel with two successive portions of 10 - 15 ml of 0.1N hydrochloric acid and finally with the same volume of water. It is important to allow the phases to become crystal clear before the next washing; this requires only two to three minutes standing. The upper phase was taken to dryness in vacuo. Frequently after removal of the ether, the wet residue has shown a tendency to froth, which can effectively
be overcome by addition of petrol (60 - 80°C) followed by further evaporation. The residue was transferred to a tared flask using petroleum ether, and the weight obtained after removal of solvent.

In the determination of the composition of the total lipids of rat tissues (Chap. 6), the chloroform:methanol (2:1 by vol.) extracts were acid-washed directly, but addition of more methanol was found necessary to reduce the extent of emulsification.

Filtration through cellulose. This was performed using Whatman Standard Grade powdered cellulose, 25 gms. of cellulose being taken for 0.5 gm. phospholipid. The column was prepared in ethanol:ether (1:9 by vol.) and the phospholipid applied in the same solvent, 200 - 250 mls. eluate being collected from a 25 gm. column. The fat recovered was subsequently weighed in the same manner as the acid-washed extract.

For analysis, the cleaned phospholipid was made to volume in petroleum ether, 40-60°C or 60-80°C.

Phosphorus analysis was performed after digestion in perchloric acid, according to the method of Berenblum and Chain (1938b). This method applies to all phosphorus analyses in the thesis.

Nitrogen analysis was by semi-micro distillation (Markham apparatus) after Kjeldahl digestion. (Conway, 1947).

Choline was determined after acid hydrolysis, by precipitation as the phosphomolybdate (Chap. 3).

Ethanolamine was determined after acid hydrolysis, as
2,4-dinitrophenyl ethanolamine (Chap. 4)

B. Experiments with Ox-Brain Cephalin Fractions.

Ox-brain cephalin was prepared according to the method of Folch (1942) and separated into ethanolamine and serine rich fractions by step-wise precipitation from chloroform solution by addition of ethanol. The individual fractions were further dialysed according to Folch's directions.

Acid-washing of the cephalin fractions was carried out without the use of ethanol, as follows: 100 - 200 mgms. fat were dissolved in about 30 mls. ether and thoroughly emulsified with about 10 mls. 0.1N hydrochloric acid (in some cases, 0.5N) in a 50 ml. centrifuge tube. The emulsion was centrifuged at 0°C. until most of the ether layer had cleared. The clear portion was siphoned off, fresh ether added and the contents again shaken and centrifuged. The process had to be repeated four to five times until there was no emulsion at the interface.

Alkali-washing of the previously acid-washed cephalin was effected in a separating funnel, using a system consisting of chloroform:methanol: 0.1N sodium hydroxide in the proportions by volume of 2:1:0.6. The chloroform layer was finally washed with water.

Nitrogen analysis was carried out after Kjeldahl digestion, by the micro-diffusion method of Conway (1947).
Ethanolamine and Serine were determined after hydrolysis and separation on Amberlite IRC-50 by the ninhydrin method (Chap. 4).

Fatty Acid analysis was a micro modification of standard titrimetric methods, described in Appendix 1.

Results.

It will be of interest to record firstly some observations on "petrol soluble" extracts which have not been submitted to further purification.

Without acid-washing, the acetone insoluble fraction of ether extracts of egg yolks has been found to be incompletely soluble in petrol. In one instance, 2.00 gms. acetone insoluble material were suspended in petrol and 1.80 gms. phospholipids recovered in the soluble portion after centrifugation. This "petrol soluble" material was freely soluble in ether, but addition of alcohol (up to 20% by vol.) gave a copious precipitate. At this low concentration of alcohol, the precipitate could not have been cephalin, which is greatly diluted by lecithin in egg yolk. Moreover, 94% of the phosphorus was recovered in the ether-alcohol soluble fraction but only 76% of the weight of the "petrol soluble" extract. Analysis showed that the insoluble material was rich in nitrogen.

The above experience did not occur with ethanol:ether extracts of the yolk residue following ether extraction. This may be because most of the impurities are removed in an initial ether extraction.
TABLE 2.

Column Washing compared with Acid Washing.

* Results expressed as molar % of total phosphorus.

<table>
<thead>
<tr>
<th></th>
<th>Petrol Soluble</th>
<th>Cellulose Column</th>
<th>Acid-Washed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight (mgms.)</td>
<td>450</td>
<td>343</td>
<td>369</td>
</tr>
<tr>
<td>P (µ moles)</td>
<td>430</td>
<td>387</td>
<td>440</td>
</tr>
<tr>
<td>%P.</td>
<td>2.96</td>
<td>3.50</td>
<td>3.70</td>
</tr>
<tr>
<td>N (µ moles)</td>
<td>1580</td>
<td>424</td>
<td>503</td>
</tr>
<tr>
<td>%N.</td>
<td>4.90</td>
<td>1.73</td>
<td>1.91</td>
</tr>
<tr>
<td>aN/P.</td>
<td>366</td>
<td>110</td>
<td>111</td>
</tr>
<tr>
<td>aCholine/P.</td>
<td>72</td>
<td>74</td>
<td>77</td>
</tr>
<tr>
<td>aEthanolamine/P.</td>
<td>25</td>
<td>25</td>
<td>24</td>
</tr>
</tbody>
</table>
or because the presence of ethanol depresses their solubility. For example, the atomic ratio of nitrogen to phosphorus in the petrol soluble portion of an ether extract was found to be 3.7, while in a subsequent ethanol: ether extract, this value was 0.92. After purification on cellulose, the first figure fell to one, while the second did not alter significantly (found 0.95). These observations are contrary to the widely held belief (e.g. Brante, 1949) that the presence of ethanol in the extraction solvent causes solution of non-lipid impurities.

Comparison of a "petrol soluble" extract after acid-washing and column washing is given in Table 2. There is a slightly lower recovery (90%) of fat after column washing as compared with acid-washing. Other differences are barely significant. An interesting observation is the apparent absence of non-lipid phosphorus in the "petrol soluble" extract in contrast to the large amount of non-lipid nitrogen. This is shown by the recovery of 105% of the phosphorus after acid-washing.

One disadvantage of acid-washing in chloroform:methanol is the need to use more alcohol than is the case with ethanol:ether, in order to prevent emulsification. This leads to a greater amount of water in the lipid phase, which involves difficulty in taking to dryness. Another inconvenience is having the lipid phase at the bottom of the separating funnel and the aqueous wash which is to be discarded, at the top. One can therefore recommend the
TABLE 3.

Comparison of the Present Ox-Brain Cephalin Preparations with those of Folch (1942).

Folch's results are shown in brackets.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>I</th>
<th>II</th>
<th>III</th>
</tr>
</thead>
<tbody>
<tr>
<td>%N</td>
<td>1.31(1.15)</td>
<td>1.71(1.62)</td>
<td>1.79(1.78)</td>
</tr>
<tr>
<td>%P</td>
<td>4.83(4.25)</td>
<td>3.24(3.58)</td>
<td>3.50(3.65)</td>
</tr>
</tbody>
</table>

Atomic Ratio,

\[ \frac{N}{P} \]

<table>
<thead>
<tr>
<th></th>
<th>I</th>
<th>II</th>
<th>III</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.60(0.60)</td>
<td>1.17(1.00)</td>
<td>1.13(1.08)</td>
<td></td>
</tr>
</tbody>
</table>
advantages of ethanol:ether over methanol:chloroform.

The present preparation of ox-brain cephalin is compared with that of Folch in Table 3. Both preparations had been dialysed for four days against distilled water. Dialysis does not remove all the ash since Folch found his preparations to contain a high proportion of ash.

The preliminary examination for bases in the fractions was carried out by two-dimensional paper chromatography of both acid and alkaline hydrolysates using in the first dimension, butanol: acetic acid:water (5:1:4 by vol., upper phase) and in the second, water-saturated phenol. The unexpected feature of chromatograms derived from hydrolysates of the dialysed cephalin was the occurrence of a large amount of ninhydrin-reactive substance which could not be identified as either serine or ethanolamine. The conditions of hydrolysis ensured the absence of any but traces of phosphorylated bases. The unknown substance was not identified beyond doubt but its position on chromatograms (Fig.1) suggested glutamic acid. It was found to be most abundant in fraction I but was also present in fraction III and absent from fraction V. Acid washing was effective in removing this material completely. Although there was no sign of gross contamination in fraction V the concentrated acid-washings showed a complex pattern of ninhydrin-reactive substances on two-dimensional paper chromatography.

Table 4 shows the effect of acid-washing on the ox-brain
Two-dimensional paper chromatography of an acid hydrolysate of dialysed fraction I of ox-brain cephalin.
TABLE 4.

Loss of Nitrogen and Phosphorus upon Acid-Washing of Dialysed Cephalin Fractions.

The loss of nitrogen and phosphorus was estimated on the acid-washings. The atomic ratios of nitrogen to phosphorus (N/P) and of amino-nitrogen to phosphorus (NH₂-N/P) refer to the acid-washed fat.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>I</th>
<th>III</th>
<th>V a</th>
</tr>
</thead>
<tbody>
<tr>
<td>N/P</td>
<td>0.76</td>
<td>1.15</td>
<td>1.12</td>
</tr>
<tr>
<td>NH₂-N/P</td>
<td>..</td>
<td>0.72</td>
<td>0.58</td>
</tr>
<tr>
<td>%N lost.</td>
<td>24</td>
<td>2.4</td>
<td>2.5</td>
</tr>
<tr>
<td>%P lost.</td>
<td>40</td>
<td>7.5</td>
<td>1.6</td>
</tr>
</tbody>
</table>

* a washed with 0.5 N hydrochloric acid. The other fractions were washed with 0.1 N hydrochloric acid.
**TABLE 5.**

Comparison of Acid- and Alkali-Washed Cephalin

*(Fraction V of Folch (1942)).*

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Fat Recovered (% by weight)</th>
<th>Phosphorus Content (% by weight)</th>
<th>(^a)N/P</th>
<th>(^b)f.a./P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acid</td>
<td>103</td>
<td>3.68</td>
<td>1.12</td>
<td>1.75</td>
</tr>
<tr>
<td>Alkali</td>
<td>90</td>
<td>2.82</td>
<td>1.22</td>
<td>2.16</td>
</tr>
</tbody>
</table>

\(^a\) Atoms of nitrogen per atom of phosphorus.

\(^b\) Moles of fatty acid per gm. atom of phosphorus.
cephalin fractions. The amount of non-lipids not removed by dialysis is clearly very large in the case of fraction I. The phosphorus appeared to be entirely inorganic phosphate. Paper chromatography of the acid-washings showed that the nitrogen was almost entirely "glutamic acid". Results for free amino-nitrogen also included in Table 4, were surprisingly low.

Alkali-washing of fraction V was carried out with the object of preparing the alkali salt of cephalin. Washing with alkali removed a great deal of the dark brown discolouration of the acid-washed fat which presumably had resulted from autoxidation on storage. However, the most notable result is the increase in the proportion of fatty acids to phosphorus (Table 5).

Discussion.

According to Kaucher, Galbraith, Button and Williams (1943) sphingolipids account for only 2.5% of the total phospholipids of egg yolk. Table 2 shows that 99 - 100% of the total phosphorus is accounted for by lecithin and cephalin on the basis of choline and ethanolamine determinations. Therefore, the atomic ratio of nitrogen to phosphorus should be very close to one if non-lipids are absent. With ether extracts this figure has ranged from 1.0 to 1.1, while for the subsequent ethanol:ether extracts, it has ranged from 0.92 to 0.98 in three different preparations.

Acid-washing and filtration through cellulose both give the
same value for the atomic ratio of nitrogen to phosphorus. A significant difference is the low recovery of fat from paper. Garcia, Lovern and Olley (1956) have reported a selective loss of inositol lipids after passage through cellulose. Bevan, Gregory, Malkin and Poole (1951) and Lea and Rhodes (1953) dissolved the fat in chloroform:methanol for passage through cellulose. In my own preliminary experiments I found no removal of non-lipid nitrogen when ether was used as developing solvent but that the addition of alcohol was necessary, but only to the extent of about 10% by volume. Apart from the partial precipitation of non-lipid nitrogen by ethanol, its presence must establish partition equilibrium between the stationary and mobile phases.

Fractions III and V of ox-brain cephalin which were predominantly phosphatidyl serine and phosphatidyl ethanolamine respectively (Chap. 4) had atomic ratios of nitrogen to phosphorus which were 10 - 15% higher than unity. Only small amounts of non-lipid nitrogen and phosphorus were removed on acid-washing and were not sufficient to alter the ratio of nitrogen to phosphorus significantly. It is possible that the excess nitrogen is in the form of cerebroside, incompletely removed in the ether separation step (see Introduction). However fraction I, a mixture of phosphatidyl serine and phosphoinositide, which had also been dialysed, contained a large amount of non-lipid nitrogen and phosphorus removable by acid-washing. The non-lipid nitrogen may have been glutamic acid
on paper chromatographic evidence. Hächt and Mink (1952), studying the paper chromatography of phospholipids and their hydrolysates, found what they believed on the basis of chromatographic evidence to be glutamic acid in brain cephalin. With very little quantitative data they suggested this may form part of an unrecognised phospholipid. Their purification consisted of dialysis which has here been shown to be inadequate as a means of removing nitrogenous non-lipids, so their glutamic acid was probably an impurity. The low ratio of amino-nitrogen to phosphorus found in fractions III and V is not in agreement with the analysis of Folch who found over 90% of the nitrogen in these fractions to be amino-nitrogen.

The alteration of the molar ratio of fatty acids to phosphorus on alkali-washing (Table 5) may be attributed to removal of lyso-phospholipid although it is not entirely clear why lysophospholipid should partition into the alkali layer. Since the fatty acid/phosphorus ratio has increased following alkali-washing, there is no indication of hydrolysis of the phospholipid, which is in accord with the degree of stability to alkali found by Spiegel-Adolf (1936). The evidence of fractionation by this means prompted further investigation of the partition of cephalin between aqueous emulsions and immiscible or partly miscible solvents. This work will be described in the following section.
c. Attempts at Further Purification of Folch's cephalin by Distribution between Solvents and a Buffered Aqueous Emulsion.

Suitable reference material was an important requirement in the appraisal of new analytical procedures for aminophospholipids. It was hoped that this requirement would be fulfilled by the brain cephalin preparation of Folch (1942), of which fractions III and V were claimed to be very nearly pure aminophospholipid. Unfortunately I was unable to confirm the results of Folch (1942) in all respects and the present and following sections record observations of the heterogeneity and anomalous titration behaviour of cephalin prepared according to the method of Folch (1942).

Experimental.

A. Distribution at pH 10.

In each of four 50 ml. centrifuge tubes, 184 mgms. cephalin (fraction V) were emulsified with 10 mls. water and the emulsion brought to pH 10 with 0.1N potassium hydroxide. 25 mls. ether were added to each tube and the mixture thoroughly shaken. The resulting emulsion could be resolved into a clear pale yellow upper layer and a clear, dark reddish brown aqueous layer with some gel at the interface by centrifuging at 0°C. The ether layer was removed with a pipette and the extraction with ether repeated several times. The combined ether extracts were taken to dryness and weighed. The dark coloured aqueous phase, still containing about 85% of the weight
of phospholipid in clear solution, was then extracted with 25 mls. n-butanol. The pale yellow butanol layer was removed and washed with water, the washings being returned to the original aqueous layer. A second butanol extract which contained only a quarter of the weight of fat in the previous extract, was treated in a similar way. Finally the aqueous layer was acidified with hydrochloric acid and extracted with ether. On this occasion most of the brown colour passed into the ether. The extraction was repeated once.

B. Distribution at pH 8.

While the above experiment was conducted at the arbitrarily chosen pH 10, it was anticipated that a more interesting fractionation might result if a pH was chosen which had some relationship to the buffering capacity of the phospholipid. Titration of the cephalin fractions, which will be reported in section d of this chapter, showed pronounced differences in buffering capacity below pH 8 of fractions I, III, and V. The procedure was the same as described for fraction V at pH 10, an ether extract, a butanol extract and an ether extract after acidification being collected from each fraction separately.

Fatty acids and Phosphorus were determined as described previously.

Nitrogen was determined by the micro-diffusion method.

(Conway, 1947)
<table>
<thead>
<tr>
<th>Fraction</th>
<th>Wt. Fraction (mgms.)</th>
<th>ZP.</th>
<th>Atomic Ratio, N/P.</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Ether Extract, pH 10</td>
<td>91</td>
<td>0.71</td>
<td>1.36</td>
</tr>
<tr>
<td>B. Butanol Extract, pH 10</td>
<td>314</td>
<td>3.02</td>
<td>1.06</td>
</tr>
<tr>
<td>C. Ether Extract after acidification</td>
<td>142</td>
<td>3.34</td>
<td>0.90</td>
</tr>
<tr>
<td>D. Residual Aqueous Phase</td>
<td>...</td>
<td>...</td>
<td>1.39</td>
</tr>
</tbody>
</table>
Bases were examined qualitatively by chromatography of acid hydrolysates in the paper chromatographic system of Levine and Chargaff (1951).

Results.

In the treatment of fraction V at pH 10, three fractions were obtained which contrasted sharply in appearance. The ether extract at pH 10 consisted of a pale yellow oil; the butanol extract was a fluffy white powder; while the acid ether extract consisted of reddish brown flakes. All fractions were soluble in petrol.

Phosphorus and nitrogen analyses are shown in Table 6. With the provision that at pH 10 and 0°C, there is no hydrolysis or re-arrangement of phosphatidyl ethanolamine, there are indications of purification with regard to the atomic ratio of nitrogen to phosphorus. The bulk of fraction V formed a clear solution in water at pH 10 from which it could not be extracted by ether. 57% by weight has been recovered in a butanol extract with an atomic ratio of nitrogen to phosphorus of 1.03, as compared with the starting material which had 1.15 to 1.20.

In contrast to its behaviour at pH 10, fraction V was almost completely extracted into ether at pH 8. Moreover the molar proportions of nitrogen and fatty acids to phosphorus were correct for phosphatidyl ethanolamine in the ether extracted portion (Table 7). On the other hand, fraction III largely concentrated in the butanol extract and it also underwent some purification with regard to the
### TABLE 7.

Partition of Cephalin Fractions at pH 8.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Sub-fraction</th>
<th>Weight (mgas)</th>
<th>N/P</th>
<th>f.a./P</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>a. Ether-sol.</td>
<td>3.5</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td></td>
<td>b. Butanol-sol.</td>
<td>158</td>
<td>0.95</td>
<td>2.07</td>
</tr>
<tr>
<td></td>
<td>c. Butanol-insol.</td>
<td>48.8</td>
<td>0.42</td>
<td>0.86</td>
</tr>
<tr>
<td>III</td>
<td>a. Ether-sol.</td>
<td>8.0</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td></td>
<td>b. Butanol-sol.</td>
<td>229</td>
<td>1.10</td>
<td>2.06</td>
</tr>
<tr>
<td></td>
<td>c. Butanol-insol.</td>
<td>42</td>
<td>1.34</td>
<td>1.64</td>
</tr>
<tr>
<td>V</td>
<td>a. Ether-sol.</td>
<td>138</td>
<td>0.95</td>
<td>1.95</td>
</tr>
<tr>
<td></td>
<td>b. Butanol-sol.</td>
<td>36.2</td>
<td>1.46</td>
<td>1.56</td>
</tr>
<tr>
<td></td>
<td>c. Butanol-insol.</td>
<td>13.8</td>
<td>0.65</td>
<td>...</td>
</tr>
</tbody>
</table>

Sub-fractions "c" refer to the material finally extracted by ether after acidification of the aqueous layer.
above ratios. A similar result was found with fraction I which, like fraction III, is predominantly phosphatidyl serine. However in this case, the material insoluble in ether or butanol must be largely inositol phospholipid, a conclusion which is in accord with the low proportions of nitrogen and fatty acids to phosphorus. A qualitative examination of the bases in the ether extract from fraction V and the butanol extract from fraction III did not reveal the same extent of partitioning, although ethanolamine predominated in the former case and serine in the latter. It was not possible to decide whether this was any better than in the starting material. Determination of free amino groups by direct reaction with 2:4-dinitrofluorobenzene (Chap. 5) showed that neither had there been any increase in the proportion of amino-nitrogen to total nitrogen.

Discussion.

The difference of behaviour between fractions III and V of the cephalin presumably depends upon the availability of acid groups in these fractions. The acidic nature of cephalin was first shown in the titration studies of Grün and Limpöcher (1927) prior to which, cephalin was formulated with a betaine structure. In discussing his fractionation of cephalin Folch (1942) points out that the low solubility in alcohol of fraction I as compared with fraction V (fraction III being intermediate) parallels the ash content of these preparations. The higher ash content of fractions I and III suggests that these are the more acidic fractions. Therefore an additional
parallel is found in behaviour of fractions I, III and V on distribution between water and ether at pH 8.

The difference in behaviour of fraction V at pH 8 as compared with pH 10 is not readily interpreted. Secondary phosphate esters are known to be quite strong acids (pK 1-2) and at pH 8 phosphatidyl ethanolamine should be present entirely as the alkali salt, which partitions into the ether phase. Why then does it remain in the aqueous phase at pH 10? The low proportion of amino-nitrogen to total nitrogen shows that the behaviour observed is not that of pure phosphatidyl ethanolamine. However this low proportion of amino-nitrogen is anomalous, since the cephalin preparation was free of choline (Chap. 3) and these sub-fractions have the correct proportions of nitrogen and fatty acids to phosphorus for a typical phosphoglyceride.

Non-ionised impurities may be expected to pass into the ether phase at pH 8-10. Such an impurity would be cerebroside. The results in Table 6 indicate however, that not much of this material is present as shown by the weight of the fraction and the atomic ratio of nitrogen to phosphorus. The phospholipid remaining after butanol extraction which passed into ether upon acidification, had a very dark colour, suggesting a concentration of the products of autoxidation. If autoxidation results in the formation of acidic groups from centres of unsaturation, the products would be more acidic than unaltered cephalin.
Problematical features of this fractionation are (a) the heterogeneity of Folch's cephalin fractions with regard to the proportion of nitrogen and fatty acids to phosphorus, and (b) the fact that free amino-nitrogen is very much less than total nitrogen even in those sub-fractions which have the correct proportions of nitrogen and fatty acids to phosphorus. The occurrence of plasmalogens, particularly in fraction V (Klenk and Böhm, 1951) is a complicating factor, especially since Klenk and Debuch (1954) have found evidence of complicated structures for plasmalogens. The question of free amino groups in "cephalin" will be discussed more fully in later chapters. In the next section it will be seen that there is also a low titratability of acid groups in Folch's cephalin.

The above indications of a promising separation of acidic phospholipids by a simple distribution procedure may seem surprising in view of the lack of success of counter-current distribution in similar attempts (Lovern, 1953; Lovern and Olley, 1953; Olley and Lovern, 1953). However of the total phospholipids submitted to counter-current distribution, the acidic phospholipids are a minor component and their behaviour must be greatly influenced by other lipids present. Moreover, a limitation of counter-current distribution is that it can only be carried out without emulsification, which imposes a considerable restriction on the choice of solvent systems so far as phospholipids are concerned.
(d) **Titration of Cephalin in an Aqueous System.**

**Experimental.**

A. Titration of Ox-Brain Cephalin Fractions.

3 - 4% dispersions of the acid-washed cephalin fractions in 10 ml. water were titrated with 0.085N potassium hydroxide under a stream of nitrogen. The alkali was added in 0.1-0.2 ml. portions, the emulsion being thoroughly stirred after each addition and a period of 2-3 minutes allowed for equilibration prior to measurement of pH with a glass electrode.

B. Titration of Egg Yolk Phospholipids.

The material studied was the acid-washed acetone insoluble fraction of an ethanol:ether extract of egg yolks. A 5% dispersion in 10 ml. water was titrated under the same conditions as the brain cephalin.

In two additional experiments, samples of the same phospholipid were incubated (a) at pH 9.8 and 37°C, and (b) at pH 3.6 and 37°C, for 24 hours in both cases. The sample incubated at pH 9.8 was acid-washed after incubation and prior to measurement of the titration curve.

**Results and Discussion.**

Titration curves for the brain cephalin fractions are shown in fig. 2. Cephalins contain both acidic and basic groups and are
Fig. 2.

Potentiometric titration of ox-brain cephalin fractions in an aqueous system.
customarily formulated as zwitterions. Consequently the initial pH should be given by the expression

\[ \text{pH} = 0.5 \left( pK_w + pK_a - pK_b \right) \] (Glasstone, 1942)

where \( pK_w \) is the negative logarithm of the ionic product of water, \( pK_a \) is the negative logarithm of the acid dissociation constant and \( pK_b \) is the negative logarithm of the basic dissociation constant.

Phosphatidyl ethanolamine and phosphatidyl serine are secondary esters of phosphoric acid, which are generally quite strong acids. If one substitutes in equation 1 the values of \( pK_a \) for di-n-butyl phosphoric acid (1.72) and \( pK_b \) (= 9.8) for the amino group of glycine one obtains

\[ \text{pH} = 7 + 0.9 - 2.1 \]

\[ = 5.8 \]

This result should be compared with phosphatidyl ethanolamine but not with phosphatidyl serine, which contains the additional carboxyl group. Contrary to this expectation, fraction V where the amino-nitrogen is almost entirely ethanolamine, registered an initial pH of 3.3 and showed only a slight degree of buffering. In the case of fractions III and I where there is marked initial buffering, a more reliable comparison with theoretical expectation might be made (the proportions of serine to ethanolamine being known) if all the available acid were titrated. However the equivalent amount of alkali consumed at pH 8 is much less than the total phosphorus in these preparations.
The low titratability of aqueous cephalin sols was first observed by Christensen and Hastings (1940) although earlier work (Rudy and Page, 1930) had shown that cephalin could be completely titrated with alkali in non-aqueous systems. Christensen and Hastings did not present an analysis of their cephalin preparations, while in the present ox-brain cephalin fractions, amino-nitrogen was equivalent to only 72% of total phosphorus in fraction III and 58% in fraction V. These analyses may be related to the low titratability of the fractions, but then one has to account for non-acidic phosphorus and the fact that on partition at alkaline pH no increase in the proportion of amino-nitrogen to total nitrogen resulted.

An important feature of the brain cephalin is its possible deterioration in the course of a lengthy extraction and precipitation procedure and subsequently upon storage. It was therefore thought necessary to examine the titration behaviour of a freshly prepared sample of phospholipid with the additional objects (a) to observe whether cephalin may be titrated in the presence of lecithin and (b) what kind of treatments may influence the titration curve.

The result for freshly prepared egg phospholipid is shown in fig. 3, curve A. The portion of this curve between pH 2.5 and pH 5.5 is clearly that of a fairly strong acid (pK = 2.7) against strong alkali. The portion between pH 8 and pH 9.3 is that of a
Fig. 3. Potentiometric titration of egg yolk phospholipid in an aqueous system.
weak protogenic base \( (pK_a = 9) \). The situation here is very similar to that experienced with the brain cephalin, inasmuch as at an acid pH there is no apparent dissociation of the basic function. The important difference is that in the case of the egg phospholipid, an independent estimate of lipid amino-nitrogen by reaction with 2:4-dinitrobenzene showed approximate agreement between the acid groups by titration (at pH 5.5, the point of maximum inflection) and amino-nitrogen (shown by arrow at 61 ueq.).

Curves B and C show the titration behaviour of the same preparation of egg phospholipid after mild alkali and acid treatment, respectively. Both treatments have virtually abolished the acid function of the cephalin to give a titration curve of a similar kind to that of fraction V of the brain cephalin. Fractions I and III appear to be intermediate between this and the untreated egg phospholipid. Thus it would seem possible that the ox-brain cephalin fractions have undergone in the course of preparation some form of derangement resembling that shown by the freshly prepared egg yolk phospholipid after the treatment described.

A further feature of curve A which requires comment are the subsidiary inflections about the equivalence point. A similar property is shown by the brain cephalin fractions. In the case of the egg phospholipid, the inflections below pH 7 cannot be accounted for by phosphatidyl serine which is not present (Chap. 6). The first impression is of the presence of additional ampholytes
with progressively weaker acidic and basic groups. This impression gains conviction from later experience with egg phospholipid (Table 4, Chap. 3 and Table 9, Chap 6), where non-choline nitrogen has always been in excess of amino-nitrogen and cephalin determined by titration in non-aqueous medium has always been in excess of cephalin determined as the N-2:4-dinitrophenyl derivative. Derivatives of nitrogen with $pK_a$ from 7 to 8 must be under the influence of inductive effects, such as are found in amides, peptides, etc. Likewise, the $pK$ of ester derivatives of phosphoric acid is markedly influenced by factors such as hydrogen bonding (Kumler and Eiler, 1943) or amide formation. In other words, the occurrence of something like the amide of phosphatidic acid might well explain the anomalous acidity of cephalin sols, as well as the occurrence of non-amino-, non-choline-nitrogen.

Previously, Christensen and Hastings (1940) observed the acidity of cephalin sols but they succeeded in titrating only a small part of the total cephalin. Mild acid and alkali treatment, intended to simulate effects on cephalin during isolation and storage have here been shown to lower the titratability of cephalin. Christensen and Hastings rightly questioned the zwitterion formulation of cephalin on the basis of their results. They suggested that "cephalins may have other formulations than those attributed to them". My own experience of cephalin provides much support for this theme, as will be shown in later chapters.
SUMMARY.

(a) Solubility of a phospholipid extract in ether or petroleum ether is not a reliable indication of the absence of water-soluble contaminants.

(b) Dialysed ex-brain cephalin contains a large proportion of non-lipid amino-nitrogen, probably glutamic acid, which can be removed by acid-washing.

(c) The removal of contaminating nitrogen compounds from an ether solution of total phospholipid by filtration through cellulose, requires the addition of a polar solvent to the ether. There is a slightly low recovery of lipid phosphorus by this procedure, when compared with acid-washing.

(d) Distribution between a buffered aqueous emulsion and ether or butanol has been found to be an effective means for the further purification of Polch's cephalin fractions, with regard to the proportions of nitrogen and fatty acids to phosphorus. However the proportion of amino-nitrogen to total nitrogen remained low.

(e) Aqueous dispersions of cephalin register a low pH, which is contrary to what may be theoretically expected from a zwitterion structure.
An aqueous sol of freshly-prepared egg phospholipid gives a sharp end-point with alkali, which is in approximate agreement with the amino-nitrogen content of the preparations. In contrast, the titratability of Folch's cephalin fractions is low. Mild treatment of phospholipids has been found to affect their titratability.
CHAPTER 3.

DETERMINATION OF CHOLINE-CONTAINING PHOSPHOLIPIDS.

Lecithin, the first phospholipid recognised (Goblely, 1850) is easily the most abundant phospholipid in Nature. Choline isolated from lecithin after hydrolysis was first characterised as the chloroplatinate by Strecker (1868).

For many years, lecithin was the only phospholipid component determined, "cephalin" being obtained by difference from the total phosphorus or nitrogen.

(a) Methods based upon Hydrolysis and Precipitation of Choline.

Introduction.

Lecithin and sphingomyelin are generally accounted for as a group, based upon choline liberated after hydrolysis (total choline-containing phospholipids). Alkaline hydrolysis usually with aqueous baryta, is a long established procedure for splitting off choline from phospholipids. The relative effectiveness of various alkalis has been studied by Brante (1949). While alkaline hydrolysis is usually carried out for about two hours at reflux
temperature, acid hydrolysis is effected by refluxing in constant boiling (about 6N) hydrochloric acid for twelve to eighteen hours. The length of hydrolysis is dictated by the greater resistance of the phosphorylcholine residue in sphingomyelin to both acid and alkaline hydrolysis than the same residue in lecithin.

In 1930, Kapfhammer and Bishchoff introduced Reinecke acid as a precipitant for choline. This reagent \((\text{Cr(NH}_3)_2(\text{SCN})_4\text{H})\) or its ammonium salt, has been widely adopted for the semi-micro determination of choline in tissues (Engel, Salmon and Ackerman 1954). The amount of choline Reineckate formed has been estimated by direct weighing or by measurement of its red colour in acetone solution. Greater sensitivity with the Reineckate method has been obtained by measurement of its optical density in the ultra-violet \((\varepsilon_{327\mu \text{m}} = 5,820)\) or by determination of the chromium content of the precipitate (0.2 - 1.0 \(\mu\) mole choline).

A precipitant less commonly used than ammonium Reineckate is potassium periodide (Best and Lucas, 1943). A maximum of nine atoms of iodine combine with one molecule of choline to form the enneaiodide. Either residual iodine in the precipitant solution or iodine in the precipitate is determined. The precipitate may vary in composition and is unstable and for these reasons, has not met with general satisfaction although improvements continue to appear (Appleton, Levy, Staele and Brodie, 1953).

Other precipitants for choline, which have not been widely
adopted owing to their lack of specificity, are phosphotungstic acid (Gakenheimer and Reguera, 1946), cadmium chloride (Seaman, Hugonet and Leibman, 1949) and mercuric chloride (Chargaff, 1942).

Levine and Chargaff (1951) introduced quantitative paper chromatography for the determination of phospholipid constituents. Choline was determined after chromatography of a lipid hydrolysate by development of a spot with aqueous phosphomolybdic acid followed by washing with iso-butanol, reduction of choline phosphomolybdate with stannous chloride and quantitative planimetry of the molybdenum blue so formed. In an examination of phospholipid fractions from brain, Levine and Chargaff (1951) detected only traces of substances other than choline in hydrolysates, which gave a spot with phosphomolybdic acid. The specificity of phosphomolybdic acid for choline on paper chromatograms suggested that this reagent should be equally specific for choline in vitro. Tests which I carried out with standard choline solutions gave evidence of quantitative precipitation of choline, while reduction of the complex to molybdenum blue furnished a sensitivity of the same order as micro-Reineckate methods.

Experimental.

Materials  Choline chloride was recrystallised from ethanol ether before use and dried over phosphorus pentoxide.

Phosphomolybdic acid whether L.R. or A.R., was
recrystallised from water. Some commercial products contained a large amount of water-insoluble material, or were contaminated with reduction products. An aqueous solution saturated at room temperature was prepared.

*Iso-Butanol* was A.R. or redistilled over alkali.

*Sulphuric acid*, 10N.

*Stannous Chloride* solution consisted of 40% stannous chloride in concentrated (10N) hydrochloric acid.

*Ethanolamine* was distilled before making up a standard solution in water.

*Serine* was B.D.H. laboratory reagent.

Lipid extracts of rat tissues, egg yolk phospholipid and ox-brain cephalin were isolated as described earlier (Chapter 2).

**Hydrolysis Procedure.**

Hydrolysis in 6N hydrochloric acid has been employed routinely for the determination of choline-containing phospholipids. The phospholipid sample (5 - 30 mgms., depending upon the lecithin content) was placed in a 25 ml. round bottomed flask, 3 or 5 mls. of ethanol and 3 or 5 mls. of concentrated hydrochloric acid added, and the flask then placed on the boiling water bath under reflux for about 18 hours.
Subsequently the still acid solution was extracted with three successive portions of ether, the ether layer being removed each time by aspiration. The residual ether in the aqueous layer was removed by cautious warming (a tendency to froth was observed) and the aqueous layer taken to dryness on the bath under a stream of air. The dry residue was made to volume by pipetting in 2.0 or 5.0 mls. water, the flask stoppered and thoroughly shaken.

In one instance hydrolysis was effected with aqueous baryta. Saturated aqueous baryta (2.0 mls.) was added to the fat sample and the mixture heated on the boiling water bath for 5 hours. After neutralisation with carbon dioxide and removal of barium carbonate by centrifugation, the hydrolysate was made acid with one drop of 10\% hydrochloric acid then extracted with ether and taken to dryness and made to volume with water.

**Procedure for Determination of Choline.**

Aliquots (0.5 ml.) of the unknown solution were placed in conical 15 mls. centrifuge tubes. Two drops of saturated phosphomolybdic acid were added, the tubes rotated between the hands and allowed to stand for at least half an hour at 5\(^\circ\)C. The yellow precipitate readily separated out and was packed by a few minutes' centrifuging. After pouring off the supernatant, the tube was stood in an inverted position for at least five minutes; residual drops adhering to the inside wall of the centrifuge tube were removed with a roll of filter paper. It was important that
all the water be drained off before adding iso-butanol (0.5 ml.) otherwise droplets of water interfered with subsequent packing of the precipitate in iso-butanol. The wash with iso-butanol was repeated once. Traces of phosphomolybdic acid remaining on the rim of the tube were removed by wiping with a piece of cotton wool moistened with ethanol. Finally, the precipitate was dissolved in acetone (1 - 2 mls.) and transferred in a total of 3 mls acetone to a test tube with a mark at 10.0 mls. Sulphuric acid (1.0 ml. 10N) was added and the mixture made to volume with ethanol. Reduction to molybdenum blue was brought about by addition of one drop of stannous chloride solution, after which the contents were shaken and the extinction measured in the "Spekker" absorptiometer incorporating Ilford filter number 608.

Results.

Standard Choline Solutions.

As little as 0.3 µ mole of choline in 0.5 ml. can be precipitated with phosphomolybdic acid, as may be seen in figure 1. However, for ease of manipulation the most convenient range is 0.5 - 2.0 µ moles choline. It may be seen in figure 1 that 1.00 density unit, measured on the "Spekker" is equivalent to 2.15 µ moles choline. The factor found independently by Dr. F. D. Collins is 2.07 and since this is based upon wider experience of standard solutions, it has been referred to for all calculations
Fig. 2. Absorption spectra of molydenum blue (open circles) measured within 5 minutes of addition of stannous chloride and of artefact (dots) formed upon allowing the same solution to stand overnight. The ordinates represent optical density in arbitrary units.
of choline in phospholipid hydrolysates.

The absorption spectrum of molybdenum blue in 0.1 N sulphuric acid is shown in figure 2 (curve A). With Ilford filter 608, the portion of the spectrum measured is in the vicinity of 650 - 750 μm. Generally on standing for a few hours and occasionally without standing, a different reduction product shown in curve B, results. In this event an approximate result can be obtained by comparison of curve B and curve A (figure 2).

**Interfering Substances.**

Substances which were expected to interfere were (a) non-lipid impurities; and (b) products of hydrolysis of phospholipids other than lecithin or sphingomyelin.

Examination of Table 2 in Chapter 2 shows that an impure preparation of egg yolk phospholipid having an atomic ratio of nitrogen to phosphorus of 3.7 gave a result not significantly different from that of the same preparation after removal of non-lipid nitrogen. However, the method has not been applied to the determination of total choline in tissues and must be presented as a means for the analysis of purified total lipids for choline.

No interference from ethanolamine or serine was detected below the level of 16 μ moles/0.5 ml. Above this level, ethanolamine forms a precipitate which is only slightly soluble in iso-butanol and is soluble in acetone; serine forms a precipitate which is freely soluble in iso-butanol, so interference is not so serious.
TABLE 1.

Recovery of Choline in the presence of Ethanolamine.

Each sample contained 1.0 μ mole choline.

<table>
<thead>
<tr>
<th>Ethanolamine Added (μ moles)</th>
<th>Optical Density (Ilford Filter No. 608)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.0</td>
<td>0.490</td>
</tr>
<tr>
<td>4.0</td>
<td>0.497</td>
</tr>
<tr>
<td>8.0</td>
<td>0.515</td>
</tr>
<tr>
<td>16.0</td>
<td>0.513</td>
</tr>
<tr>
<td>20.0</td>
<td>0.605</td>
</tr>
</tbody>
</table>
as with ethanolamine. In Table 1 are shown the recoveries of 1.0 \( \mu \) mole of choline in the presence of 2 - 20 \( \mu \) moles of ethanolamine.

Serious interference is due to ammonia. Ammonium phosphomolybdate is insoluble in water and iso-butanol but soluble in acetone. However the solubility in acetone is repressed by the presence of butanol to a much greater extent than that of choline phosphomolybdate. In iso-butanol:acetone (1:4 by vol.) at room temperature, the solubility of ammonium phosphomolybdate, assuming the same factor as for the choline, is 0.42 \( \mu \) moles/ml., while in acetone it is 4.5 \( \mu \) moles/ml. Fortunately ammonia occurs only in traces in phospholipid acid hydrolysates, provided care is taken to avoid contamination from the atmosphere and ammonia should be absent from baryta hydrolysates.

Qualitative tests showed that both acetylcholine and phosphoryl-choline are precipitated by phosphomolybdic acid. The latter substance should not be present under the recommended conditions of hydrolysis.

**Choline in Phospholipid Hydrolysates.**

Choline analyses of both total and fractionated egg phospholipids and the phospholipids of rat tissues are considered in detail in chapter 6 in connection with estimates of non-choline-containing phospholipids. A typical result is illustrated in the
Following comparison of acid and alkaline hydrolysis.

Alkaline Hydrolysis: A sample of egg phospholipid (18.0 mgms.) after barbta hydrolysis, was made to 5.0 mls. Aliquots of 0.5 ml. were taken for choline determination.

Density: 0.69, 0.71

μ moles choline/mgm. phospholipid: 0.80, 0.82.

Acid Hydrolysis: Another sample (10.8 mgms.) of the above preparation of phospholipid after acid hydrolysis, was made to 2.0 mls. Aliquots of 0.5 ml. were taken for choline analysis.

Density: 0.98, 1.06

μ moles choline/mgm. phospholipid: 0.75, 0.81.

Specificity with respect to Phospholipids: Analyses of Cephalin.

Application of the phosphomolybdate procedure to an hydrolysate of Folch's ox-brain cephalin demonstrated that there is negligible interference at a level of 25 μ moles of cephalin-phosphorus/0.5 ml. hydrolysate. The preparation of unfractionated cephalin had not been acid-washed and contained an amino-acid impurity (Chapter 2) in addition to serine and ethanolamine.

A sample of cephalin (37.3 mgms.) containing 4.15% phosphorus, after acid hydrolysis, was made to 1.0 ml. and 0.5ml. was taken for precipitation of choline. A yellowish white precipitate which appeared upon addition of phosphomolybdic acid was insoluble in acetone. The analysis based on the acetone-soluble portion, was as follows:-
Density: 0.125

µ moles choline/µ mole phosphorus: 0.01.

While Folch's cephalin has a high content of amino-acids, the cephalin of egg yolk contains only ethanolamine (Chapter 6). The isolation of egg yolk cephalin by chromatography on silica gel (Chapter 6) permitted a direct test of interference from the cephalin fraction of egg yolk phospholipid.

A sample of the cephalin fraction (5.6 mgms.) after acid hydrolysis, was made to 2.0 mls. A 0.5 ml aliquot gave no sign of a precipitate after addition of phosphomolybdic acid, even after standing for two days at 5°C.

Recovery of Choline in a Lipid Hydrolysate.

The recovery of varying amounts of choline added to an acid hydrolysate of rat liver lipids is shown in Table 2.

Comparison with the Reineckate Procedure.

The photometric Reineckate procedure of Glick (1944) was applied to acid hydrolysates of liver and brain phospholipid of the rat (Table 3) which were also analysed by the phosphomolybdic acid method.

For both Tables 2 and 3 I am indebted to Dr. F. D. Collins, who has jointly studied the method.

Discussion.

The manner in which choline combines with phosphomolybdic acid
<table>
<thead>
<tr>
<th>Choline in Hydrolysate</th>
<th>Choline Added</th>
<th>Total Found</th>
<th>% Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>(µ moles)</td>
<td>(µ moles)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.06</td>
<td>0.28</td>
<td>1.34</td>
<td>1.34</td>
</tr>
<tr>
<td>&quot;</td>
<td>0.83</td>
<td>1.69</td>
<td>1.88</td>
</tr>
<tr>
<td>&quot;</td>
<td>1.11</td>
<td>2.16</td>
<td>2.13</td>
</tr>
<tr>
<td>&quot;</td>
<td>1.39</td>
<td>2.45</td>
<td>2.44</td>
</tr>
<tr>
<td>&quot;</td>
<td>1.67</td>
<td>2.73</td>
<td>2.68</td>
</tr>
</tbody>
</table>
Comparison of Phosphomolybdate and Reineckate Methods.

(with the permission of Dr. F.D. Collins)

<table>
<thead>
<tr>
<th>Material</th>
<th>Phosphomolybdate</th>
<th>Reineckate</th>
</tr>
</thead>
<tbody>
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<td>Liver</td>
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<td></td>
</tr>
<tr>
<td>1.</td>
<td>156</td>
<td>160</td>
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<tr>
<td>2.</td>
<td>49.3</td>
<td>51.3</td>
</tr>
<tr>
<td>3.</td>
<td>49.3</td>
<td>53.3</td>
</tr>
<tr>
<td>Brain</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.</td>
<td>24.1</td>
<td>26.7</td>
</tr>
<tr>
<td>2.</td>
<td>21.0</td>
<td>22.1</td>
</tr>
<tr>
<td>3.</td>
<td>22.6</td>
<td>23.6</td>
</tr>
</tbody>
</table>
is not known for certain. It was observed that under conditions where phosphomolybdic acid is quantitatively extracted from water into iso-butanol (0.1 N sulphuric acid), the choline phosphomolybdic acid complex remains in the aqueous phase. This suggests that the compound is not simply a salt, but for simplicity it will be referred to as "choline phosphomolybdate". Conversion of inorganic phosphate to phosphomolybdic acid followed by reduction to molybdenum blue is the basis of the method of Berenblum and Chain (1938 b) for determination of phosphorus. In a study of the conditions affecting the yield of molybdenum blue, Berenblum and Chain (1938 a) found maximum ease of reproducibility in 1.0N - 1.2N sulphuric acid. Below this concentration of acid, the yield of colour is greater, but much more critically dependent upon the concentration of acid.

If the density of a solution of molybdenum blue is measured with Ilford filter 508, 1.00 density unit is equivalent to 0.69 μ mole phosphorus/10 mls. by the method of Berenblum and Chain and 2.07 μ moles choline/10 mls. by the present method for choline. The ratio 2.07/0.69 = 3.0 implies that three molecules of choline combine with one "molecule" of phosphomolybdic acid. The "molecular weight" of phosphomolybdic acid being 3940, the equivalent weight for choline is 1313 gms./mole choline. This is about four times the equivalent weight of Reinecke acid (337 gms./mole choline) and is of the same order as iodine in the enneaiodide
All three methods require the isolation of a precipitate, the quantity of which is the limiting factor in the sensitivity of the determination.

The specificity of phosphomolybdic acid as a reagent for choline in phospholipid hydrolysates is attested by the paper chromatographic analyses of Levine and Chargaff (1951) who analysed a variety of factions of brain phospholipid. Under the conditions of the present method, ethanolamine begins to precipitate at the level of 20 μ moles/0.5 ml., there being no interference when 16 μ moles are present. This means that 3% lecithin in the presence of 97% phosphatidyl ethanolamine can be estimated, provided that the aliquot in which choline is to be precipitated contains no more than 0.5 μ mole choline.

In no preparation of phospholipid or lipid extract that I have analysed have I been able to account for total nitrogen in terms of choline- and amino- nitrogen. One would not expect this to be the case if phosphomolybdic acid lacked specificity as a precipitant for choline.

Table 3 shows that there is good agreement between the phosphomolybdate and Reineckate procedures, for the phospholipid of liver and brain. The Reineckate method is generally regarded as superior to the enneaiodide which requires very careful controls for stoichiometric reaction. Advantages which phosphomolybdic acid possesses over Reinecke acid are (a) greater quantity of precipitate
formed; (b) the stability of the phosphomolybdate compared with the Reineckate, which is sensitive to light and (c) reliable photometric measurement with visible light, while for comparable sensitivity the light absorption of the Reineckate must be measured at 327 μm where there is a greater possibility of error due to lipid decomposition products, etc. The disadvantage of the present method is the interference of ammonia, which is not precipitated by Reinecke acid.

Quantitative aspects of the precipitation of phosphorylcholine by phosphomolybdic acid were not studied. However, in conjunction with the use of lecithinase D which removes phosphorylcholine from lecithin, the conversion of phosphorylcholine to the phosphomolybdate promises a valuable approach to the determination of the specific radioactivity of lecithin labelled with P³². All that is required is reduction of the phosphorylcholine-phosphomolybdic acid complex to molybdenum blue, followed by photometric and radioactivity (liquid counter) measurements on the same sample.

(b) Alternative Procedures for the Determination of Choline-containing Phospholipids.

Lecithin may be obtained pure by chromatography, but the procedure is not quantitative and is time-consuming (Hanahan, Turner and Jayko, 1951). A simplified adsorption procedure, whereby the choline-containing phospholipids are eluted from magnesium oxide with methanol (Taurog, Enterman, Fries and
Chaikoff; 1944) requires differential saponification for the
determination of the lecithin component (Schmidt, Benotti, Hershman
and Thanhauser, 1945). In the present work, elution from magnesium
oxide was examined to obtain an estimate of the specific radioactivity
of the choline-containing phospholipids in a rat liver labelled
in vivo with $^{32}$P, but was not employed routinely.

Non-aqueous titration, which has been applied to the
determination of cephalin, has not yet been successfully adapted
to the determination of lecithin. Fishgold and Chain (1935)
who discussed non-aqueous titration of phospholipids with both acid
and alkali, found that all phospholipids have the property of
binding one mole of hydrogen ions in acid reaction but in alkaline
reaction, they only give up hydrogen ions so far as they possess
amino groups. Since Fishgold and Chain (1935), there have been
considerable advances in the practice of non-aqueous titrations
(for example a review by Riddick, 1954). Direct determination of
total phospholipids by acid titration, followed by cephalin by
alkali titration, from which data choline-containing phospholipids
may be calculated by difference, represents a rapid and simple
procedure for ascertaining the approximate phospholipid composition.

**Experimental.**

**Materials.** Dioxane, redistilled.

Acetic Acid, A.R.
Perchloric Acid, 72%, A.R.

Mercuric Acetate. A 6% solution in glacial acetic acid.

**Technique of Titrations.**

For acid titration a sample containing 0.5 - 1.0 mM total phospholipid, was dissolved in 5.0 mls. glacial acetic acid or acetic acid: dioxane (3:17 by vol.) to which was added 1.0 ml mercuric acetate solution. The titrant consisting of 0.05 - 0.1 M perchloric acid in dioxane, was added in portions, the E.M.F. being read after each addition of acid with a glass electrode. The perchloric acid solution was standardised potentiometrically with potassium hydrogen phthalate.

Alkali titration was generally carried out by the procedure of Rudy and Page (1930). A 2.0 ml. aliquot of the phospholipid solution in benzene was titrated with 0.01 - 0.02 N alcoholic potassium hydroxide, employing phenolphthalein as indicator.

The phospholipid preparation employed in this work was fraction 7 of Table 9, Chapter 6 and was obtained from egg yolk lipid by solvent fractionation of the cadmium chloride complex.

**Results and Discussion.**

Differential plots of the titration of total nitrogen are shown in figures 3 and 4. In fig. 3 the solvent containing the fat was acetic acid; in fig. 4, acetic acid : dioxane (3:17). Mercuric
Fig. 3.

Potentiometric titration of egg yolk phospholipid dissolved in glacial acetic acid, with perchloric acid in dioxane as titrant. $\Delta E$ is the change in potential (m. volts) measured with the glass electrode, brought about by addition of $V$ mls. of 0.074 N perchloric acid.
Comparison of Titration and Other Analytical Data

on a sample of Egg Yolk Phospholipid

The data refers to fraction 7 of the third fractionation of egg yolk phospholipids, Table 9, Chapter 6. Results are here expressed as total μ moles in the preparation.

<table>
<thead>
<tr>
<th>Component</th>
<th>Method of Determination</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>P.</td>
<td></td>
<td>1090</td>
</tr>
<tr>
<td>N.</td>
<td></td>
<td>1250</td>
</tr>
<tr>
<td>Total Phospholipid</td>
<td>Perchloric acid Titn.</td>
<td>1210</td>
</tr>
<tr>
<td>Cephalin.</td>
<td>Alkali Titn.</td>
<td>660</td>
</tr>
<tr>
<td>Choline-containing</td>
<td>As 2,4-dinitrophenyl deriv.</td>
<td>478</td>
</tr>
<tr>
<td>Phospholipid.</td>
<td>From titration data, by difference.</td>
<td>550</td>
</tr>
<tr>
<td>Choline-containing</td>
<td>As choline phosphomolybdate, after hydrolysis.</td>
<td>545.</td>
</tr>
</tbody>
</table>
Fig. 3.

Potentiometric titration of egg yolk phospholipid dissolved in glacial acetic acid, with perchloric acid in dioxane as titrant. $\Delta E$ is the change in potential (m. volts) measured with the glass electrode, brought about by addition of $V$ mls. of 0.074 N perchloric acid.
chloride was added to the sample for titration in order to remove from the system any anions which may possibly be present e.g. chloride, as a result of washing with hydrochloric acid; although in view of the strong dissociation of the secondary phosphate ester, it is doubtful whether extraneous anions would be present in significant amount. The first maximum at approximately half the titre when the fat is titrated in glacial acetic acid, is reduced to an inflection when 85% dioxane is used as solvent. This is in accord with the presence of two bases, the difference in pK being magnified in the more protogenic solvent acetic acid. Since the pK\textsubscript{b} of choline may be expected to be lower than that of the amino group, the first peak presumably records the lecithin content and the second, the total nitrogen of the sample. Perchloric acid was chosen for the titration because it is the strongest of acids and completely dissociated even in protogenic solvents. However, owing to the fairly strong acidic properties of the secondary phosphate ester, the difference in apparent pK\textsubscript{b} of lecithin and cephalin is not great enough to permit an accurate estimate of the two main components of egg phospholipid in the solvent systems tested here. Consequently, an independent estimate of cephalin has been obtained by titration with alkali in a largely aprotic solvent which represses the acidic dissociation of the amino group but has no conspicuous effect on the dissociation of the quaternary ammonium base.
In table 4 are given some data on the composition of fraction 7 together with titration data.

Perchloric acid titration gives a result for total phospholipid in close agreement with lipid nitrogen which is about 15% higher than lipid phosphorus. The total nitrogen comprises roughly equal amounts of choline (after hydrolysis) and amino groups (by direct reaction with 2,4-dinitrofluorobenzene, Chapter 6). This is in agreement with the titration curve which shows approximately equal amounts of two bases. The estimate of cephalin by titration is somewhat in excess of free amino groups and this has been found to be generally the case (Chapter 6). However if cephalin by titration is subtracted from the total nitrogen, the difference representing non-amino nitrogen, is in close agreement with choline-nitrogen, determined by precipitation of the phosphomolybdate after hydrolysis.

There have been no improvements in the titration of lipid nitrogen, since Fishgold and Chain (1935) employed benzene-alcohol mixtures with perchloric acid as titrant and dimethyl yellow as indicator. By this means the nitrogen of lecithin and cephalin and half the nitrogen (presumably choline-nitrogen) of sphingomyelin could be titrated. The present work, although not carried out routinely, has confirmed that a good estimate of lipid nitrogen can be obtained by direct titration; moreover, by carrying out the titration potentiometrically in a suitable solvent, there is an
indication that lecithin and cephalin may be titrated separately. An improvement in the direct titration of lecithin in the presence of cephalin should result when native cephalin is converted to its 2,4-dinitrophenyl derivative which is an exceedingly weak base, but this possibility has not been examined.
CHAPTER 3.

SUMMARY.

(a) A new method for the determination of choline in hydrolysates of acid-washed lipid extracts has been described.

(b) There is substantial agreement in choline analysis between:

1. Acid and Alkaline hydrolysates;
2. The present method and the Reineckate method described by Glick (1944).

(c) The method has limitations,

1. When ammonia is present;
2. If phosphatidyl ethanolamine makes up more than 95% of the total phospholipid.

(d) A potentiometric study of the titration of phospholipid with perchloric acid has shown promise of a rapid method for the simultaneous determination of choline-containing and amino-nitrogen-containing phospholipids.
CHAPTER 4.

THE DETERMINATION OF ETHANOLAMINE AND SERINE IN PHOSPHOLIPID HYDROLYSATES.

The term "cephalin" was coined by Thudichum (1884) to name a lipid fraction which gave ethanolamine on hydrolysis. He believed the ethanolamine to be an artefact formed from choline, which had already been known for some thirty years to be present in lipid hydrolysates. It was not until the work of Renall (1913) and Baumann (1913) that the base in the cephalin fraction was shown to be largely ethanolamine. Only in recent years has the amino-acid serine been shown to be a component of phospholipids. (Folch, 1942).

Introduction.

If choline is determined as an estimate of choline-containing phospholipids then the difference between total phosphorus and choline on a molar basis has commonly been taken as an analytical figure for aminophospholipid (cephalin). This procedure has been applied to blood by Hack (1947) and Sinclair (1948) and to animal tissues by Kaucher, Galbraith, Button and Williams (1943). The results of these workers are frequently quoted but there is evidence to believe that they are misleading. - Urban (1937)
found that in extracts of heart tissue 22% of the total phospholipid nitrogen could not be accounted for as choline or amino-nitrogen. Employing an isotope dilution technique, Chargaff, Ziff and Rittenberg (1942) found compelling evidence of the existence of non-amino and non-choline nitrogen in carefully purified extracts of liver, brain and heart. These authors also found a decrease in amino-nitrogen on storage of lipid extracts.

As opposed to the indirect determination of cephalin, a more reliable estimate is only obtained by determination of the characteristic moiety, viz., the free amino group. This has generally been carried out after hydrolysis of the phospholipid.

In the past, a variety of methods for the hydrolysis of lipids has been used for the liberation of the amino-nitrogen but without any definite knowledge of the resistance to hydrolysis of the bonds concerned. Hydrolysis was effected at reflux temperature in the following examples: 3 hrs. in 6N methanolic HCl (Artom, 1945); 24 hrs. in 6N HCl (Levine and Chargaff, 1951); 18 hrs. in 1.0 N aqueous HCl (Chargaff, Ziff and Rittenberg, 1942). It is only very recently that attempts have been made to elucidate the mechanism of hydrolysis of phosphoglycerides. Schmidt, Bessman and Thannhauser (1953) found that the ester bond from ethanolamine to phosphoric acid is much more labile in glycerclylphosphoryl-ethanolamine than in phosphorylethanolamine both in acid and alkali and that the ethanolamine of glycercylphosphorylethanolamine is released without
formation of phosphoric acid. Baer, Stancer and Korman (1953) studying the hydrolysis of synthetic cephalin and glycerylphosphorylethanolamine, suggested that acid and alkaline hydrolyses proceed via cyclic ortho esters.

The longest-standing method for amino-nitrogen is the gasometric procedure of van Slyke (1911). This has been used frequently for analysis of amino-nitrogen in phospholipid hydrolysates (Levene and Rolf, 1925; Rudy and Page, 1930; Folch, 1942; Klenk and Debuch, 1954). The method may be applied on a scale of 5 - 50 \( \mu \) moles of amino-nitrogen (van Slyke, 1929).

Following the discovery of serine in phospholipids (Folch, 1942), Artom (1945a) introduced a semi-micro method for the determination of both serine and ethanolamine in phospholipid hydrolysates, in which serine is separated from ethanolamine by selective adsorption on permutit. By the action of periodic acid which is a specific reagent for vicinal hydroxyl and amino groups, the bases were decomposed to formaldehyde which was determined iodometrically. The method requires 10 - 50 \( \mu \) moles of base and is thus suitable for the analysis of tissue lipid extracts. The analyses by Artom (1945b) of rat tissues employing this method, are in fact the only comprehensive direct analyses for aminophospholipids in tissues that are available.

A more sensitive variation of periodate oxidation is the method of Burmaster (1946) who estimated ammonia liberated instead of formaldehyde, by the micro-diffusion technique of Conway (1947).
Burmaster's method may be used for the determination of amino-
nitrogen but serine must be determined separately. This makes
the overall sensitivity considerably less, serine being generally
a minor component of phospholipids.

The gasometric method for amino-acids described by van Slyke
and Dillon (1938) may be used for the separate determination of
serine, either on the intact phospholipid (Pfolch, 1948) or on the
hydrolysate (Chargaff, Ziff and Rittenberg, 1942). The principle
of this method is the determination of carbon dioxide formed by the
decarboxylation of an amino acid or its derivative (ester or mono-
substituted nitrogen) by ninhydrin. By suitable variation of
apparatus the gasometric method is capable of a wide range of
sensitivity, with a macro range of 0.8 - 4.1 mgms. carboxyl-
nitrogen and micro range of 0.04 - 0.21 mgms. carboxyl-nitrogen.
This method may have special advantages if one wished to recover
the carboxyl-carbon or carboxyl-oxygen of serine since no separation
of the serine-containing phospholipid from a mixture of phospholipids
would be called for.

The introduction of paper chromatography as an aid to the
analysis of aminophospholipids (Levine and Chargaff, 1951) has
greatly improved the sensitivity of the determination of ethanolamine
and serine but is subject to considerable errors unless great care
is taken. The method will estimate 5 - 20 μgms. amino-nitrogen was
successfully applied by Levine and Chargaff (1952) from mitochondria
and microsomes. Separation of the bases on a paper chromatogram is followed by elution and estimation of amino-nitrogen by the ninhydrin reaction of Moore and Stein (1953).

The work to be described commenced with a study of the ninhydrin method which was the most sensitive method available for the determination of amino-nitrogen. For reasons which will be discussed, paper chromatography as a means of separating serine and ethanolamine in hydrolysates was abandoned in favour of ion-exchange.

Following the successful application of fluoro-2,4-dinitrobenzene (FDNB) to the direct determination of lipid amino-nitrogen (Chap. 6.), this reagent was applied in a similar way to the determination of amino-nitrogen in hydrolysates. The FDNB method is very much simpler than previously described methods for the determination of serine and ethanolamine in phospholipid hydrolysates, with practically no loss of sensitivity by comparison with the ninhydrin method.

Experimental.

A. Determination of Amino-nitrogen by Reaction with Ninhydrin.

Materials.

Amberlite Resins IRC-50 and IR-100 (H);
Phosphate buffer 0.1 M, pH 6.8;
Sodium hydroxide, 0.5 M;
Standard solutions, each 0.0025 M, of ethanolamine
and serine in the above phosphate buffer; Ninhydrin, A.R., 5% solution in ethanol or cellosolve; Pyridine-cellosolve-potassium cyanide reagent. This was prepared from ethyl cellosolve, which had been refluxed over aluminium filings and potassium hydroxide and distilled in vacuo. The redistilled cellosolve (125 mls.) was mixed with pyridine, A.R. (80 mls.) and water added to make 250 mls. The mixture was run through a column of IR-100 (H) about 12" x 0.66", with a flow rate of about 2 mls./min. The resin was regenerated with dilute HCl immediately after use, as it slowly dissolves on standing in a mixture of cellosolve and pyridine. Finally 2.5 mls. 0.01 M potassium cyanide /250 mls. was added to the resin-treated solvent mixture. The reagent was freshly prepared every three days.

Procedures.


Hydrolysis was effected with either 6 N methanolic HCl, prepared by passing dry HCl into cooled methanol or with equal parts of 11 N HCl and ethanol, at reflux temperature for 3 hours unless otherwise stated. Fatty acids were removed in the manner described in Chapter 3 for the determination of choline and the residue was made to volume in phosphate buffer. Sufficient lipid was taken for
hydrolysis so that the buffer solution contained a total of 1 - 2 
μ moles of amino groups/. 0.2 ml.

2. Preparation and Use of Ion-exchange Resin.

The resin Amberlite IRC-50 was sifted and the fraction passing
commercial mesh size 40 was used. The resin was cleaned by
allowing it to stand (a) in 0.5 N sodium hydroxide, 15 mins.; (b)
in two successive portions of water; (c) in 1.0 N hydrochloric acid,
15 mins.; (d) in water, until the supernatant was only weakly acid.
The moist resin was then suspended in the phosphate buffer and
sufficient 0.5 N sodium hydroxide added in portions to maintain
the pH of the buffer for 15 mins. The resin was finally rinsed
with buffer and suspended in the buffer for the preparation of the
column. A column of resin 4" long was prepared in a chromatography
tube of 0.25" internal diameter by pouring in a slurry in four or
five portions, each portion being carefully packed in with a glass
rod. The columns prepared have had a flow rate of about 1.0 ml./
min. They did not run dry, so there was no need for a rubber tube
and clamp.

A 0.2 ml. aliquot of the unknown solution was placed on the
column and washed down with 0.3 ml. buffer, so that 0.5 ml. effluent
passed into the first of 24 test-tubes (with a 10 mls. mark) contained
in a test-tube rack beneath the column. The rack was then moved so
that the next tube came under the column and a further 0.5 ml.
buffer added to the top of the column. This process was repeated
for the first 8 tubes, when the buffer was replaced by 0.5 N sodium
hydroxide until a total of 24 tubes had been collected. The process took no more than 15 mins.

2. Determination of Amino Groups.

Each eluate was made acid by the addition of one drop of 11 N hydrochloric acid followed by the addition of 2.0 mls. pyridine-cellosolve-potassium cyanide reagent and 0.2 ml. ninhydrin solution. The tubes were then covered and placed in a beaker of boiling water for 15 mins., subsequently transferred to a beaker of cold water and the contents of each tube made to 10 mls. with distilled water. The optical density was read in the "Spekker" absorption meter Ilford filter 606. Amino-nitrogen (µ moles) was calculated from the optical density by use of the factor 0.46. The blank correction was obtained from the trough between the serine and ethanolamine peaks.

B. Determination of Amino-nitrogen by Reaction with Fluoro-2,4-dinitrobenzene.

Materials.

Fluoro-2,4-dinitrobenzene was made by L. Light and Co.

For use in kinetic experiments it was re-crystallised from ethanol. Serine and ethanolamine were the same material as used previously and ethanolamine phosphoric acid was a sample prepared by Dr. F.D. Collins, who used the method of Cherbuliez and Weniger (1946).

Triethylamine was distilled under a fractionating column and
the middle fraction collected. A 0.1 M solution in ethanol was prepared.

2,4-Dinitrophenyl serine (DNP-serine) was prepared according to the method of Porter and Sanger (1948) who gave the melting point as 199°C. The preparation upon which figure 7 is based had a melting point (corrected) of 195°C.

2,4-Dinitrophenyl ethanolamine (DNP-ethanolamine) was prepared by the procedure described in Appendix 2. The melting point was 90°C., which agrees with that found by Axelrod, Reichental and Brodie (1953) who prepared it using the method of Sanger (1945).

Celite used in the chromatography was "hyflo-supercel" manufactured by the Johns-Manville Co. of New York.

Magnesium trisilicate was the B.D.H. product for chromatographic purposes.

Solvent A consisted of petroleum ether (60°-80°): acetone, 95:5 (by vol.).

Solvent B consisted of petroleum ether (60°-80°): acetone, 4:1 (by vol.).

Solvent C was acetone.

Solvent D consisted of acetone:acetic acid, 98:2 (by vol.)

Solvent E consisted of ethanol:water, 1:1 (by vol.).

Dimethylformamide and Tetramethylammonium hydroxide were B.D.H. L.R. grade. The latter was an aqueous solution (25%, w/v).
1. **Hydrolysis Procedure.**

A sample of fat expected to contain 2 - 10 μ moles amino-nitrogen was heated on the boiling water bath for a period of three hours in about 5 mls. of 6 N hydrochloric in equal parts of ethanol and water. After removal of fatty acids and evaporation of the acid layer to dryness, the residue was made to volume in water.

2. **Reaction with Fluoro-2,4-dinitrobenzene (FDNB).**

To 0.5 ml. hydrolysate containing 1 - 5 μ moles total amino groups, were added 0.5 ml. ethanol, 1.0 ml. 0.1 N triethylamine and one drop of FDNB. The mixture contained in a covered 1" boiling tube was placed in a water bath at about 50°C. for 5 - 10 mins., when it was taken to dryness in vacuo in order to remove triethylamine. The residue was then taken up in 1.0 ml. 1.0 N ethanolic hydrochloric acid and again taken to dryness in vacuo.

3. **Preparation and Use of Column.**

Chromatography of the DNP bases was carried out on a mixed-bed adsorbent made up of celite (2 gms.) and magnesium trisilicate (1 gm.). When the reaction products were chromatographed on this adsorbent, dinitro-phenol (side-product from FDNB) formed a yellow band which was not easily separated from DNP-ethanolamine. Since the reaction products had previously been taken to dryness in the presence of
Fig. 1.

Jacket employed for protection of chromatography column from daylight.
hydrochloric acid, the formation of a coloured band by
dinitrophenol indicated that the adsorbent must be capable
of reacting as a base under the conditions of chromatography.
In later work the adsorbent was modified by acid-washing with
great improvement in the separation of dinitrophenol. However
the behaviour of the adsorbent in its original form will be
described by reference to the analysis of a standard solution
of serine and ethanolamine.

Serine (2.02 μ moles) and ethanolamine (2.64 μ moles) in
0.5 ml. 0.02 N hydrochloric acid were reacted with FDNB and the
reaction products transferred to a column of mixed adsorbent
(3 gms.) in Solvent A. Dinitrophenol was eluted as a coloured
band in petroleum ether : acetone (1:1 by vol.) Acetone eluted:
DNP-ethanolamine while DNP-serine was removed with a solvent
consisting of acetic acid : acetone : petroleum ether (6 : 12 : 82 by vol.). All stages of the chromatography were carried out
with careful protection from light using a jacket of the design
shown in figure 1. Low recoveries of DNP-serine with evidence
of breakdown products in the absorption curve, resulted when
the chromatography was carried out in light but DNP-ethanolamine
was unaffected. After taking to dryness the last two eluates
were dissolved in 0.1 N hydrochloric acid in ethanol and the
extinction was measured at 350 and 345 mμ respectively.

The acid-washed adsorbent was employed for most analyses of
lipid hydrolysates. Acid-washing was carried out by suspension of the adsorbents in roughly equal parts of ethanol, glacial acetic acid and water, warming on the boiling water bath for a few minutes and filtration through a sintered glass funnel under suction. The mixed adsorbent was finally washed several times with acetone, the suspension being thoroughly mixed each time and rapidly sucked dry, then placed in an oven at 110°C. for at least an hour before use.

A column about 2" high and 0.5" in diameter was prepared by suspension of the mixed adsorbents in Solvent A. If a fairly thick slurry is used there is little tendency for the adsorbents to separate. The column was packed in two or three sections with the aid of a plunger and air pressure from the compressed air mains (5 lbs./in.²).

In an analysis of sheep brain fat (chloroform : methanol extract, acid-washed) a quantity of hydrolysate derived from 10.8 mgms. total fat was reacted with FDNB and the products transferred to a column of acid-washed adsorbent (3 gms.) in Solvent A. Dinitrophenol did not form a coloured band on the acid-washed adsorbent but was eluted in undissociated form along with excess FDNB and dinitrophenyl ethyl ether in Solvent A. The column was developed with this solvent until the DNP-ethanolamine band almost reached the bottom of the column, when the receiver was changed and DNP-ethanolamine quantitatively eluted with Solvent B. (about 25 mls.). Solvent C did
not elute any coloured material and did not cause any movement of the remaining yellow band at the top of the column but Solvent D eluted a yellow band. Preliminary experiments showed that DNP-serine is also eluted by Solvent D, 15 - 20 mls being sufficient for quantitative elution. When ethanolamine phosphoric acid was added in recovery experiments, the DNP-derivative was eluted from the column with Solvent E (10 - 15 mls).

4. Simultaneous Determination of DNP-Serine and DNP-Ethanolamine without Chromatography.

This procedure may only be applied to mixtures of DNP-serine and DNP-ethanolamine in the absence of other DNP derivatives, for example to hydrolysates of DNP-lipid (Chap. 5). The sample containing 0.5 - 1.0 µ mole of DNP groups, is dissolved in 9.9 mls. dimethylformamide : ethanol (4:1 by vol.) and the optical density at 400 µm recorded; after addition of tetramethylammonium hydroxide (0.10 ml. of 10% w/v aqueous solution) the contents are mixed and the optical density recorded at 500 µm. The proportion of DNP serine to DNP ethanolamine may be obtained from the ratio of the optical densities at these two wave-lengths. The total amount of DNP base present may be calculated from the molecular extinction coefficients of DNP serine and DNP ethanolamine in the above mixture of dimethylformamide and ethanol (figs. 10 & 12).
Results.

A. Results with the Ninhydrin Method.


The reagent described in the experimental section is based upon that of Troll and Cannan (1953), which is an improvement on the original ninhydrin reagent of Moore and Stein (1948) for the estimation of amino groups. The present reagent is more stable, gives more reproducible results and a higher yield of colour than that of Moore and Stein (1948). It differs from that of Troll and Cannan (1953) in that phenol has been replaced by cellolose, there being no need in the present analyses for a buffer with reserve acid; moreover, the presence of phenol has an adverse effect on Amberlite IR-100 (H) which was employed to remove ammonia and metallic cations from the reagent. In a systematic examination of the method of Troll and Cannan (1953), I found that phenol could be replaced by a number of solvents including ethanol and glacial acetic acid and also, that much less pyridine might be used; further, that potassium cyanide as reducing reagent could be replaced by potassium ferrocyanide. The most critical feature was the proportion of water in the reagent since both the rate of colour development and the size of the blank increase with increasing water content. The blank value tends to increase as the reagents stands and is lowest immediately after ion-exchange treatment.
The product of the reaction between ninhydrin and primary amines is diketohydrindamine-diketohydrindylidene. The anion of this substance (Ruhemann's Purple) has $\lambda_{\text{max}}$ at 570 $\mu$m. The optical density at this wave-length on the spectrophotometer equals 1.15 x the optical density measured with Ilford filter 606 in the "Spekker" absorptiometer. With standard solutions of serine and ethanolamine made up in 0.1 M aqueous hydrochloric acid, the apparent $\varepsilon_{\text{max}}$ of Ruhemann's Purple assuming quantitative yield, varied from 16,000 to 18,000.

When approximately 0.5 $\mu$ mole serine was placed on the column of IRC-50, the elution peak occurred in the third tube and 95% was recovered in the first 8 tubes, the remainder forming a very small peak in the ninth tube with the change from phosphate buffer to 0.50 M sodium hydroxide. The blank value, taken as the mean value for the subsequent four tubes, was 0.119 $\pm$ 0.003. The apparent $\varepsilon_{\text{max}}$ of Ruhemann's Purple was 25,000.

Whereas serine passed straight through the column, ethanolamine was retained at pH 6.8 and required alkali for elution. When 1.0 $\mu$ mole ethanolamine was applied to the column in 0.20 ml. phosphate buffer and development carried out as described in the experimental section, the elution peak occurred in tube 16 and all of the ethanolamine was recovered in tubes 14 - 19. The mean blank value in the remaining tubes was 0.070 $\pm$ 0.006. The apparent $\varepsilon_{\text{max}}$ of Ruhemann's Purple was 24,800.
**TABLE 1.**

**Amino Groups in Acid Hydrolysates of Sheep Liver Fat.**

<table>
<thead>
<tr>
<th>Period of Hydrolysis (hrs.)</th>
<th>0.5</th>
<th>1.0</th>
<th>3.0</th>
<th>5.0</th>
<th>8.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amino-nitrogen (µ moles/mgm.)</td>
<td>0.324</td>
<td>0.318</td>
<td>0.290</td>
<td>0.296</td>
<td>0.286</td>
</tr>
<tr>
<td>total fat.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a. Acetone-insoluble fraction.
Fig. 3.

Ion-exchange analysis of acid hydrolysates (after 3 hour's refluxing in 6 N HCl) of ox-brain cephalin fractions III and V, the optical density having been recorded after reaction of each eluate with ninhydrin.
It was found that at pH lower than 6.8, the capacity of the resin fell rapidly, resulting in poorly compact elution peaks for ethanolamine. Also, a distorted elution peak and low recovery of ethanolamine in some cases was attributed to repeated regeneration of the resin. Repetition of the analysis with a fresh batch of resin resulted in a well-defined elution peak.

2. Analysis of Lipid Hydrolysates.

In view of the confusing variety of hydrolysis procedures for aminophospholipids described in the literature, the rate of liberation of amino-nitrogen on acid hydrolysis in 6 N ethanolic hydrochloric acid was first studied. The lipid taken for this purpose was obtained by ether:ethanol extraction of sheep liver, precipitation of the phospholipids with acetone and separation of this fraction into the petroleum ether soluble portion. At the time of this study the importance of acid-washing was not realised, nor was a method established for the determination of choline.

The rate of liberation of amino-nitrogen, expressed on a weight basis, is shown in Table 1 and the distribution of bases obtained by chromatography on IRC-50, in figure 2. It may be seen that there is a small but steady decrease in amino groups liberated when refluxing is continued beyond 0.5 hour. Chromatography on Amberlite IRC-50 has generally shown a small peak immediately behind ethanolamine, which is probably ammonia. In table 1 the serine fraction accounted for 19% of total amino groups at 0.5 hour;
16% at 3 hours; and 16% at 8 hours.

In figure 3 are shown the analyses of fractions III and V of ox-brain cephalin prepared according to the procedure of Felch (1942). The results of these analyses have already been recorded in Table 4 of Chapter 2.

B. Results with FDNB Method.

1. The Reaction of Serine and Ethanolamine with FDNB.

The kinetics of the reaction of serine and ethanolamine with FDNB in aqueous ethanol in the presence of triethylamine have been examined (a) to ascertain the limiting conditions necessary for quantitative reaction of the amino groups and (b) to be assured that one product, corresponding to the introduction of one DNP substituent per molecule, is formed under these conditions. The latter assurance was necessary in view of recent evidence that polymerisation reactions occur in the reaction of FDNB with amino-acids in aqueous ethanol in the presence of sodium bicarbonate (Heikens, Hermanns and van Velden, 1954); this would be manifest by incomplete reaction of available amine-nitrogen.

In the study of the reaction rates at room temperature (figures 4 and 5), standard ethanolamine was made up from the hydrochloride while serine was weighed out as the free base; extinctions were read at 420 m\(\mu\) after addition at each time interval, of 1.0 ml. of 1.0 M aqueous hydrochloric acid and ethanol to bring the volume to 10.0 mls. The final blank readings were
Fig. 4.

Rate curves for the formation of DNP-ethanolamine (triangles), DNP-serine (dots) and DNP-ethanolamine phosphoric acid (open circles) in the presence of 0.012 M FDNB and 0.04 M triethylamine.
Fig. 4.
Differential plot (as in fig. 3) of the potentiometric titration of egg yolk phospholipid dissolved in acetic acid : dioxane (3:17 by vol.)
Fig. 5.
Rate curves for the formation of DNP-ethanolamine (triangles), DNP-serine (dots) and DNP-ethanolamine phosphoric acid (open circles) in the presence of 0.060 M FDNB and 0.04 M triethylamine.
**Fig. 5.**

Rate curves for the formation of DNP-ethanolamine (triangles), DNP-serine (dots) and DNP-ethanolamine phosphoric acid (open circles) in the presence of 0.060 M FDNB and 0.04 M triethylamine.
of the order 0.10 for 0.012 M FDNB and 0.15 for 0.05 M FDNB.

The extinctions attained may be referred to figures 6 and 7 for calculation of the yield of DNP derivative. In 0.1 M ethanolic hydrochloric acid, \( E_{420\mu} \) for DNP-serine is 4,000 while \( E_{420\mu} \) for DNP-ethanolamine is 4,400. Therefore in figures 4 and 5, the final extinctions represent quantitative yields within the error of measurement. A five-fold difference in the concentration of FDNB does not affect the final result.

The conditions of reaction described in the experimental section approximate to 0.05 M, with respect to both FDNB and triethylamine.


Referring to the analysis described in the experimental section, the extinctions at \( \lambda_{\text{max}} \) of the recovered DNP-ethanolamine and DNP-serine in 50.0 mls. and 30.0 mls. respectively of 0.1 M hydrochloric acid, were 0.95 and 1.03, corresponding to 2.75 \( \mu \) moles (104% recovery) and 1.95 \( \mu \) moles (97.5% recovery) calculated from the \( E_{\text{max}} \), which, as may be seen in figures 6 and 7, are 17,200 and 15,700 respectively.

3. Analysis of Brain Lipid Hydrolysate.

The results of this analysis which has been described in the experimental section, are presented in Table 2 together with the recovery of serine and ethanolamine which were added to the hydrolysate prior to reaction with FDNB.
Fig. 7.
Extinction curves of a molar solution of DNP-ethanolamine in 0.1 N hydrochloric acid in ethanol (open circles) and in 0.1 N sodium hydroxide in ethanol (dots).
4. **Analysis of Hydrolysates of Egg Phospholipids.**

Analyses of both fractionated and total egg yolk phospholipid are considered in detail in Chapter 6. The only DNP derivative found in hydrolysates of total egg yolk phospholipid which had been reacted with FDNB, was DNP-ethanolamine but in some preparations obtained by solvent fractionation of the cadmium chloride complex, a yellow derivative eluted from the column by acetone was found which had properties quite different from DNP-ethanolamine and DNP-serine. The yellow colour was discharged by acid but in acetone the absorption spectrum was as shown in figure 8.

5. **Determination of DNP-serine in the Presence of DNP-ethanolamine.**

In figures 6 and 7, it may be seen that the light absorption of DNP-serine in acid solution is displaced to the ultra-violet with respect to that of DNP-ethanolamine. Also the indicator effect in alkali is greater with DNP-serine than with DNP-ethanolamine. Further a greater stability of DNP-ethanolamine under alkaline conditions was found: after refluxing in 0.05 N potassium hydroxide in ethanol for 20 minutes, the loss of extinction at 400 mp for DNP-serine was 95%, but for DNP-ethanolamine the loss was 20%. In an attempt to establish a differential method for the determination of the two DNP derivatives, attention was given to the Janovsky reaction (Janovsky, 1891) which is a test for aromatic nitro compounds by the colour produced on reaction with acetone in the presence of alkali.
Fig. 8.

Spectrum in acetone of the acetone eluate referred to in the text. The ordinates represent optical density in arbitrary units.
With the present derivatives the colour produced in the Janovsky reaction was red, the maximum absorption being at 540 μm. It was found that as the colour yield was increased by increasing the concentration of acetone or alkali, the stability of the colour was less. A compromise system consisting of 0.01 M sodium ethoxide in equal parts of acetone and ethanol was found to give the highest colour yield consistent with a fair degree of stability (figure 9). In this system the colour yield of DNP-serine at the point of maximum development is exactly half that of DNP-ethanol-amine. While this observation might be applied with some success to the determination of DNP-serine in the presence of DNP-ethanol-amine, a recent development of the Janovsky reaction (Porter, 1955) was found to have considerable advantages.

In the procedure of Porter (1955) the sample is dissolved in 9.9 mls. dimethylformamide and 0.10 ml. 10% aqueous tetramethylammonium hydroxide is added. With the two bases in question the colour developed is red, but the absorption maximum in this case is 430 μm, unlike the product in the Janovsky test system. Dimethylformamide, apart from possessing good general solvent properties, has a stabilising effect on the red coloured derivative which was found to be very sensitive to even small additions of ethanol or acetone. Five per cent of acetone by volume when added to the test system, causes a slow change in colour to mauve;
Yield and stability of extinction at 540 mμ from DNP-ethanolamine (open circles) and DNP-serine (dots) under the conditions of the Janovský reaction.
Fig. 10.

Absorption spectra of products from DNP-ethanolamine in Porter's system (dots) and in Porter's system diluted with an equal volume of acetone (open circles).
with an equal volume of acetone the change is immediate but the colour fades on standing. In figure 10 are shown the absorption spectra of the red ($\lambda_{\text{max.}}$ 430 m$\mu$) and mauve ($\lambda_{\text{max.}}$ 570) products from DNP-ethanolamine. With DNP-serine the same products are formed but the yield is lower (see Table 3). The extinction at 430 m$\mu$ derived from DNP-ethanolamine was stable for at least an hour while that from DNP-serine showed a detectable drop only after half an hour.

Addition of alcohol to the test system did not alter $\lambda_{\text{max.}}$ as did acetone but repressed colour development much more in the case of DNP-serine than in the case of DNP-ethanolamine. Addition of 5% (by vol.) of ethanol to Porter's system containing DNP-serine caused 25% reduction in optical density at 430 m$\mu$; 56% reduction with 10% (by vol.) of ethanol and with 20% (by vol.) of ethanol $\varepsilon_{430\ m\mu}$ was 6,640, which is almost identical with DNP-serine in dimethylformamide (figure 11). In contrast $\varepsilon_{430\ m\mu}$ for DNP-ethanolamine in Porter's system containing 20% ethanol (by vol.) was 16,000 which is 76% of the value in the absence of ethanol. The absorption curves shown in figures 11 and 12 demonstrate the magnitude of the difference in behaviour of DNP-serine and DNP-ethanolamine in this system. Visually the difference is obvious, inasmuch as DNP-ethanolamine furnishes a red colour while DNP-serine remains yellow. The stability of the red colour from DNP-ethanolamine in this system is indicated in the following values for the optical density of a sample of DNP-ethanolamine measured
Fig. 12.

Absorption spectra of DNP-ethanolamine in ethanol: N,N-diethylformamide (1:4 by vol.) before (open circles) and after (dots) addition of tetramethylammonium hydroxide.
TABLE 3.

Apparent $\varepsilon_{\text{max}}$ of some Derivatives of DNP-Serine and DNP-Ethanolamine.

<table>
<thead>
<tr>
<th>System</th>
<th>Colour</th>
<th>$\lambda_{\text{max}}$</th>
<th>DNP-Serine</th>
<th>DNP-Ethanolamine</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1 M HCl (ethanol)</td>
<td>yellow</td>
<td>345 $\text{m}<em>{E}^S$ 350 $\text{m}</em>{E}^S$</td>
<td>15,700</td>
<td>17,200</td>
</tr>
<tr>
<td>0.1 M NaOH (water)</td>
<td>yellow</td>
<td>358 $\text{m}<em>{E}^S$ 352 $\text{m}</em>{E}^S$</td>
<td>17,500</td>
<td>18,500</td>
</tr>
<tr>
<td>A</td>
<td>red</td>
<td>540 $\text{m}_{E}^S$</td>
<td>6,100</td>
<td>12,000</td>
</tr>
<tr>
<td>B</td>
<td>red</td>
<td>430 $\text{m}_{E}^S$</td>
<td>14,000</td>
<td>21,000</td>
</tr>
<tr>
<td>C</td>
<td>mauve</td>
<td>570 $\text{m}_{E}^S$</td>
<td>25,000</td>
<td>30,000</td>
</tr>
<tr>
<td>D</td>
<td>red $^E$</td>
<td>430 $\text{m}_{E}^E$</td>
<td>$\varepsilon$ 430 $\text{m}_{E}^S$ = 6,640</td>
<td>16,000</td>
</tr>
<tr>
<td>yellow $^S$</td>
<td></td>
<td>370 $\text{m}_{E}^S$</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$S$=DNP-Serine; $E$=DNP-Ethanolamine.

A: 0.01 M sodium ethoxide in ethanol:acetone (1:1 by vol.).
B: System of Porter (1955) (see text).
C: Porter's system diluted with an equal volume of acetone.
D: Porter's system plus ethanol (20% by vol.).
at intervals (indicated in brackets) after addition of
tetramethylammonium hydroxide: 0.945 (1'); 0.940 (2');
0.930 (4'); 0.925 (6'); 0.915 (10'). Therefore quite accurate
determinations can be made provided the alkali is added immediately
before measurement.

The determination of DNP-ethanolamine in the presence of
DNP-serine by the use of the above differential colour reaction
is best accomplished by measurement of optical density at 500 μm
where there is very little absorption due to DNP-serine. It was
observed that the absorption at 500 μm obeys Beer's Law. By
measurement of the extinction at 400 μm say, of a mixture of
DNP-serine and DNP-ethanolamine in ethanol:N,N-dimethyl formamide
before addition of alkali and that at 500 μm after the addition of
alkali, it should be possible to determine the proportion of DNP-
serine in admixture with DNP-ethanolamine. The calculation may
be made from the following simultaneous equations:

\[
E_{400 \text{ μm}} = E_{400 \text{ μm}}^S e + E_{400 \text{ μm}}^E t
\]
\[
E_{500 \text{ μm}} = E_{500 \text{ μm}}^S e + E_{500 \text{ μm}}^E t
\]

where \(E\) is the optical density of the unknown, \(S e\) is the molar
concentration of DNP-serine and \(E t\) is the molar concentration of
DNP-ethanolamine. The present results indicate that the following
values of \(E_{400 \text{ μm}}\) and \(E_{500 \text{ μm}}\) may be substituted:
Unfortunately, there was insufficient time to apply these principles to hydrolysates of 2,4-dinitrophenylaminophospholipids, formed by direct reaction of lipid sample with PDNB (Chapter 5).

Discussion.

At the outset of the work described in this chapter, it was believed that ninhydrin possessed advantages over other reagents previously used for the determination of amino-nitrogen lipid hydrolysates, with regard to its sensitivity and ease of application. The paper chromatographic procedure of Levine and Chargaff (1951) seemed to be a simple and convenient method for the separation of serine and ethanolamine. Although the latter method represents a distinct advance in refinement of technique, it was found to have a number of disconcerting weaknesses. (a) The separation of serine and ethanolamine in lipid hydrolysates compared very poorly with the separation of synthetic mixtures, with the result that guide strips were not an accurate means of locating the bases for estimation. This may have been due to distortion of the ethanolamine spot by choline which runs to the same position; and more especially, distortion of the serine spot ($R_p$, 0.20) by salt, glyceryl phosphate, etc. Application of the spray reagent of Hanes and Isherwood (1949) showed evidence of phosphate esters in the same position as
serine. (b) Owing to the low and variable yield of Ruhemann’s Purple with the reagent of Moore and Stein (1948) which is due partly to impurities in the chromatography paper bearing the amino compound (Boissonnas, 1950), it was necessary to carry out analyses of standard mixtures on lanes adjacent to those carrying the unknown. (c) The blank value was frequently high, perhaps due to retention of atmospheric ammonia by the paper.

That metallic cations were a cause of the low and variable yield of colour in the ninhydrin reaction was suggested by the work of Meyer and Ricklis (1953) and Troll and Cannan (1953). The latter introduced a new reagent consisting of a pyridine-phenol buffer incorporating potassium cyanide as reducing reagent in place of stannous chloride which was used by Moore and Stein (1948). Troll and Cannan (1953) also stressed the importance of removing contaminating cations from the pyridine and phenol by treatment with permutit.

Considering the results quoted above, it was decided that more effective use of the ninhydrin reagent would be made if the lipid hydrolysate were submitted to ion-exchange analysis, thereby removing interfering cations at the same time as effecting a separation of acidic and basic components. A modified form of the reagent of Troll and Cannan (1953) when applied to standard solutions of serine and ethanolamine made up in once distilled water 0.1 N with respect to hydrochloric acid, gave yields of Ruhemann's
Purple equivalent to an apparent $\varepsilon_{\text{max.}}$ of Ruhemann's Purple (based on the quantity of amino-nitrogen taken) of 16,000 to 18,000. After chromatography on Amberlite IRC-50, the apparent $\varepsilon_{\text{max.}}$ rose to 25,000 for both serine and ethanolamine. Troll and Cannan (1953) found an apparent $\varepsilon_{\text{max.}}$ of 21,000 - 22,000 for amino-acid solutions without ion-exchange treatment and claimed the same $\varepsilon_{\text{max.}}$ for the sodium salt of diketohydrindamine-diketohydrindylidene which they prepared. However an independent estimate by McFadyen and Fowler (1950) was 25,000. Comparison with my own results indicates that it is possible to obtain quantitative yields of Ruhemann's Purple if the bases in question are separated by ion-exchange chromatography, thereby dispensing with the controls which are necessary in paper chromatographic analysis.

The method developed in the present section is not without its own shortcomings. These are (a) the collection of a large number of eluates and (b) variation in the "trough" values between serine and ethanolamine, which impairs the accuracy of determination of serine when it is present in small quantities in relation to ethanolamine.

If there is a need to recover serine and ethanolamine in phospholipid hydrolysates, for example in the event of turnover studies with isotopic carbon or nitrogen then an advantage is to be gained by their conversion to stable derivatives which can be
estimated spectrophotometrically. In the present application
of the reagent fluoro-2,4-dinitrobenzene, the reaction system
employed is different from that introduced by Sanger (1945) and
since widely adopted by other workers. The use of sodium bicar-
bonate in Sanger's reaction system is accompanied by numerous
difficulties not the least of which is the removal of the salt
at the completion of the reaction. A careful study of the
limiting conditions necessary for quantitative reaction in the
presence of triethylamine as base has reduced side-products to
a level where 0.5 - 2.0 μ moles of amino-nitrogen can be determined
directly.

There is a number of published methods for the separation
of N-2,4-dinitrophenyl derivatives. The most generally useful
method for the separation of DNP-amino-acids is the two-
dimensional paper chromatography of Levy (1954). DNP-amino-
alcohols generally require quite different solvent systems for
separation by paper chromatography, for example those of Jatzkewitz
and Nguyen-Dang Tam (1954). Column chromatography of DNP-amino-
alcohols has been studied by Green and Kay (1952) and Grassmann,
Hörman and Endres (1954). Since DNP-amino-alcohols are much
less strongly adsorbed than DNP-amino-acids, the main difficulty
in the present application of column chromatography was the
separation of DNP-ethanolamine from dinitrophenol. Experimenta-
tion with eluants based upon those introduced by Green and Kay
(1952) lead to the choice of a mixture of magnesium trisilicate and celite as adsorbent. Treatment of the reaction products with acid prior to transfer to the column was undertaken to convert dinitrophenol to its undissociated form in which it was expected to be least strongly adsorbed. Unless the column itself was also acid-washed, dinitrophenol formed a coloured band which was only with difficulty separated from DNP-ethanolamine. The procedure is much simpler than ninhydrin methods but has one disadvantage in the instability of DNP-serine if chromatographed in daylight.

Fluro-2,4-dinitrobenzene was applied independently to the analysis of amino-nitrogen in lipid hydrolysates by Axelrod, Reichental and Brodie (1953). These authors carried out the reaction in the system of Sanger (1945) and employed a somewhat complicated series of solvent distributions in order to separate DNP-ethanolamine from DNP-serine. The latter was not separated from dinitrophenol so that extinction measurements were made in acid solution at 420 μ and a blank correction made. Because of the necessity of making four successive transfers of DNP-serine, at each transfer an aliquot being taken, the method suffers a marked loss of sensitivity as well as failing to isolate the derivatives in a pure state.

With the development of a method for the preparation of the DNP derivative of intact cephalin, which is readily separated
from excess reagent and side-products by application of a high vacuum (Chapter 5), a need was anticipated for the determination of DNP-serine and DNP-ethanolamine in the intact derivative. Acid hydrolysis of DNP-lipid would provide DNP-serine and DNP-ethanolamine as the only DNP derivatives present provided there is no decomposition; these might then be separated by column or paper chromatography.

If there is no need to recover the split DNP-bases, their simultaneous determination by a suitable differential method would furnish a rapid and elegant procedure for the estimation of serine and ethanolamine in lipid extracts. A method of this nature is especially suited to following the resolution of DNP-lipids by column chromatography or counter-current distribution where a large number of analyses is necessary.

A study of the behaviour of DNP-serine and DNP-ethanolamine in various modifications of the Janovsky reaction (Janovsky, 1891) has shown a way to accomplish the simultaneous determination of DNP-ethanolamine and DNP-serine in hydrolysates of DNP-lipid. The basic requirements of this reaction appear to be an aromatic nitro group, an active methylene group and alkali. The absorption spectra may be referred to a variety of ionised quinonoid forms but owing to their extreme lability (except in certain solvents, such as dimethylformamide), it is doubtful that they could be isolated. Experimentation with the system of Porter (1955)
based on the Janovsky reaction has resulted in a test system in which DNP-ethanolamine develops a red colour while DNP-serine remains yellow.

Ideally, such a test system should be applied to DNP-lipid without hydrolysis; it is probable that similar experimentation with samples of DNP-phosphatidyl ethanolamine and DNP-phosphatidyl serine would provide the answer. However limitations of time prevented the application of this test system to the analysis of DNP-lipid.
(a) An improved application of the ninhydrin method to the analysis of phospholipid hydrolysates has been described.

(b) There is no further liberation of amino-nitrogen from phospholipids following 0.5 hours' refluxing in 6 M ethanolic hydrochloric acid; there is a significant loss of amino-nitrogen after 8 hours' hydrolysis.

(c) Isolation of the amino-nitrogen of phospholipid hydrolysates has been accomplished by quantitative conversion to the 2,4-dinitrophenyl derivatives and adsorption chromatography.

(d) A method has been developed for the simultaneous determination of DNP-ethanolamine and DNP-serine without separation from one another.
CHAPTER 5

The Preparation of 2,4-Dinitrophenyl-Cephalin.

Introduction.

The history of phospholipid chemistry is to a very large extent an account of isolation and purification of native phospholipids from naturally occurring complex mixtures. In this work, little advantage has been taken of altering the functional groups of a particular species in such a way as to effect a marked change in physical properties and so facilitate a separation. The customary addition of magnesium chloride to bring about quantitative precipitation of phospholipids by acetone is the most common example of the application of this principle. Hydrogenation has been used to obtain pure hydrogenated lecithin (Levene and Rolf, 1926 b) and cephalin (Gray, 1940). This step however, has only been found of assistance at a late stage in the purification process.

Addition of halogens to the unsaturated residues (Levene and Rolf, 1926 a) has been carried out as a means of characterisation; it has not been recommended to facilitate isolation. Numerous reactions have been described in the patent literature but they are very obscure and of doubtful use for the isolation and characterisation of phospholipids.

The idea of forming an intact derivative of native cephalin was suggested in the first place by the example of protein chemistry.
Moreover of the known phospholipids, cephalin lends itself best to chemical modification, being the only phospholipid in which there occurs a functional group namely the amino group, with a sufficient degree of reactivity to render the idea feasible.

Of the numerous reagents which are in use for the modification of the free aminogroup in proteins, fluoro-2,4-dinitrobenzene was the obvious choice, being fat soluble. The reactivity of the amino group in cephalin was realised as long ago as 1916 when Levene and West reacted a cephalin fraction from brain with phenyl- and anphthyl isocyanates to obtain what appeared to be phenyl- and naphthyl ureido derivatives of cephalin. Witanowski (1928) provided indirect evidence of methylation by formaldehyde of the amino group of cephalin thereby obtaining lecithin.

A. The Reaction of Cephalin with Fluoro-2,4-dinitrobenzene.

1. Reaction of Lipids with FDNB in Sanger's System.

Experimental and Results

In early experiments use was made of the reaction system of Sanger (1945), originally designed for water-soluble substances. The material used in these experiments was the petroleum ether-soluble portion of an ethanol:ether extract of rat liver. The extract was not acid-washed.

In a typical experiment, 26.5 mgms. total rat liver fat was dissolved in 1.0 ml. chloroform in a blackened flask. Ethanol (6.0 mls.) 5% aqueous sodium bicarbonate (1.0 ml.) and one drop of FDNB were added and the flask shaken for two hours at room temperature. The excess sodium bicarbonate was removed by centrifugation, washed with ethanol and the supernatant and washings taken to dryness in vacuo. The residue
was taken up in 2.0 mls. water-saturated amyl alcohol and placed on a column which consisted of 2.5 gms. Whatman standard grade paper powder, prepared in the same solvent. Development with water-saturated n-amyl alcohol removed the bulk of the yellow colour at the front, leaving behind a yellow band which could be eluted with ethanol:water (4:1 by vol.) containing a 0.1% ammonia.

The amyl alcohol eluate after evaporation to dryness, was dissolved in ether and the ether solution washed with dilute sodium bicarbonate until no more yellow colour passed into the aqueous phase. The residual ether solution still had a yellow colour which was taken as evidence of the formation of DNP-cephalin.

The aqueous ethanol eluate contained phosphorus and on chromatography on Whatman No. 1 paper sheets in the upper layer of a system consisting of tert.-amin alcohol:pyridine:water (5:1:5), moved to the position of DNP-ethanolamine phosphoric acid (Rf 0.15). It therefore appeared that a certain degree of hydrolysis had taken place in the reaction system of Sanger (1945).

Other experiments were aimed at bringing about more complete hydrolysis of cephalin in this system without regard to the isolation of DNP-lipid. For example by heating a reaction mixture on the boiling water bath for 30 mins., roughly 30% of the total phosphorus could be obtained in water-soluble form. After removal of sodium bicarbonate by ethanol precipitation, followed by concentration of the ethanol-soluble fraction, the DNP derivatives were precipitated with ether and chromatographed on paper sheets in the system described above. The chromatograms revealed DNP-ethanolamine phosphoric acid, yellow material at the front which could be DNP-ethanolamine and an unidentified DNP-derivative,
the RF of which was 0.51 as compared with DNP-serine, the RF of which was 0.62. Also the DNP-serine spot darkened on exposure to light while the unknown remained yellow. Control experiments in which serine and ethanolamine were submitted to the same treatment as the lipid, did not result in the appearance of the unidentified material.

2. The Reaction of Lipids with FDNB in an Anhydrous System.

Experimental.

Materials.

FDNB was re-crystallised from ethanol. Stock solutions of FDNB were made up in benzene. Benzene, A.R., was dried over sodium wire. Triethylamine was distilled in a fractionating column and the middle fraction was used. Both triethylamine and ethanol were dried over calcium oxide. Acid-washed fractions V and III of the ox-brain cephalin of Folch (1942) which has been described in Chapter 2, were used in the experiments.

Procedure.

Reaction rates were determined by measurement of the optical density at 420 m\(\mu\) where there was negligible absorption due to FDNB. Control systems from which blank corrections were made, contained everything except the lipid.

Results.

The reaction of FDNB with lipid amino-groups has been studied in relation to (a) the effect of solvent on reaction rate; and (b) the
effect of base on the reaction rate. The aim of this study was to establish limiting conditions for the quantitative reaction of FDNB with lipid amino groups while keeping side-products to a minimum.

In contrast to the similar study with serine and ethanolamine (Chapter 4), no well characterised reference material was available. Synthetic cephalin had it been available, may have been the most suitable reference material but even this is unstable and undergoes loss of amino-nitrogen on storage (Lea and Rhodes, 1955; Scholfield and Dutton, 1955). The preparation of cephalin according to Folch (1942) was undertaken with the objective of securing reference material but unlike that of Folch (1942), the present preparation had a low proportion of amino-nitrogen to total nitrogen (Chapter 2) when analysed by the ninhydrin method, after hydrolysis. Nevertheless important information could be gained regarding the rate of the reaction and factors which may influence it.

In figures 1 and 2 the effect of solvent and base respectively, are shown. In figure 2 the open circles indicate the optical density obtained after adding two drops of 1.0 N hydrochloric acid in order to obtain a result which could be referred to standard conditions. It is evident in figure 2 that under three different sets of conditions the final yield was the same. The same result was obtained when similar systems were allowed to stand overnight at room temperature. The fact that curves 1 and 3 had not flattened out at the time the reaction was stopped is apparently due to the slower formation of dinitrophenol from traces of moisture in the fat. This conclusion is supported by the shape of curve 2 where the fat sample was dried over phosphorus pentoxide. Dinitrophenol as the triethylamine salt has strong absorption at 420 mp, but in acid solution it has no absorption at 420 mp.
Fig. 1.

Effect of solvent on the rate of formation of DNP-cephalin in the presence of 0.04 M FDNB. The reaction was carried out in the following solvents: (1) benzene, (2) ethanol : benzene (1:1 by vol.) and (3) ethanol : benzene (4:1 by vol.).
Fig. 2.

Effect of base on the rate of formation of DNP-cephalin in the presence of 0.04 M FDNB. The rates of reaction are shown for (1) benzene containing 0.04 M triethylamine; (2) ethanol:benzene (4:1 by vol.) containing 0.01 M triethylamine; and (3) ethanol:benzene (4:1 by vol.) containing 0.05 M triethylamine. The open circles indicate the optical density obtained after acidification with 1 N hydrochloric acid, in order to obtain a result which could be referred to standard conditions. The sample of cephalin in reaction (2) had been dried over phosphorus pentoxide.
Discussion.

Since FDNB is a reagent soluble in fat solvents, it is obviously more suitable to fat chemistry than to protein chemistry. Thus a reaction system quite different to that of Sanger (1945) was indicated. It was thought that this system deserved study in exploratory experiments.

In an independent study of the reaction of FDNB with lipid from Neurospora crassa, Ellman and Mitchell (1954) employed a modification of Sanger's system similar to that described here but made no attempt to separate the DNP-lipid from excess FDNB and side-products. They submitted the total reaction mixture to acid hydrolysis and isolated the DNP-derivative of a new base, amino-2-methyl-2-propanol, from the hydrolysate. The present study has demonstrated an unexpected lability of cephalin in Sanger's system whereby hydrolysis occurs with the splitting off of DNP-ethanolamine and DNP-ethanolamine phosphoric acid. The formation of DNP-ethanolamine phosphoric acid is surprising in view of the fact that in glycerylphosphorylethanolamine, the base-phosphate bond is more labile than the glycerol-phosphate bond (Schmidt, Bessman and Thannhauser, 1953). It therefore appears likely that DNP-ethanolamine phosphoric acid results from the hydrolysis of DNP-cephalin and that the mechanism of hydrolysis of DNP-cephalin is different from that of native cephalin. The formation of phosphorylated base using very mild alkaline hydrolysis may serve a useful purpose in turnover studies with radioactive phosphorus (p32).

The reaction of halogeno-2,4-dinitrobenzenes with amines in anhydrous solvents has been investigated kinetically by several authors and provides a basis for applying the reaction to aminophospholipids. Brady and Cropper (1950) pointed out that chlorodinitrobenzene (CDNB)
reacts with only half an equivalent of base since it is unreactive towards the positively charged portion formed as a side-product:

\[ 2 \text{RNH}_2 + \text{Cl.C}_6\text{H}_3(\text{NO}_2)_2 \rightarrow \text{R.NH.C}_6\text{H}_3(\text{NO}_2)_2 + \text{R.NH}_3^+ + \text{Cl}^- \]

Fluorodinitrobenzene (FDNB) however, is capable of reacting with the ionically charged base. Thus, while CDNB consumes two moles of base per mole of CDNB, FDNB consumes only one mole of base per mole FDNB. Moreover FDNB reacts 100 - 200 times as fast as CDNB at 30°C. FDNB is clearly the more suitable reagent for the preparation of dinitrophenyl derivatives but there are aspects of the reaction which have only been studied with CDNB and it is instructive to consider these before applying the reaction to new substances.

The reaction with aliphatic amines has been studied only with CDNB. Brady and Cropper (1950) found steric effects to exert a considerable influence on the rate of reaction. The rate with methylamine is only one tenth the rate with dimethylamine which is a stronger base. However the rate with ethylamine is 4.5 times the rate with diethylamine, presumably because the ethyl group provides steric hindrance while the methyl group does not. Di-isopropylamine is only $10^{-2}$ times as reactive as diethylamine. On the other hand, while ethylamine is a third as reactive as methylamine, further linear extension of the alkyl chain does not greatly influence the reaction rate.

Chapman and Parker (1951) have ascribed the increased reactivity of FDNB with aromatic amines as compared with CDNB, to greater solvation of a relatively stable transition state and this was claimed to be the essential role of the proton acceptor. The pronounced effect of ethanol on the reaction rate which must be due to solvation, is evident in figure 1.
However as may be seen in figure 2, the effect of triethylamine is of much greater significance than that of ethanol. Thus it is possible to obtain a rapid reaction of FDNB with lipid amino groups in a non-polar (and hence non-reactive) solvent such as benzene, provided triethylamine is present. The spectrophotometric nature of the measurements makes it difficult to go beyond these simple comparative deductions. It is probable that the extinctions measured are the sum of that due to the transition state and that due to the product. Since the absorption curve of the transition state is probably quite different from that of the DNP derivative to which it decomposes, the information obtained is not sufficient in itself to enable any conclusions to be made about the actual mechanism of the reaction.

B. The Preparation of DNP-Cephalin.

Experimental.

It is evident from the preceding section that the reaction of aminophospholipid with FDNB provided triethylamine is present, is quite rapid. The routine procedure for the preparation of DNP-lipid has been as follows:

The fat sample containing 5 - 50 μ moles of amino-nitrogen, was dissolved in 5 - 10 mls. benzene, 2 drops of FDNB (approx. 0.2 m mole) and 2 drops of triethylamine (approx. 5 m moles) added, allowed to stand 1 - 2 hours at room temperature and taken to dryness at the water pump, being maintained at 50°C. for a few minutes to ensure complete removal of triethylamine. The residue consists of DNP-lipid, FDNB and a small amount of side-products consisting of dinitrophenol (formed from moisture in fat and reagents) and products formed by reaction of
FDNB with triethylamine. Even triethylamine which had been carefully distilled in a large (4" x 5'') fractionating column, was found still to give a slight reaction with FDNB.

The removal of FDNB and blank has been accomplished in two ways, the first being that generally adopted:

(a) Distillation on to a cold finger at a temperature of 80 - 100°C. (boiling water bath) and a pressure of 0.05 - 0.01 mm. mercury. While FDNB distills readily under these conditions, for complete removal of blank it is necessary to redistribute the fat on the walls of the flask at least once. A flask of the dimensions shown in figure 3 (about 60 mls. capacity) sufficed for samples of fat from 5 to 50 mgms. With larger samples of fat (and larger amounts of FDNB) it was found preferable to use a larger flask and so obtain a more rapid removal of the blank.

(b) Chromatography of the reaction mixture (from which triethylamine had been removed) on talc or celite. This procedure will be described more fully in a later section. It is not recommended for analytical work since one cannot always be certain of quantitative elution of the DNP-phospholipid, which depends upon the amount of cephalin in the fat sample and the ratio of fat to adsorbent.

Having isolated DNP-lipid free of non-lipid DNP material, the amount present was determined from the extinction in petroleum ether at $\lambda_{\text{max}}$, which is 345 mu (figure 4). $E_{\text{max}}$ has been assumed to be 16,500, an approximate figure which is based upon the following facts: (a) $E_{\text{max}}$ of DNP-serine in acid ethanol ($\lambda_{\text{max}}$ = 345 mu) is 15,700 and $E_{\text{max}}$ of DNP-octadecylamine in ethanol ($\lambda_{\text{max}}$ = 348 mu) is 17,300. (b) the highest $E_{\text{max}}$. (P) (extinction in 1 litre per gm. atom of phosphorus) obtained for DNP-cepahalin after chromatography, was 17,000. (c) the above
Fig. 4. Absorption spectrum of a sample of DNP-cephalin measured in petroleum either (dots) and in ethanol (open circles).
a free acid than the native phospholipid. As pointed out in Chapter 3, this should enable more accurate titration of lecithin in the presence of cephalin. It also permits modification of the acid group (Chapter 7) with less ambiguity than in the native phospholipid.

(e) It should be possible by mild hydrolytic means, to obtain the phosphorylated base and glycerylphosphoryl base which could be separated by paper chromatography and determined spectrophotometrically without the need for markers as in the procedure of Dawson (1954b) for native cephalin.

Not all of the advantages inherent in the formation of DNP-cephalin have been exploited. Attempts at the isolation of the DNP derivative by chromatography will be described in the following section. The direct determination of lipid amino-nitrogen in this form is the subject of Chapter 6 while the esterification of DNP-cephalin will be considered in Chapter 7.

C. The Chromatography of DNP-cephalin.

Experimental and Results.

Initial experiments carried out with DNP-lipid from rat liver showed, as might be expected, that glycerides and vitamin A are much less strongly adsorbed than phospholipids and are thus readily separated by adsorption chromatography. The present account will be confined to the behaviour of the fractionated cephalin of Folch (1942).

Adsorbents tested were silica (B.D.H.) sucrose (Baker's sugar) talc (pharmaceutical), celite ("Hyflo-supercel") and "siliconed" celite (Jutisz, Privat de Garilhe, Suquet and Fromageot, 1954). A series of eluants of increasing polar strength consisted of benzene, chloroform, mixtures of chloroform and ethanol, and ethanol or ethanol:water.
1. The Relative Strengths of Adsorbents.

Columns of adsorbent were prepared in petroleum ether or benzene and the DNP-lipid applied in the same solvent, the ratio of adsorbent to phospholipid being of the order 1 - 2 gms./mgm. of phospholipid. Early exploratory work consisted of chromatographing DNP-lipid prepared from rat liver fat on sucrose, by which means it was found possible to separate vitamin A which was eluted by petroleum ether, from DNP-lipid fractions which were eluted by chloroform and acetone. The light absorption of the coloured eluates when measured in petroleum ether, was identical with that of DNP-cephalin prepared from the ox-brain fractions. However not all of the DNP derivative could be recovered from sucrose unless ethanol was employed as eluant, with consequent solution of the adsorbent.

A survey of alternative weak adsorbents taking the same ratio by weight of adsorbent to phospholipid, led to the following order of adsorptive strengths beginning with the weakest: "siliconed" celite, sucrose, celite, talc and silica. This series was found by development of the chromatogram with the following solvents of increasing power of elution: petroleum ether, benzene, chloroform and ethanol. For example, an eluate from talc obtained with ethanol: chloroform (1:4 by vol.) when transferred to an equal weight of "siliconed" celite, was almost completely eluted by benzene.

Band formation was frequently observed upon development with weakly polar solvents such as benzene and chloroform but without careful adjustment of the proportion of adsorbent to phospholipid, elution of these bands required the use of an ethanol-containing eluant which caused the slower fraction to spread and be partly eluted with the faster.
Fig. 5.
2. **Chromatography on Talc.**

Chromatography on talc was found to be an effective means of isolating DNP-cephalin from a reaction mixture containing FDNB and side-products. This is illustrated in the following experiment.

A reaction mixture containing 7.35 mgms. fraction V (acid-washed) was taken to dryness in vacuo to remove triethylamine, dissolved in benzene and transferred to a column of talc (5 gms.) which had been prepared in benzene. The yellow zone of adsorption extended about half-way down the column and was not affected by washing the column with benzene; but it was resolved into two bands by chloroform, one band moving at the front. Each band was collected separately.

Ethanol:chloroform (1:4 by vol.) and ethanol:water (9:1 by vol.) removed further yellow material but there was no distinct band formation. The absorption spectra of these eluates, measured in ethanol, may be seen in figure 5. Both chloroform eluates were predominantly side-products, the second (curve A) being dinitrophenol apparently contaminated with a small amount of DNP-lipid. The subsequent two eluates (curves B and C respectively) both contained phosphorus, \( \varepsilon_{\text{max}} \) (P) in ethanol being 19,000 and 14,000 respectively. The total phosphorus recovered was 55%, 40% being recovered in the last eluate. The recovery of phosphorus thus compares well with the atomic ratio of amino-nitrogen to phosphorus in acid-washed fraction V, which was 0.59 (Table 4, Chapter 2).

In another experiment, talc which had been washed with 10% (w/v) aqueous acetic acid followed by ethanol and benzene, was employed for chromatography. A similar aliquot of fraction V as taken in the previous experiment, after reaction FDNB and removal of triethylamine, was transferred to 5 gms. acid-washed talc in benzene; development
with benzene in this case removed all the blank (dinitrophenol being in undissociated form) and resulted in the appearance of two well separated and well defined zones of adsorption. The first (A) was eluted with chloroform:benzene (1:4 by vol.) and had $\lambda_{\text{max}} = 340 \text{ mu}$ (cyclohexane); the second (B) was largely eluted with ethanol:benzene (1:19 by vol.) and had $\lambda_{\text{max}} = 345 \text{ mu}$ (cyclohexane); the remainder (C) was eluted with ethanol:water (1:9 by vol.) and had $\lambda_{\text{max}} = 350 \text{ mu}$ (cyclohexane). Based on extinctions at $\lambda_{\text{max}}$, the proportion A:B:C was 30:40:30, $\varepsilon_{\text{max}} (P)$ being respectively 13,800, 14,500 and 5,000. Recovery of phosphorus was 63%, compared with 55% in the previous experiment. Further comparison with the previous experiment shows an improved ease of elution of the derivatives as well as better resolution, there being a difference of 10 mu between $\lambda_{\text{max}}$ of the least and most strongly adsorbed fractions but the separation from non-DNP-lipid phosphorus is poorer.

3. Efficiency of Adsorption Chromatography.

The low content of amino-nitrogen in acid-washed fraction V was a disadvantage for the preparation of pure DNP-cephalin. The recovery of phosphorus and extinction in the above two experiments shows that a good measure of purification of the derivative has been achieved by chromatography. An additional approach was further purification of the native cephalin before reaction with FDNB. This was attempted by distribution between solvents and a buffered aqueous emulsion (Chapter 2) but the material obtained had an even lower proportion of amino-nitrogen to total nitrogen than the acid-washed preparation. When the butanol extract at pH 10 of fraction V (Table 6, Chapter 2) was reacted with FDNB (using the cold finger for removal of blank) the yield of DNP derivative taking $\varepsilon_{\text{max}}$ (petroleum ether)
was only 22% based on total nitrogen as compared with 59% yield with the acid-washed starting material. The following experiment describes an attempt to isolate DNP-cephalin from the above butanol extract after reaction with FDNB.

A quantity of material containing 4.76 \( \mu \) moles nitrogen was reacted with FDNB and after removal of triethylamine, the reaction products were dissolved in ether and washed with 0.1 M hydrochloric acid followed by water then taken to dryness and transferred to a column of talc (5 gms.) in benzene. After washing the column with benzene and chloroform to effect removal of side-products the chromatogram was developed with a 0.5% solution of trimethylamine hydrochloride in chloroform. Two well-defined bands resulted the faster (A) having \( \lambda_{\text{max.}} \) (petrol) at 340 mp and \( \varepsilon_{\text{max.}} \) (P) = 15,000 while the slower (B) had \( \lambda_{\text{max.}} \) (petrol) at 340 mp and \( \varepsilon_{\text{max.}} \) (P) = 17,000. Some yellow colour remained on the column and was eluted with ethanol. This material (C) had \( \lambda_{\text{max.}} \) at 342 mp and \( \varepsilon_{\text{max.}} \) (P) = 9,700. Calculated on the basis of the extinctions at \( \lambda_{\text{max.}} \), the proportion A:B:C = 61:25:14. The recovery of phosphorus was 25%.

When the above experiment was repeated with an incomplete reaction mixture (absence of triethylamine) so as to ensure incomplete reaction of amino groups with FDNB, eluates A and B had \( \varepsilon_{\text{max.}} \) (P) of 12,100 and 12,500 respectively while for C, \( \varepsilon_{\text{max.}} \) (P) was 2,020. The proportion A:B:C was 69:21:10. The recovery of phosphorus was 32%. In this experiment the proportions of the three eluates and the total extinction recovered were of the same order as in the analysis of the complete reaction mixture, but the recovery of phosphorus is higher and this is shown in lower values for \( \varepsilon_{\text{max.}} \) (P) of the fractions.

If the true \( \varepsilon_{\text{max.}} \) (P) in petroleum ether is taken as 16,500 it may be
calculated that for the first of the above two experiments the DNP-cephalin recovered is 23% of total lipid-nitrogen, which is in good agreement with the result obtained when the blank was removed by means of the cold finger (22% of total nitrogen). The two experiments suggest that while chromatography effectively separates DNP-cephalin from non-aminophospholipid, there is little separation from unreacted aminophospholipid.

4. Resolution of DNP-cephalin, with regard to Spectroscopic Properties.

In previous chromatographies (e.g., section 1) there was evidence that the more easily eluted DNP-cephalin fractions had λ\text{max} somewhat lower than the less readily eluted fractions when measured in petroleum ether. In order to obtain better evidence of the possible heterogeneity of DNP-cephalin, an experiment was designed without particular regard to recoveries but with the aim of isolating an enriched "fast-running" fraction, a "middle" fraction and a "slow-running" fraction.

The material taken for this experiment was fraction C of Table 6, Chapter 2. The products of reaction with FDNB, after removal of triethylamine were washed in ether solution with 0.1 N hydrochloric acid, followed by water. After taking to dryness, they were transferred in benzene to a column of talc (10 gms.). A benzene eluate containing DNP-lipid due to overloading, was reserved for further purification. The adsorbed material was eluted with ethanol:chloroform (1:4 by vol.) and ethanol:water (9:1 by vol.). The combined ethanol:chloroform and ethanol:water eluates were then placed on 15 gms. celite in benzene. Benzene and chloroform removed two fast-running bands which were discarded. The bulk of the remaining material was eluted by ethanol:chloroform (1:4 by vol.), and was labelled "middle fraction". Ethanol:water (9:1 by vol.) eluted further material which was
Fig. 6.
Absorption spectrum in petroleum ether of "fast-running" material from fraction V, referred to in text.
labelled "slow-moving fraction". The benzene eluate from the first chromatography on talc was placed on 10 gms. celite in benzene. Benzene eluted a fast-running band, presumably blank, which was discarded. Subsequently the ethanol:chloroform (1:4 by vol.) eluate was collected and labelled "fast-running fraction".

The most interesting fraction was the "fast-running fraction". The absorption spectrum measured in petroleum ether showed $\lambda_{\text{max}}$ at 340 mp with evidence of a subsidiary maximum at 405 mp (figure 6). This is quite different from the absorption spectrum in petroleum ether of total DNP-cephalin (figure 4). $\varepsilon_{\text{max}}$ (P) of this fraction was 9,900 and the ratio of fatty acids to phosphorus (the method for fatty acid determination is described in Appendix 1) was 2.8. The "middle fraction" had the usual absorption characteristics of DNP-cephalin in petrol. $\varepsilon_{\text{max}}$ (P) was 11,400 and the fatty acid to phosphorus ratio, 2.2. The "slow-running fraction" was insoluble in petrol and had $\lambda_{\text{max}}$ at 348 mp in ethanol.

5. Re-chromatography of DNP-cephalin Fractions.

The starting material in this experiment was sub-fraction IIIb of Table 7, Chapter 2. One of the objects was to see whether a fast-running component similar to that prepared from fraction V could be found in fraction III of the brain cephalin and at the same time to observe whether constant $\varepsilon_{\text{max}}$ (P) could be obtained on repeated chromatography.

A quantity of material containing 31 μ moles nitrogen was reacted with FDNB and the reaction products after acid-washing, transferred in benzene solution to a column of celite (15 gms.). After elution of side-products with benzene, DNP-lipid was eluted with ethanol:chloroform (1:4 by vol.) and ethanol:water (9:1 by vol.); the yield of DNP-cephalin was 70%
Fig. 7.
Absorption spectrum in petroleum ether of "fast running" material from fraction III, referred to in text.
of total nitrogen on an equivalent basis. Re-chromatography was carried out on 20 gms. celite. No band formation was observed. Chloroform spread the zone of adsorption but did not elute any material. Elution was effected with ethanol:chloroform (1:9 and 1:4 by vol.) to give fraction J. Ethanol:water (9:1 and 2:3 by vol.) provided fractions B and C. The absorption spectra of A and B were measured in petroleum ether but that of C in benzene, since it was insoluble in petrol. All had $\lambda_{\text{max}}$ at 345 mp and their respective $\varepsilon_{\text{max.}} (P)$ were 11,700, 14,800 and 8,300. The molar ratios of fatty acids to phosphorus were respectively 2.58, 2.04 and 1.92. The recovery of extinction in the second chromatography was 90%.

When B was re-chromatographed on siliconed celite (20 gms.), most of it moved at the front in benzene to give D. A chloroform eluate (E) and an ethanol eluate (F) were collected for comparison. All fractions had $\lambda_{\text{max.}}$ at 345 mp measured in benzene; in the case of D, the inflection at 400 - 420 mp took on the appearance of a maximum (figure 7) similar to that in the "fast-running" material from fraction V. Their respective $\varepsilon_{\text{max.}} (P)$ were 14,500, 14,600 and 15,000. Based on their extinctions at 345 mp, the proportion D:E:F = 85:9.5:5.5. The recovery of extinction was 88%.

**Discussion.**

At the time this work was commenced (1953) there were no reports of the successful isolation of cephalin by chromatography. The experience of Borgstrom (1952) who studied chromatography of phospholipids on sucrose, silica gel and magnesium oxide, first indicated the possibility of recovering cephalin by adsorption methods. While Borgstrom (1952) was able to recover
the total phospholipids from silica in a methanol eluate, he did not attempt the separation of cephalin from choline-containing phospholipids. Some time after my own work commenced, Lea and Rhodes (1954) reported the separation of cephalin from choline-containing phospholipids by chromatography on silica.

The formation of a coloured derivative was implemented mainly as an aid to the study of the chromatographic behaviour of cephalin. At the same time it was hoped that dinitrophenylation of the amino group, thereby abolishing its basic property, would lead to greater ease of elution as compared with the native phospholipid. However the experiments described in Section 3 (under Experimental and Results) show that the latter expectation was not confirmed. Nevertheless, in spite of very impure starting material the greater part of the derivative has been isolated with a molar ratio of phosphorous to DNP substituents of the order expected. In chromatographies of fraction V of the ox-brain cephalin on talc, the phosphorus recovered along with DNP-amino-nitrogen was equivalent to amino-nitrogen, determined independently by the ninhydrin method after hydrolysis.

In order to appreciate what has been achieved in these chromatographies, one has to consider the possible nature of the non-amino-phospholipid contaminant in the ox-brain cephalin. Whatever it is, it is certainly phospholipid and with similar solubility properties to those of cephalin. It may therefore be expected in other sources of cephalin and would not be readily separated by counter-current distribution or other processes which are the same in principle, such as partition chromatography which has been employed in a number of instances (Hecht and Mink, 1952; Amelung and Bohm, 1954; Lea, Rhodes and Stoll, 1955) to resolve phosphatidyl-ethanolamine and -serine or to separate lecithin and cephalin. Unfortunately
in none of the instances cited, have the fractions obtained been analysed for amino-nitrogen. The analyses of Artom (1945) as well as other examples discussed in Chapter 4, have shown that total phospholipids are not always accounted for by aminophospholipid and choline-containing phospholipids. Choline was shown to be absent from an acid hydrolysate of the cephalin employed in this work (Chapter 3); and a final attempt at the purification of the cephalin fractions, while it yielded material with the correct proportions of nitrogen, fatty acids and phosphorus, resulted in a lowering of the proportion of amino-nitrogen to total nitrogen in the case of fraction V. The contaminant must therefore be an unknown nitrogen-containing phosphoric or a re-arrangement product of cephalin. The existence of unidentified nitrogenous phospholipids in brain has been shown conclusively in the analysis by isotope dilution, which were carried out by Chargaff, Ziff and Rittenberg (1942).

Phospholipids present such complex mixtures, varying in composition from one source to another, that any attempt to establish a procedure for the isolation of a particular component would require wide application before it could be adequately appraised. Moreover, analyses of the supposedly purified component will have to be of a higher order of accuracy than generally accepted by biochemists if reliable conclusions can be made from turnover studies. The importance of purity and analytical accuracy is emphasised by the work of Dawson (1954b), who found that when inorganic p³² is incorporated into the lipid fraction of a brain homogenate, the inositol diphosphate, which is recovered as a relatively minor component of the total lipid phosphorus, has a specific activity ten times that of the total phospholipid. Recently, Lea, Rhodes and Stoll (1955) have described the isolation of cephalin from egg yolk phospholipid by chromatography on silica gel. The material gave an atomic ratio of nitrogen
to phosphorus of 0.96 and amino-nitrogen was 96% of total nitrogen. The latter was determined by a method which has been employed only by the authors themselves and consists of direct reaction of the lipid sample with ninhydrin. The uncertainties of this method will be discussed in the next chapter.

However, supposing that this material is 92% aminophospholipid, the experience of other workers who have employed silica gel indicates that the same success would not be achieved with other sources of cephalin. McKibbin and Taylor (1952) took as their starting material the alcohol-insoluble lipid fraction of dog liver. This was chromatographed on silica gel using the same eluants as Lea, Rhodes and Stoll (1955) used, although the silica was from a different source. The readily eluted fraction (corresponding to cephalin in the case of the egg yolk phospholipid) which was eluted with methanol:chloroform (11:89 by vol.), was found to be free of choline but rich in inositol and contained a sphingolipid which was not sphingomyelin. By submitting this eluate to another alcohol precipitation followed by re-chromatography of the supernatant, a fraction was obtained with an atomic ratio of nitrogen to phosphorus of 0.07 and with glycerol, phosphorus and fatty acids in the molar ratio 3:2:3 (“polyglycerol phosphatide”). Since this latter fraction accounted for only 0.5% of the original lipid phosphorus, its presence would not be conspicuous in a "purified" cephalin preparation. If its rate of turnover were several hundred-fold that of cephalin, erroneous conclusions might be made from a claim of purity in the cephalin isolated.

Evidence of complexity even in that portion of the ox-brain cephalin fractions which reacts with FDNB has been found in the absorption spectra of eluates. The "fast-running" fractions with a subsidiary maximum in the vicinity of 400 μm also have an unusual phosphorus and fatty acid composition.

In experiments where there was no attempt to obtain an enriched "fast-running"
fraction, the more easily eluted fractions had the highest $\varepsilon_{\text{max.}}$ (P), of the order 16,000 to 17,000 while $\varepsilon_{\text{max.}}$ (P) of the less readily eluted fractions was lower, possibly because of incomplete separation from non-DNP-lipid phosphorus. It is surprising therefore, that an enriched "fast-running" fraction should have a molar proportion of fatty acids to phosphorus of 2.8 and $\varepsilon_{\text{max.}}$ (P) of 9,900, suggesting the predominance of a component with a molar ratio of fatty acids to phosphorus of 3 and of DNP groups to phosphorus of 0.5. Apparently insufficient of this material was present in DNP-cephalin to markedly affect the absorption curve or the ratio of DNP groups to phosphorus atoms in the fraction readily isolated by chromatography. It is conceivable that the "polyglycerol phosphatides" of McKibbin and Taylor (1952) referred to in the previous paragraph is a building stone of such a complex amino-phospholipid, since it contained the same proportion of fatty acids to phosphorus although it was free of nitrogen. More recently, the counter-current distribution studies of Garcia, Lovern and Olley (1956) have lead to similar conclusions. Unidentified phospholipid fractions obtained by this means had a composition approximating to a molar ratio of fatty acids:glycerol:phosphorus of 4:2:1. Nitrogen was also present, although no analytical figure was given. The bases were not identified.

In the face of such evident complexity, it is impossible to speak of "cephalin" or "DNP-cephalin" as a single entity. For this reason it may be too much to expect that $\varepsilon_{\text{max.}}$ (P) of all eluates should be the same as that of model compounds such as DNP-serine or DNP-octadecylamine, or even exactly a multiple. Small departures from 16,000 - 17,000 may be due to varying electromeric effects in different complex forms of DNP-cephalin. Re-chromatography of a DNP-cephalin fraction with $\varepsilon_{\text{max.}}$ (P) of 14,800 gave
three fractions with successive \( \varepsilon_{max} \) of 14,500, 14,600 and 15,000. (Section 5).

The study of the chromatography of DNP-cephalin was not carried beyond this exploratory stage since at the same time considerable advantage was found in further modification of cephalin by esterification of the free acid group (or groups). This work will be described in Chapter 7. Nevertheless it was thought necessary to establish the direct reaction of lipids with FDNB as a means for the direct determination of lipid amino-nitrogen and this will be the subject of Chapter 6.
(a) The system of Sanger (1945) does not provide a satisfactory means for the preparation of DNP-lipids, which undergo hydrolytic decomposition in this system.

(b) The conditions affecting the reaction of lipids with fluoro-2,4-dinitrobenzene in anhydrous systems have been studied and from these, a simple and rapid method for the preparation of DNP-lipids has been worked out.

(c) Adsorption chromatography of DNP-cephalin derived from the ethanol-insoluble fraction of ox-brain lipids has been shown to effect quantitative recovery of DNP-cephalin from a reaction mixture, with a useful degree of separation from non-aminophospholipid impurity. Evidence has been found of complex forms of aminophospholipid.
CHAPTER 6.

THE DETERMINATION OF AMINOPHOSPHOLIPIDS WITH

FLUORO-2,4-DINITROBENZENE.

Introduction.

The determination of lipid amino-nitrogen without hydrolysis presents at least two advantages: firstly, of being able to dispense with the tedious preparation of an hydrolysate and secondly, of obtaining a more valid estimate of aminophospholipid, since there is the possibility of occurrence of bound forms which liberate amino-nitrogen on hydrolysis.

There are three methods which have been used for the direct determination of lipid amino groups, all of which involve loss of the lipid sample and none of which has met with general satisfaction. For many years the van Slyke procedure (van Slyke, 1911, 1929) was the only method available for the determination of amino groups and it has frequently been applied without hydrolysis. However it was generally found that results by direct method in which the lipid sample is dissolved in glacial acetic acid, were 10 - 20% higher than those obtained on an hydrolysate (for example, Rudy and Page, 1930). Polich (1946) found that the direct van Slyke method gave erratic results in his hands. Contamination with non-lipid amino-nitrogen was for some time thought to be the cause of high and variable results by this procedure. However the recent work of Lea and Rhodes (1954) has shown pretty conclusively that the excess nitrogen evolved is due to reaction of unsaturated fatty acids with the nitrous
acid but they do not suggest a mechanism for this reaction.

The ninhydrin reaction, which has been discussed fully in Chapter 4, has been studied by Lea and Rhodes (1956) as a micro-method for the direct determination of cephalin. They employed the reaction systems of Moore and Stein (1946) and Powden (1951) and found both reagents to give variable colour yields from one batch to another. It was therefore necessary to refer constantly to standardised solutions in each run of analyses. Reference standards consisted of synthetic phosphatidyl ethanalamine and egg phospholipid preparations which had been independently analysed after hydrolysis (6 N hydrochloric acid in sealed tube at 100°C, for 15 hours) by the van Slyke procedure. Lea and Rhodes have stated in a later paper (1955) that synthetic phospholipid as well as native phospholipid, undergoes loss of amino-nitrogen on storage. Consequently the availability of suitable reference material becomes a serious set-back to the use of the ninhydrin reaction. It remains a valuable qualitative test for the presence of traces of aminophospholipid in preparations of other phospholipids, for example lecithin.

The third procedure which has been applied to the estimation of cephalin without hydrolysis, is the titration of acid groups. The acidic nature of aminophospholipids has already been discussed in Chapter 2; here it was shown that quite an accurate end-point may be obtained by titration of cephalin in aqueous dispersion. Although this fact is not widely realised, the titration of phospholipids dissolved in aprotic solvents such as benzene has been demonstrated as evidence of the switchoverionic nature of cephalin (Fishgold and Chain, 1935; Rudy and Page, 1930). The acid group of lecithin is not titrated with alkali owing to the presence of an equivalent amount of the strong base, choline.
Titration of phospholipids with alkali has not been generally adopted as a method for the direct determination of cephalin although it is far simpler than methods depending upon the determination of amino-nitrogen and may be expected to give results of equal significance, provided free fatty acids are not present. In the present work, it was thought of value to compare titration estimates of cephalin with those obtained by formation of the 2,4-dinitrophenyl derivative. Since the solvent mixture of Rudy and Page (1930) was found to give a satisfactory end-point with phenolphthalein, the titration was not carried out potentiometrically as in the acid titration of lecithin (Chapter 3).

In the attempt to appraise the reaction of lipids with FMIB as a reliable method for the direct determination of amino-nitrogen, attention has been given to both total phospholipid preparations and fractionated phospholipids. By applying the direct method to the analysis of total lipid extracts of rat tissues, it has been possible to compare results by this method with those of independent investigators.

Experimental.

Materials.

**Total Phospholipids.**

The preparation of egg yolk phospholipid and total lipid extracts of rat tissues has been described in Chapter 2. All preparations were acid-washed (Chapter 2) before analysis, unless otherwise stated.

**Fractionated Phospholipids.**

The fractionation procedures employed were those of Polch (1942) for
ox-brain cephalin; Lea, Rhodes and Stoll (1933) for egg yolk cephalin; and solvent fractionation of the cadmium chloride complex of egg yolk phospholipid, based upon the ether solubility of the cephalin complex and the insolubility in ether of the lecithin complex (Trier, 1913).

Lea, Rhodes and Stoll (1933) isolated egg yolk cephalin by chromatography on silica gel. The authors were not able to prepare batches of silica gel which consistently gave the best results. The separation of cephalin and lecithin was not sharp, necessitating trial experiments and the use of a fraction collector. In the present attempt to prepare cephalin by this method, silica gel (B.D.H., "for chromatography") was employed as adsorbent. The silica was dried at 124°C for four days prior to use. The material chromatographed was the acid-washed acetone-insoluble fraction of a chloroform:ethanol extract of egg yolks. Of this, 0.99 gm. was transferred in ethanol:chloroform (1:4 by vol.) to a column of 24 gm. silica prepared in the same solvent. The column was developed with the same solvent and approximately 15 mls. fractions collected with an automatic fraction collector. Upon weighing the fractions after removal of solvent in vacuo, no peak in the elution curve was discernible. A quantity of fat was slowly leached from the column, amounting to 116 mgs. in 700 mls. of effluent, after which the rate of elution became too slow to warrant further elution.

Solvent fractionation of a mixed complex with cadmium chloride has been used for the purification of lecithin since the time of MacLean (1915). Since the customary methods for the preparation of cephalin make use of precipitation with ethanol, that fraction of the cephalin which remains in solution along with the lecithin has been neglected. Cadmium chloride was
used in the present work to enable a partial separation of cephalin from lecithin in the ethanol-soluble fraction of the phospholipids of egg yolk. Three experiments of this nature were carried out and the details will be given in order, since a progressively larger number of fractions was prepared in each successive experiment.

1st Fractionation.

The yolks of ten eggs were homogenised with 2 litres of acetone. The residue after washing with acetone, was extracted with 500 mls. chloroform:ethanol (1:9 by vol.) and subsequently with four changes of 100 mls. ethanol. The chloroform:ethanol extracts were then concentrated in vacuo to about 200 mls., filtered and made to 1 litre with acetone. The precipitate thus formed was dissolved in a small volume of ethanol and re-precipitated with acetone. After collecting the precipitate and removing the acetone in vacuo, it was found to be incompletely soluble in petroleum ether. The petrol-soluble portion weighed 3.21 gms. and was insoluble in ethanol. (Ethanol-insoluble fraction).

The ethanol-acetone supernatant from the first acetone precipitation was concentrated in vacuo and more acetone added to give a further precipitate which gave a turbid solution in petroleum ether. The petrol-soluble portion recovered after centrifugation, weighed 6.23 gms. This was soluble in ethanol. (Ethanol-soluble fraction).

The acetone-extracted cake was suspended in petroleum ether overnight, separated by filtration and washed with petrol. After allowing the petrol-ether soluble fraction to stand in ether solution at 5°C. for five days, a small quantity of material precipitated out and this was filtered off. The ether-soluble material after acid-washing weighed 0.252 gms. (Petrol extract).

Cadmium chloride treatment of the first two fractions was carried out as follows:
The alcohol-insoluble fraction was dissolved in ether:ethanol (not sufficient ethanol to cause turbidity) and a saturated solution of cadmium chloride in methanol added until no further precipitate was formed. The precipitate which was removed by centrifugation, appeared to be partially soluble in ether but was nevertheless discarded. The supernatant, after being acid-washed to remove cadmium chloride, weighed 0.921 gm.

The ethanol-soluble fraction was dissolved in ethanol and similarly treated with cadmium chloride. The precipitate obtained was easily sedimentable in contrast to the previous fraction, and appeared to be quite insoluble in ether. Upon concentrating the ethanol supernatant in vacuo, a precipitate was obtained which appeared to be largely soluble in ether. This and the ether wash of the first precipitate were combined and acid-washed. The weight of phospholipid recovered was 0.315 gm.

2nd Fractionation Experiment.

In this experiment attention was confined to the ethanol-soluble portion of the total phospholipid, which was fractionated by extraction of the cadmium chloride complex with ether.

Each of 10 eggs was homogenised in about 50 ml. chloroform and sufficient ethanol added to the combined extracts to dehydrate the protein and so facilitate its filtration. The clear filtrate was washed several times with 0.1 N hydrochloric acid and then with water and finally dried to some extent over anhydrous sodium sulphate. After taking to dryness in vacuo, the fat was dissolved in about 1 litre of warm acetone and the solution allowed to cool at 5°C. The precipitated phospholipid was dissolved in 200 ml. of hot ethanol and the precipitate which formed on cooling was washed with a little ethanol and discarded. The soluble portion was treated with cadmium chloride (saturated solution in methanol) until completely precipitated. The cadmium chloride complex was washed three times with
ethanol saturated with cadmium chloride and finally extracted with successive 50 ml. portions of ether to which sufficient cadmium chloride was added to facilitate sedimentation of the precipitate in the centrifuge (about 3,500 g. for half an hour). Each of the ether extracts (fractions 1, 2 and 3, successively) was washed with 0.1 M hydrochloric acid and water, taken to dryness, weighed and made to volume in petroleum ether. After the third extraction very little of the cadmium chloride complex dissolved in ether.

3rd Fractionation.

This fractionation was carried out on a larger scale than the previous two, in order to obtain a larger number of fractions. The procedure was similar to that used in the second experiment. It was found that ether alone tended to dissolve too much of the complex at once. By adding a proportion of ethanolic cadmium chloride to the ether employed for extraction, a larger number of fractions could be obtained. However there was no attempt at a careful control of these conditions.

Egg yolks (24) were extracted with a total volume of about 2 litres of ethanol:chloroform (1:5 by vol.). Further ethanol was added to the extract to facilitate filtration. The residue was washed with a small quantity of ethanol:chloroform and the combined extracts were saturated with nitrogen, shaken up with 0.1 M hydrochloric acid and the emulsion allowed to stand overnight at 5 °C. The lower layer was subsequently removed and washed three times with a saturated aqueous solution of sodium sulphate. The washed chloroform:ethanol extract was finally dried over anhydrous sodium sulphate and taken to dryness under nitrogen at a temperature no higher than 40 °C.
The washed lipid extract was dissolved in 2 litres warm acetone and the acetone-insoluble material collected after cooling the solution to 5°C. The precipitate was washed with acetone and the acetone removed in vacuo. Ethanol-insoluble material was removed by solution in 1 litre of ethanol at room temperature and allowed to stand overnight at 5°C. under nitrogen. The ethanol-soluble fraction was taken up in a small volume of dry ether and allowed to stand under nitrogen at -10°C. for several days. The ether-insoluble fraction was discarded. The remainder after taking to dryness, weighed 17.3 gms.

Fractionation with cadmium chloride was effected in the following manner:

Fraction 1. Addition of a total of 175 mls. of a saturated solution of cadmium chloride in ethanol to a solution of the phospholipid in 100 mls. ethanol brought about complete precipitation of the cadmium chloride complex. The precipitate was removed by centrifugation and washed with a small quantity of ethanol. The combined pale yellow supernatant and wash were concentrated to about 25 mls. at reduced pressure and kept at -10°C. for a week. The precipitate which formed was discarded and the supernatant was diluted with ether and washed with 0.1 N hydrochloric acid. The fat recovered (0.162 gms.) was characterised by its solubility in ethanol in the presence of excess cadmium chloride.

Fraction 2. The first ether extraction was carried out by suspending the complex in 100 - 150 mls. ether to which 10 mls. ethanol saturated with cadmium chloride were added. After centrifugation this extract was diluted with ether and washed with 0.1 N hydrochloric acid to give 0.747 gms. phospholipid.
Subsequent Fractions. Two further extracts were obtained in the same way as Fraction 2. Fraction 3 weighed 0.222 gm. and Fraction 4, 2.17 gms. Less cadmium chloride was present in the latter extraction, which condition accounts for the increased weight of fat extracted. Subsequent extraction with ethanol:ether (1:10 by vol.) removed a negligible weight of lipid so the final fractions were obtained by extraction with ether alone. Fractions 5, 6, 7, 8 and 9 weighed 0.133 gms., 0.645 gms., 0.954 gms., 0.261 gms. and 0.163 gms. respectively. The residue which was in effect a purified lecithin preparation, weighed 10.3 gms. (Fraction 10).

Toxin Treatment.

The presence of a specific lecithinase in Clostridium welchii α-toxin suggested that enzyme treatment may be an effective means of separating cephalin from lecithin. The method used was based upon the test system described by MacFarlane and Knight (1941), which was claimed to bring about quantitative hydrolysis of lecithin. The preparation of toxin employed in this work was obtained from the Commonwealth Serum Laboratories, Melbourne.

Preparation 5 of the second fractionation of egg yolk phospholipids (72 mgms.) was emulsified in 5.0 ml. water and the pH adjusted to 8 with 0.1 N sodium hydroxide. Calcium chloride (0.5 ml. of 0.11 N solution) and about 5 mgms. toxin were added and the mixture incubated at 40°C. In the space of 10-15 mins., the opalescent emulsion had become clear with a quantity of fat floating on the surface. Titration with 0.1 N sodium hydroxide to pH 3 (employing an external indicator) showed that there was no further liberation of acid after an hour. The digest was finally acidified with hydrochloric acid, extracted with ether and the ether extract washed several times with 0.1 N hydrochloric acid and water.
Hydroxylamine Treatment:

Hydroxylamine was freshly prepared by decomposition of hydroxylamine hydrochloride with sodium ethoxide followed by distillation in vacuo. It was stored at -70°C. The lipid sample (10 - 50 mgms.) was dissolved in ether:ethanol, one drop of hydroxylamine added and the mixture taken to dryness at the water pump. Hydroxylamine was subsequently removed at a pressure of 0.01 - 0.05 mm. mercury at the temperature of the boiling water bath.

Analytical Methods.

Phosphorus and nitrogen were determined as described in previous chapters.

Amino-nitrogen was determined as the 2,4-dinitrophenyl derivative both directly (Chapter 5) and after hydrolysis (Chapter 4). In the direct method the blank was removed by means of the cold finger. The extinction was measured at 345 mµ in petroleum ether solution where ε_{max.} was taken as 16,500. In one case the ninhydrin reagent described in Chapter 4 was applied directly to the lipid sample.

Choline-containing phospholipids were determined after hydrolysis as choline phosphomolybdate (Chapter 3).

Acidic phospholipids were determined by non-aqueous titration employing the method of Rudy and Page (1930). The phospholipid sample was dissolved 15 ml. benzene, 2.0 ml. ethanol added and the solution titrated to an end-point with phenol phthalein against standard (0.02 M) ethanolic potassium hydroxide. The blank titration was 0.1 - 0.2 ml.

Plasmalogens was determined by the method of Feulgen and Grünberg (1930).
Results.

The analytical methods developed in this thesis for choline (Chapter 3), serine and ethanolamine (Chapter 4) and lipid amino-nitrogen (Chapter 5) were jointly applied to the study of the composition of egg yolk phospholipid, which is readily prepared and of relatively simple composition. Leclíthin is reported to make up 60% of egg yolk phospholipid and sphingomyelin and cerebrosides are minor components, accounting for 2.5% and 1.0% of total lipid nitrogen respectively (Calbraith, Sutton and Williams, 1943). The present investigation was planned to provide a comprehensive appraisal of the direct method for the determination of amino-phospholipid with special attention to the purification of lipid extracts and the stability of amino-nitrogen in extraction procedures; and by comparison with alternative estimates, for example as non-choline-containing phospholipid (the procedure which has been most commonly adopted), as serine and ethanolamine obtained after hydrolysis and by titration. Analyses of both total and fractionated egg yolk phospholipid will be presented, the latter because they reveal better the complexity of the phospholipid composition which is not so evident when a total extract is analysed. This fractionation was carried out primarily for another purpose which will be described in Chapter 7.

The determination of the phospholipid composition of rat organs is finally presented to demonstrate the application of the methods and enable a comparison to be made with the results of other workers.

Analyses of Total Phospholipid from Egg Yolk.

Two methods which have been used in this work for the purification of phospholipids, acid-washing and filtration through cellulose, have been
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>unwashed</td>
<td>938</td>
<td>1060</td>
<td>370</td>
<td>164</td>
<td>140</td>
</tr>
<tr>
<td>paper column</td>
<td>517</td>
<td>600</td>
<td>390</td>
<td>131</td>
<td>...</td>
</tr>
</tbody>
</table>

|            |            |          |         |                      |                        |
| unwashed   | 935        | 3500     | 680     | 260                   | 242                    |
| paper column| 940        | 940      | 640     | 226                   | 213                    |
| acid-washed| 930        | 1060     | 690     | 224                   | ...                    |
### TABLE 2

**Aminophospholipids in Serial Extracts of Egg Yolks.**

<table>
<thead>
<tr>
<th>Extraction Solvent</th>
<th>L/P</th>
<th>Atomic Ratio, Choline/L/P</th>
<th>Atomic Ratio, Direct Hydrolyzate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ether, 15°C.</td>
<td>3.57</td>
<td>1.02</td>
<td>0.63</td>
</tr>
<tr>
<td>Ethanol:ether (3:1 by vol.), -10 to 0°C.</td>
<td>2.94</td>
<td>0.94</td>
<td>0.72</td>
</tr>
<tr>
<td>Ethanol:ether (1:1 by vol.), 60°C.</td>
<td>3.01</td>
<td>0.96</td>
<td>0.67</td>
</tr>
<tr>
<td>Weighted Mean</td>
<td>3.74</td>
<td>0.99</td>
<td>0.67</td>
</tr>
</tbody>
</table>

See also Table 1 of Chapter 2. Lipids insoluble in ether were removed prior to acid-washing of each preparation.
discussed and compared in Chapter 2. The analyses shown in Table 1 were obtained on the petroleum ether-soluble portion of an ether extract of egg yolks. As pointed out in Chapter 2, solubility of a lipid extract in petroleum ether is not a reliable criterion of the absence of impurities; however in these preparations from egg yolks, the impurities removable by acid-washing and filtration through cellulose have not interfered significantly with the chlorine determination and have led to only slightly higher values for amino-nitrogen.

In securing phospholipid preparations for a variety of purposes (such as experiments to be described in the next chapter), ether extraction was employed for convenience in evaporating to dryness, as well as to avoid possible loss of amino nitrogen when using polar solvents, which require higher temperatures for evaporation. As shown in Table 1 of Chapter 2, ether extracts little more than one third of the phospholipid which may be extracted from egg yolk by ethanol:ether. Since it was of interest to know the proportion of cephalin to total phospholipid in a complete extract, an analysis of serial extracts was carried out as shown in Table 2. This table shows a much higher proportion of cephalin to total phospholipid in an initial ether extract as compared with the subsequent ethanol:ether extracts, a result which could be due to selective extraction of cephalin by a weekly polar solvent or to destruction of amino groups under the conditions of extraction in the presence of ethanol.

The latter contingency was tested by submitting the same preparation of phospholipid to various treatments intended to simulate extraction procedures. There was no significant loss of amino-nitrogen when a sample of total egg yolk phospholipid was (a) heated in ethanol:ether (1:1 by vol.)
<table>
<thead>
<tr>
<th>Arino-Nitrogen as P% of Choline-Nitrogen</th>
<th>Arino-Nitrogen + Choline-Nitrogen, as P% of Total Nitrogen</th>
<th>Arino-Nitrogen, as P% of Non-Choline-Nitrogen</th>
</tr>
</thead>
<tbody>
<tr>
<td>21.2</td>
<td>67</td>
<td>64</td>
</tr>
<tr>
<td>16.0</td>
<td>93</td>
<td>52</td>
</tr>
<tr>
<td>20.6</td>
<td>93</td>
<td>74</td>
</tr>
<tr>
<td>19.5</td>
<td>89</td>
<td>64</td>
</tr>
<tr>
<td>23.8</td>
<td>90</td>
<td>64</td>
</tr>
<tr>
<td>21.2</td>
<td>95</td>
<td>71</td>
</tr>
<tr>
<td>22.6</td>
<td>96</td>
<td>71</td>
</tr>
<tr>
<td>21.6</td>
<td>93</td>
<td>76</td>
</tr>
<tr>
<td>21.0</td>
<td>98</td>
<td>68</td>
</tr>
</tbody>
</table>
in a sealed tube at 50°C. for 6 hours; (b) heated in ethanol-ether (1:5 by vol., saturated with water) in a sealed tube at 50°C. for 6 hours; (c) emulsified in a solution of purified egg albumin (0.5 gm. albumin and 3 gm. phospholipid in 5.0 ml.) and heated at 50°C. for 6 hours.

These test systems do not exactly replicate the conditions of extraction but rule out the extraction solvent and protein, to which tissue phospholipids are largely bound, as possible causes of low recovery of amino-nitrogen.

In Table 3 are collected details of preparation and analytical results for a number of egg yolk phospholipid preparations which have been used during the course of this work.

It may be seen that in all the analyses thus far presented, there is good agreement between determinations of amino-nitrogen both directly and after hydrolysis. In these hydrolysates all amino groups were found in the form of DPI-ethanolamine, in agreement with the previously reported absence of serine from egg yolk phospholipid (Chayes, Ziff and Rittenberg, 1942). The proportion of total nitrogen found in the form of amino-nitrogen is of the same order as found by other workers; Ise, Rhodes and Stoll (1935) reported 18.4% of total phospholipid to be cephalin while Kaucher and co-workers (1943) by subtraction of choline from equivalents of total phosphorus, found 29%. The latter method of determination if applied to my own results, provides a considerably higher estimate of cephalin than direct determination. This has been made explicit in Table 3 where in no case is total lipid phosphorus accounted for by equivalents of choline and amino-nitrogen. Thus amino-nitrogen averages only 60% of non-choline-nitrogen, total nitrogen being almost equivalent to total phosphorus; the implication
### TABLE 4.

**Analysis of Ox-Brain Cerebrin for Amino-Nitrogen.**

(Amino-Nitrogen expressed as % Total Phosphorus)

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Kinhydrin (direct)</th>
<th>Kinhydrin (hydrolysate)</th>
<th>F.N.E. (direct)</th>
</tr>
</thead>
<tbody>
<tr>
<td>III</td>
<td>34</td>
<td>72</td>
<td>70</td>
</tr>
<tr>
<td>V</td>
<td>58</td>
<td>57</td>
<td>58</td>
</tr>
</tbody>
</table>
**TABLE 5.**

Composition of Egg Yolk Cephalin prepared according to Lea, Rhodes and Stoll (1955).

<table>
<thead>
<tr>
<th>A.P.</th>
<th>N/P</th>
<th>Choline-N/P</th>
<th>Amino-N/P (^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.13</td>
<td>1.02</td>
<td>0.0</td>
<td>0.72</td>
</tr>
</tbody>
</table>

\(^a\) Determined by direct reaction with PNDB.
of these results is the occurrence in egg yolks of a non-choline-containing, non-amino-nitrogen-containing phospholipid fraction. While this is an in-conspicuous part of the total phospholipid, it is present in amounts comparable to the aminophospholipid fraction.

Analyses of Fractionated Phospholipid.

Estimates of amino-nitrogen in ox-brain cephalin fractionated according to Folch (1942) have already been considered, both after hydrolysis (Chapter 4) and by preparation of the EEP derivative (Chapter 5). The ninhydrin reagent described in Chapter 4 was also applied directly to samples of fractions III and V employing ethanalamine as standard. The collected results are shown in Table 4.

The preparation of cephalin obtained by chromatography of egg yolk phospholipid on silica had the composition shown in Table 5. For a similar preparation Lee, Rhodes and Stell (1955) found an atomic ratio of amino-nitrogen to total nitrogen of 0.96 by direct reaction with ninhydrin.

The fractionation of egg yolk phospholipid into alcohol-insoluble and alcohol-soluble cephalin fractions, the latter being partially removed from lecithin by conversion to the cadmium chloride complex, provided an opportunity to study the distribution of the unidentified fraction in which nitrogen was apparently neither choline-nitrogen nor amino-nitrogen. It was hoped that a fraction might be obtained in which total lipid phosphorus, or even total lipid nitrogen, might be accounted for by equivalents of amino-nitrogen and choline. At the same time additional factors in the direct determination of cephalin not dealt with in the analyses of total phospholipid preparations, have been examined.
<table>
<thead>
<tr>
<th>Non-Choline-N/V</th>
<th>(d)</th>
<th>(a + b)</th>
<th>(a + c)</th>
<th>(c/3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.29</td>
<td>0.29</td>
<td>1.09</td>
<td>0.71</td>
<td>0.68</td>
</tr>
<tr>
<td>0.44</td>
<td>0.93</td>
<td>0.80</td>
<td>0.55</td>
<td></td>
</tr>
<tr>
<td>0.43</td>
<td>1.00</td>
<td>0.91</td>
<td>0.56</td>
<td></td>
</tr>
<tr>
<td>Equiv. $\text{H}^+/\text{P.}$</td>
<td>Amino-$\text{H}/\text{P.}$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>------------------</td>
<td>-----------------</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.05</td>
<td>0.59</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.37</td>
<td>0.35</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.20</td>
<td>0.24</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
The analytical results of the first fractionation of egg yolk phospholipids may be seen in Table 6. As was expected, the alcohol-insoluble fraction was low in choline-containing phospholipids. Although the alcohol-soluble fraction contained more lecithin than cephalin in spite of the calcium chloride treatment, nevertheless the proportion of cephalin was higher than in unfractionated egg phospholipid. It is probably quite accidental that the petrol extract is similar in composition to the alcohol-soluble fraction. All fractions contain nitrogen which is neither choline- nor amino-nitrogen and there is no great difference in the amount of unidentified nitrogen present in the "alcohol-insoluble" and "alcohol-soluble" fractions, in contrast to the large difference in cephalin content. Consequently the proportion of amino-nitrogen to unidentified nitrogen is higher in the "alcohol-insoluble" fraction than in the "alcohol-soluble". On the other hand, column (a+b) shows that if one determines cephalin as equivalents of acid, a fairly complete account of total nitrogen is obtained.

The analytical results of the second fractionation are shown in Table 7. These analyses are of such an enigmatic nature as to suggest at first sight, some derangement of the phospholipid; but if this is so, it is difficult to imagine what kind of re-arrangement or interaction of cephalin and lecithin would account for the composition of fractions 2 and 3, no form of hydrolysis is consistent with a residual atomic ratio of nitrogen to phosphorus higher than one, since the non-fatty fragment would have been removed by acid-washing. Moreover a control experiment in which a sample of total egg phospholipid was heated with excess calcium chloride in ethanol:ether solution at 60°C. for 15 minutes, provided no evidence of instability of phospholipid in the presence of calcium chloride: the fat
### TABLE 8.

**Effect of Toxin Treatment of an Egg Yolk Phospholipid Fraction.**

The material referred to is fraction 3 of Table 7.

<table>
<thead>
<tr>
<th></th>
<th>Before Toxin Treatment</th>
<th>After Toxin Treatment (Followed by acid-washing)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight (mgms.)</td>
<td>72</td>
<td>61</td>
</tr>
<tr>
<td>P (μ moles)</td>
<td>64</td>
<td>18</td>
</tr>
<tr>
<td>N (&quot; )</td>
<td>105</td>
<td>32</td>
</tr>
<tr>
<td>Amino-N (&quot; )</td>
<td>15</td>
<td>13</td>
</tr>
<tr>
<td>K/P</td>
<td>1.65</td>
<td>1.80</td>
</tr>
<tr>
<td>Amino-K/2</td>
<td>0.24</td>
<td>0.72</td>
</tr>
</tbody>
</table>
131.

recovered after acid-washing had the same composition as initially.

Further information about the composition of fraction 3 was obtained in an attempt to remove lecithin by the action of \( \alpha \)-toxin of *Clostridium welchii*, which is a source of lecithinase D, an enzyme which catalyzes hydrolysis of lecithin to choline phosphoric acid and diglyceride. According to MacFarlane and Knight (1921), the crude toxin is free of lipase and MacFarlane (1943) has found that it has no activity towards cephalin. The recovery of lipid constituents after toxin treatment and acid-washing is shown in Table 8. Analyses for choline and acid groups were not performed on the toxin treated fat; however, the analysis of the starting material (Table 7) showed that all non-choline nitrogen was amino-nitrogen. It is perhaps significant that the atomic ratio of nitrogen to phosphorus has risen to 1.3 while there has been very little loss of amino-nitrogen. The atomic ratio of amino-nitrogen to phosphorus has become 0.72 as compared with 0.24 before toxin treatment. From the data in Table 8, it is evident that the split products (removed by acid-washing) had an atomic ratio of nitrogen to phosphorus of 1.6. That the excess of nitrogen over phosphorus should in fact increase following toxin treatment and repeated acid-washing suggests that the excess nitrogen, apparently choline, is an integral part of a complex molecule and is bound in a form resistant to the action of lecithinase D.

A further interesting feature of fraction 3 is the approximate equivalence of acid groups and amino-nitrogen, in contrast to fractions 1 and 2 where acid groups are greatly in excess of amino-nitrogen.

The results of the third fractionation are collected in Table 9. With the exception of fraction 1 which was only a small fraction of the
<table>
<thead>
<tr>
<th>Amino-N/P, (direct)</th>
<th>Amino-N/P, (hydrolysate)</th>
<th>Amin+ + Choline-N/P.</th>
<th>Eq.</th>
<th>Plasma (mals/at F)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.72</td>
<td>0.81</td>
<td>0.30</td>
<td>3.02</td>
<td>0.146</td>
</tr>
<tr>
<td>0.50</td>
<td>0.52</td>
<td>0.60</td>
<td>1.14</td>
<td>0.145</td>
</tr>
<tr>
<td>0.47</td>
<td>0.52</td>
<td>0.71</td>
<td>1.24</td>
<td>0.126</td>
</tr>
<tr>
<td>0.41</td>
<td>0.37</td>
<td>0.90</td>
<td>1.08</td>
<td>0.058</td>
</tr>
<tr>
<td>0.46</td>
<td>0.45</td>
<td>1.08</td>
<td>1.07</td>
<td>0.103</td>
</tr>
<tr>
<td>0.49</td>
<td>0.50</td>
<td>0.96</td>
<td>1.14</td>
<td>0.070</td>
</tr>
<tr>
<td>0.44</td>
<td>0.43</td>
<td>0.94</td>
<td>1.10</td>
<td>0.053</td>
</tr>
<tr>
<td>0.50</td>
<td>0.51</td>
<td>0.75</td>
<td>0.94</td>
<td>0.071</td>
</tr>
<tr>
<td>0.54</td>
<td>0.55</td>
<td>0.92</td>
<td>1.13</td>
<td>0.096</td>
</tr>
<tr>
<td>0.624</td>
<td>0.019</td>
<td>0.019</td>
<td></td>
<td>0.180</td>
</tr>
</tbody>
</table>
total phospholipid and fraction 10 which is very nearly pure lecithin, the 
fractions obtained in this experiment were all rather similar in composition. 
This is in contrast to the previous fractionation. The explanation must 
lie in the arbitrary manner in which the fractionation of the cadmium 
chloride complex was carried out. While the cadmium chloride complex of 
fraction 10 was virtually insoluble in ether, the initial mixed complex of 
cephalin and lecithin was readily soluble in ether and an arbitrary amount 
of ethanol was added at each extraction to repress the solubility of the 
lecithin component. It would seem that the cadmium chloride complex of 
cephalin exerts a solubilising effect on the lecithin complex, reflecting 
the behaviour of the native phospholipids.

The determination of amino-nitrogen after hydrolysis in this experi- 
ment shows that there is uniformly good agreement between direct and in-
direct estimates with NBD. In fractions 5 and 6, the sum of choline-
and amino-nitrogen is equivalent to phosphorus but in no fraction is total 
nitrogen accounted for. Amino-nitrogen in hydrolysates of most fractions 
was found by reaction with NBD to be present entirely as ethanalamine but 
in some fractions there occurred an additional DNP derivative with absorp-
tion maxima at 365 m\(\mu\) and 420 m\(\mu\) in acetone (figure 9, Chapter 4), the 
colour being discharged in acid. There were only traces of this material 
present by comparison with the extinction of DNP-ethanalamine.

Reviewing the results of the above three experiments as a whole, the 
most striking observation is the inconsistency of the analyses with the 
belief that egg yolk phospholipid is simply a mixture of cephalin and leci-
thin, as is suggested for example, in the table set forth by Wittooff (1951). 
The presence of sphingolipids, amounting to only 3\% of the total phospho-
lipids (Kaufer and co-workers, 1943) cannot assist the interpretation of the present results. Most of this fraction must, in any case, have been removed in the ether separation. Throughout all three fractionations, acid equivalents have been found to be in excess of amino-nitrogen though frequently of the same order as non-choline nitrogen; and amino-nitrogen in some preparations has shown agreement with non-lecithin phosphorus but has generally been less than non-lecithin nitrogen.

Before accepting the results presented above as a valid basis for speculation about the composition of egg yolk phospholipid, some obvious sceptical interpretations were considered. Firstly there is the possibility that the high titration values were due to contamination of the fractions with free fatty acids. A determination of free fatty acids would therefore have been desirable but was not carried out. The following considerations may be weighed against this possibility: (a) free fatty acids if present, would have been removed upon acetone precipitation of the total phospholipids; (b) frequent agreement between acid equivalents and atoms of non-choline nitrogen (for example, fractions 4 - 3, Table 9) suggests a relationship not likely to arise from accidental determination.

Secondly there exists the possibility of an incomplete yield of MP derivative in the reaction with PHMB. This might result through side-reactions which may take place under the basic conditions of the reaction system. For example, it is conceivable that acid-washing may have resulted in some liberation of aldehydes from plasmalogen; in the presence of tri-ethylamine, these would react with amino groups to form Schiff's bases. For this reason plasmalogen determinations were carried out on the preparations obtained in the third fractionation (Table 9). However it can be
seen that the plasmalogens content, supposing complete degradation to aldehydes on acid-washing, is insufficient in most cases to account for the difference between amino-nitrogen and non-choline nitrogen. Yet another consideration which argues against this possibility is the agreement found between amino-nitrogen before and after hydrolysis. Since fatty aldehydes would be removed upon ether extraction of the hydrolysate, a higher estimate would be found on the hydrolysate if fatty aldehydes interfered with the direct determination.

Apart from the above considerations arising from the instability of plasmalogens, there remained the possibility of loss of amino-nitrogen in the FAB reaction system as a result of interaction of amine groups with unknown substituents in the fat, which may also occur in the hydrolysate. This kind of interference may be more conspicuous in the analysis of fractionated phospholipids as compared with a total phospholipid preparation because of possible concentration of the interfering substance in a particular fraction.

Since hydroxylamine is generally more reactive than aliphatic amines in reactions with carbonyl compounds the effect of hydroxylamine treatment prior to reaction with FAB was examined with fractions 2 and 4 of Table 2. Fraction 2 in which non-choline nitrogen is 15% of amino-nitrogen, underwent only 11% increase in amino-nitrogen after treatment with hydroxylamine; fraction 4, in which non-choline nitrogen is 13% of amino-nitrogen, also showed 11% increase. In order to examine the likelihood that the apparent increase in the yield of FAB derivative was merely due to reaction of FAB with incompletely removed hydroxylamine, a sample of hydroxylamine was reacted with FAB and the more volatile products removed in vacuo by use
Fig. 1.

Absorption spectrum in benzene, of product of reaction of FDNB and hydroxylamine.
Effect of treatment of a sample of egg yolk phospholipid with hydroxylamine on the yield of DNP derivative. Curves A and A': absorption spectra in petroleum ether of DNP-lipid and product of methylation of DNP derivative, without hydroxylamine treatment. Curves B and B': absorption spectra of DNP derivative of hydroxylamine-treated lipid before and after reaction with diazomethane. It may be seen that there is no distortion of B and B' with respect to A and A', which would indicate contamination of B and B' with the product of reaction of FDNB with hydroxylamine (fig. 1).
of the cold finger. The residue had the absorption spectrum in benzene, shown in Figure 1. The difference between treated and untreated samples of DNP-cephalin (curves 1 and E, Figure 2) showed no evidence of this material. A clearer examination of the difference was obtained by treatment of the DNP-lipid with diisoxomethane, whereby the absorption maxima were displaced to 330 m (Chapter 7). Curves A' and B' of Figure 2 represent treated and untreated samples after methylation, measured in petroleum ether. Thus there is an indication that the yield of DNP-cephalin may be low because of the occurrence of side reactions in the presence of triethylamine; but at least in the two fractions examined, this does not seem to account for the difference between amino-nitrogen and non-choline nitrogen.

**Analyses of Total Lipid Extracts of Rat Tissues.**

The phospholipid composition of lipid extracts (chloroform:ethanol, subsequently acid-washed) of a number of rat tissues, determined by methods described in this thesis, is shown in Table 10. These results may be compared with those of Arton (1945) shown in Table 11. The latter analyses are the only comprehensive analyses comparable with my own, insofar as the cephalin fraction has been determined as amino-nitrogen and not as the difference between total nitrogen and choline-nitrogen as has been a more common practice (for example, tables in Witcoff's monograph, 1951).

The atomic ratio of nitrogen to phosphorus is higher than unity in acid-washed lipid extracts of all tissues examined but with the exception of liver, is no higher than can be accounted for by published analyses for sphingomyelin and cerebrosides (data given by Witcoff, 1951). The atomic ratio of choline- plus amino-nitrogen to phosphorus is of the same order
as found for egg yolk phospholipid with the exception of heart lipid. In the choline analysis of hydrolysates of kidney and brain lipids, a portion of the phosphomolybdate precipitate (Chapter 3) was insoluble in acetone and thus cannot have been choline phosphomolybdate. It may have been derived from the non-choline, non-amino-nitrogen (not sphingosine, which should be absent from an ether-extracted hydrolysate), in which case it is of interest to assume the same factor as for choline and compare the sum of amino groups, choline and unknown with total phosphorus. Even this estimate however, is not sufficient to account for the total phosphorus in the kidney lipid.

Discussion.

Analyses of Total Phospholipid Preparations and Total Lipid Extracts.

Procedures formerly introduced for the estimation of amino-nitrogen in lipids after hydrolysis have been discussed in Chapter 4. With the exception of Aron's (1954a), none of the earlier methods has been applied to the analysis of total lipid extracts of tissues. Therefore in the present chapter, the work described was undertaken in order to gain a perspective of the application of the present techniques for the determination of amino-nitrogen by comparison with non-choline-nitrogen in total and fractionated phospholipid preparations.

The first requirement for the reliable analysis of a lipid extract is the absence of non-lipid contaminants. This aspect of the determination of phospholipids was dealt with in Chapter 2, where it was shown that acid-washing or filtration through cellulose will remove contaminants soluble in petroleum ether solutions of lipids. In ether extracts of egg yolks the contaminants were almost entirely nitrogenous, which is probably
true of impure lipid extracts of most tissues. For this reason, one may expect a phosphorus determination to be a more reliable estimate of total phospholipids than nitrogen. The variation in the atomic ratio of nitrogen to phosphorus in the total egg phospholipid preparations described in Table 3 would be due to a number of factors, including the difference in composition of an ether extract as compared with a subsequent ethanol-ether extract (Chapter 2), possibly incomplete removal of contaminating nitrogen in some cases, a differential loss of phospholipids on filtration through cellulose and analytical error. An atomic ratio of nitrogen to phosphorus higher than unity in a purified phospholipid preparation may reflect the presence of sphingoeyelin and cerebrosides. From the data of Kaucher and co-workers (1943) it may be calculated that the atomic ratio of nitrogen to phosphorus of a purified total lipid extract of egg yolk should be of the order 1.1. On the other hand, the content of cerebrosides and sphingoeyelin is higher in the organs of animals; taking data from the source quoted above, one may calculate the following values for the atomic ratio of nitrogen to phosphorus in the lipids of liver, brain, kidney and lung, respectively, of beef: 1.1; 1.6; 1.2; and 1.2. These values may be compared with those shown for rat tissues in Table 10. Kaucher and co-workers (1943) do not report nitrogen analyses, which would have provided an interesting comparison with their estimates of nitrogenous lipids.

Since sphingoeyelin like lecithin, contains a mole of choline per atom of phosphorus and cerebrosides are free of phosphorus, the sum of choline- and amino-nitrogen should be equivalent to total phosphorus, although it may be less than total nitrogen if lecithin, cephalin and sphingoeyelin are the only phospholipids present. This assumption has been made in the
calculation of cephalin by difference between total phosphorus and equivalents of choline, for example by Kucher and others (1943) and Cardini and Serantes (1943). However such an assumption takes no account of the occurrence of non-nitrogenous phospholipids, such as phosphatidic acid (Chibnall and Channer, 1927), diposphoinositol (Welch, 1942) and "polyglycerol phosphatides" (McKibbin and Taylor, 1952) as well as possibly others as yet unknown. In animal tissues, phosphatidic acid is known to occur in polymer form as "cardiolipin" (Fonbron, 1947), of which heart muscle is the richest source. Examination of Table 10 will show that considerably less lipid phosphorus was accounted for as cephalin and choline-containing phospholipid in the case of heart than with the other organs of the rat. This finding may be due to the predominance of "cardiolipin" in heart. A similar finding is shown in the analyses of Arton (1945).

The phospholipid of egg yolk received the most intensive study by the present method because of the very low content of sphingomyelin and cerebrosides reported in this source (Kucher and others, 1943, reported that these sphingolipids account for only 2.5% of total nitrogen). This is reflected in Table 3 where the mean percentage of total nitrogen accounted for was 90, as compared with 33% of total phosphorus. Owing to the absence of complete analyses of egg yolk phospholipid, it was not possible to decide whether the remaining 7% of total phosphorus was due to the occurrence of non-nitrogenous phospholipids in egg yolk. It is clear that the remaining 10% of total nitrogen is not accounted for by sphingolipids. However, fractionation of the calcium chloride complex led to the isolation of some fractions in which the sum of choline- and amino-nitrogen was equivalent to total phosphorus, although the latter was less than total nitrogen.
(Table 9). Appraisal of the present estimate of amino phospholipid in egg yolk (Tables 2 and 3) may be made by comparison with the estimate of Lea, Rhodes and Stoll (1955) who found 13% of total nitrogen to be cephalin-nitrogen by van Slyke analysis of an acid hydrolysate.

Analysis of Fractionated Phospholipid.

The complete description of a novel analytical procedure requires application to well defined reference material. In the field of phospholipid analysis, the obstacles to that requirement have been emphasized in Chapter 2 and Chapter 5. In Chapter 5 it was found that determination of amino-nitrogen by direct reaction with EDTA in ox-brain cephalin prepared according to Folch (1942) gave a low result, which was attributed to contamination with an unknown nitroguanous phospholipid with an elementary composition similar to that of authentic cephalin. Cephalin prepared from egg yolk by the method of Lea, Rhodes and Stoll (1955) gave a similar analysis to the brain cephalin inasmuch as only 72% of the nitrogen was amino-nitrogen while choline-containing phospholipids were absent and the atomic ratio of nitrogen to phosphorus was close to one. These findings are not the same as those of the authors quoted above, who reported a quantitative relationship between amino-nitrogen and total nitrogen for their preparations.

A possible explanation of the present results may be found in the work of Chargaff, Ziff and Rittenberg (1942). The latter prepared petroleumpetroleum ether extracts of acetone-dried tissues, which were washed by precipitation from aqueous emulsion with acetone and further submitted to ether separation to remove sphingolipids. Preparations from liver, brain and heart had atomic ratios of nitrogen to phosphorus of one. Acid hydrolysates of
these preparations were analysed both by the van Slyke method and by isotope dilution with $^{15}$-labelled ethanalamine and choline. Amino-acid was determined by a titrimetric ninhydrin method. Of pig liver phospholipid, only 35.6% of the amino-nitrogen could be characterised as ethanalamine or amino-acid but all of the non-amino-nitrogen was present as choline; in a preparation of beef brain phospholipid, all of the amino-nitrogen was characterised but only 52% of the non-amino-nitrogen was choline; in pig heart phospholipid, 56.3% of the amino-nitrogen was accounted for as ethanalamine or amino-acid but only 49.3% of the non-amino-nitrogen as choline. Considering their method of isolation and purification and the elementary analysis of their starting material, it is improbable that these results could be due to the presence of impurities. Chargaff, Ziff and Ritterberg (1942) concluded the presence of unrecognized bases in phospholipids. That contamination with material of this nature may account for my experience with brain cephalin has been suggested in Chapter 5.

The experience with egg yolk cephalin might be similarly explained, since there was agreement between my own analysis for amino-nitrogen and that of Lea, Rhodes and Stoll (1955) so far as the total phospholipid was concerned; however it may be seen in Table 2 that amino-nitrogen in total egg yolk phospholipid is equivalent to only 52% of non-choline nitrogen. Perhaps Lea, Rhodes and Stoll (1955) were successful in separating cephalin by chromatography from the non-choline, non-amino nitrogen, while in the present attempt at repeating their work, only a partial separation was achieved (Table 5).

There are many examples in the early literature on cephalin of unsuccessful attempts to prepare material with all its nitrogen in the form
of amino-nitrogen (Levene and Ingwallson, 1920; Levene and Holf, 1921, 1925). The first success was achieved by Rudy and Page (1930) who isolated cephalin from the ethanol-soluble fraction of brain phospholipid by conversion to its barium salt. In fractionating egg yolk phospholipid with cadmium chloride, my object was not the isolation of pure cephalin but to see whether a differential distribution of amino-nitrogen and non-choline-, non-amine-nitrogen would take place. Examination of Tables 7 and 8 shows that no very even distribution resulted but the overall picture reveals complexity in the composition of egg yolk phospholipid not only with regard to the composition of the non-choline-nitrogen, but also in the atomic ratio of nitrogen to phosphorus and of acid equivalents to phosphorus. That both acid equivalents and nitrogen were generally slightly higher than cephalin-phosphorus was also found by Rudy and Page (1930) for their preparations. Whether there may be a connection between the unidentified nitrogen and the excess acid is a matter of speculation; however this situation recalls the results of potentiometric titration of an aqueous emulsion of egg yolk phospholipid described in Chapter 2, where evidence was found of a weaker acid than the secondary phosphate ester and a weaker base than the amino group. On this evidence, the possible occurrence of the amide of phosphatidic acid was suggested.

A study of egg yolk cephalin comparable to my own is that of Burmester (1946) who examined the fractionation procedure of Suzuki and others (1932) for the preparation of $\alpha$- and $\beta$-phospholipids. Solvent fractionation of the lead salts gave $\alpha$-cephalin preparations with 2.44 to 3.62% phosphorus and $\beta$-cephalin with 1.5 to 4.42% phosphorus. In a preparation of $\alpha$-cephalin, 57% of the total nitrogen was neither amino-nitrogen
(determined by the method of Barrer, 1946) nor choline, but in preparations of \( \beta \)-cephalin, all the non-choline nitrogen was amino-nitrogen. "\( \alpha \)" and "\( \beta \)" in this context refer to the position of attachment of phosphoric acid to glycerol. Although it has since been shown that cephalin (or lecithin) corresponding to the "\( \beta \)" formulation does not occur (Jaer and Kates, 1950), it would appear that this procedure has led to the separation of two forms of cephalin, one form ("\( \alpha \)-cephalin") being associated with the unidentified nitrogen. Of similar interest is the work of Brante (1949) who found evidence of two forms of cephalin of which one, predominating in the ethanol-soluble fraction, did not liberate acid-soluble phosphorus under conditions of mild saponification in contrast to the behaviour of synthetic phosphoglyceroles.

Much of the foregoing discussion has been concerned with the complexity of non-choline-containing phospholipids, insofar as it has a bearing on the interpretation of analytical data. This leaves aside the question of the composition of the aminophospholipid fraction itself, which has been assumed to contain amino-nitrogen and acid groups equivalent to phosphorus. However part of the apparent complexity of the non-choline-containing phospholipids may be found in the aminophospholipids, i.e., there may be species other than the customarily formulated phosphatidyl ethanolamine, etc., which contain an amino group. A similar complexity may also be attributed to the choline- and sphingosine-containing phospholipids. Conjecture of this kind might be based upon the composition of fractions 2 and 3 of Table 7; especially of fraction 3 where the removal of lecithin by toxin treatment gave a residue probably contaminated with some non-nitrogenous phospholipid but whose composition approximated one atom each of
choline- and amino-nitrogen percentage of phosphorus (Table 3). Indications of complexity in the composition of "purified" cephalin have been described in Chapter 5, concerning the isolation of MEP-cephalin. If this is so, then determination of amino-nitrogen merely provides an estimate of a group of phospholipids in the same manner as the determination of cephalin as the "non-choline-containing" fraction. Further evidence of the complexity of amino-phospholipid fraction will be presented in the next chapter.

Analysis of Rat Tissue Lipid Extracts.

The analysis of rat tissues reported in Table 10 illustrates the scope of the direct method for the determination of cephalin but comparable results in the literature are meager. The proportion of total phospholipid in the form of cephalin has been compared with other published results in Table 11. From the monograph of Vittoff (1951) it seems that both Kiushch and co-workers (1943) and Cordini and Serantes (1942) determined cephalin simply as non-choline containing phospholipid. The method employed for purification of the lipid extracts and determination of choline may have differed. One may expect the methods employed by these two groups of workers to provide a maximal estimate of cephalin although in the case of brain, the estimate of Cordini and Serantes (1942) is probably low due to an error in their determination of choline, a suggestion which was made by Vittoff (1951). On the whole, these indirect estimates of cephalin show better agreement with my own determinations than those of Aron (1945) whose method for the determination of ethanolamine and serine in lipid hydrolysates has already been described in Chapter 4.

Since Aron's work represents the only direct analyses for amino-phospholipids in rat tissues independent of those reported in this thesis,
it is necessary to compare the limitations of the two methods. The extensive application of the FDNE methods to total and fractionated egg yolk phospholipid discussed in the previous two sections, has established the facts:

(a) There is uniformly good agreement between determinations by the FDNE method before and after hydrolysis.

(b) Amino-nitrogen is less than non-choline-nitrogen.

(c) An apparent increase of the order 10% in amino-nitrogen could be obtained by treatment of the phospholipid sample with hydroxylamine prior to reaction with FDNE.

The last observation suggests that if the results with the FDNE method were in error, they would be low.

On the other hand Arton (1945b) has admitted the possibility of error in his procedure which leads to high results. This is due to interference from sphingosine, if sphingolipids are not removed prior to hydrolysis of the lipid extract. For example in an extract of lung from which sphingolipids had not been removed, the sum of choline- and amino-nitrogen was found to be equivalent to 128% of lipid phosphorus. In the ether-soluble fraction the same ratio was 107%. However it is certainly too much to expect complete removal of sphingolipids by a solubility procedure and in the extracts of liver and heart which were not submitted to ether separation, Arton's estimate of amino-nitrogen may also be expected to be too high.

The direct determination of lipid amino-nitrogen with FDNE cannot be subject to interference from sphingolipids since they do not contain a free amino group. One may conclude therefore, that the true value for lipid amino-nitrogen may be slightly higher than the present estimates but lower than those of Arton (1945b).
A desirable addition to Table 4 is the determination of serine and ethanolamine after hydrolysis as outlined in Chapter 4 but there was insufficient time to carry this out. It is evident from Table 2 of Chapter 4 that the ratio of atoms of amino-nitrogen to atoms of phosphorus in an acid hydrolysate of sheep brain lipid (0.51) determined by analysis with FDNB, compares well with the direct estimate for rat brain lipid (0.54). This comparison suggests that the analysis of sheep brain lipid which has a high content of sphingolipids, was not subject to interference from sphingosine. DTF-sphingosine was presumably eluted ahead of DNP-ethanolamine and DTF-serine, if indeed sphingosine is present in ether-extracted hydrolysates as suggested by Arton (1945b).
(a) Estimates of lipid amino-nitrogen by direct reaction with fluoro-2,4-dinitrobenzene showed good agreement with estimates obtained after hydrolysis by both FMIB and ninhydrin methods.

(b) Amino-nitrogen was less than total nitrogen in preparations of brain cephalin (prepared by the method of Folch, 1942) and egg yolk cephalin (prepared by the method of Lea, Rhodes and Stoll, 1955). In preparations of egg yolk phospholipid and total lipid extracts of rat tissues, aminophospholipids were less than "cephalin" determined by difference between lipid phosphorus and equivalents of choline-containing phospholipids.

(c) By solvent fractionation of the calcium chloride complex of egg yolk phospholipid, some fractions were obtained in which there was agreement between amino-nitrogen and equivalents of phosphorus, less choline-nitrogen.

(d) The occurrence of nitrogenous phospholipids, in which nitrogen is neither amino-nitrogen nor choline, has been suggested.

(e) Unlike the method of Artus (1945a) for the determination of aminophospholipids, the FMIB methods are not subject to interference from sphingolipids.
CHAPTER 7

THE PRODUCTS OF THE REACTION OF DIAZOMETHANE WITH

2,4-DINITROPHENYL-CEPHALIN.

Introduction.

In previous chapters, the utility of the reaction of DME with aminophospholipids has been stressed chiefly as a means for the direct determination of "cephalin". Chromatography of DNP-cephalin was not by any means studied exhaustively but it was shown that DNP-cephalin could be isolated from an impure cephalin preparation with an atomic ratio of DNP-amino-nitrogen to phosphorus of unity, by use of weak adsorbents such as celite and talc. Experience with DNP-lipid from rat liver where a large amount of lecithin is present in addition to cephalin and other lipids, showed that low recoveries of DNP groups resulted where it was necessary to employ higher ratios of adsorbent to fat. Moreover it was evident that the difference in strength of adsorption between DNP-cephalin and native cephalin was not as great as was hoped. The substituent common to both these forms is the tertiary phosphoric acid group; a polar substituent such as this must play an important part in the adsorption of the phospholipid, perhaps a more important part than the amino group. Therefore the principle of chemical modification of the native structure
of cephalin has been extended to the esterification of acid groups. The results of this research, which will be described in the present chapter, include not only a highly successful method for the isolation of "classical" cephalin from total lipid extracts, but also evidence of the occurrence of complex forms of aminophospholipid.

In order to justify an additional modification of the phospholipid, the following requirements were essential: (a) a reaction which proceeds rapidly and quantitatively under mild conditions; (b) an unambiguous reaction mechanism; (c) no reaction with other lipids, e.g., lecithin; (d) no loss of DNP groups; (e) a stable product; (f) a reagent which is soluble in lipid solvents and of which any excess is readily removed.

The first choice of an esterifying reagent which might fulfill the above conditions was diazomethane, CH₂N₂, being a gas, soluble in ether, benzene, etc., readily prepared on a small scale, though not stable. The reagent has been found to give quantitative yields of methyl esters with a large number of organic and inorganic acids under mild conditions (Rodd, 1951). The work of Atherton, Howard and Todd (1943) is an example of the esterification of primary and secondary phosphate esters under mild conditions, by diazocalkanes. The reaction proceeds rapidly at room temperature with evolution of nitrogen, the reactants being generally in ether or benzene solution, sometimes ethanol:

\[ :\text{P}(\text{C})\text{OH} + \text{R}_2\text{N}_2 \rightarrow :\text{P}(\text{C})\text{OCH}_2\text{R} + \text{N}_2 \]

Frankel and Katchalski (1943) have found that in addition to the free acid, diazocalkanes will react with the salts of amines, the yield of ester being unimpaired. Quaternary amines were not tested; however it
is certain that the ester formed, if any, would rapidly decompose in the presence of strong base. For this reason it was anticipated that lecithin would not react.

On the other hand, the acidic nature of cephalin has already been demonstrated by titration against alkali both in aqueous (Chapter 2) and non-aqueous (Chapter 6) systems. Provided cations are adequately removed by acid-washing (Chapter 2), one may expect a quantitative yield of methyl ester upon reaction of diazomethane with cephalin. The particular advantage of applying the reaction to DMP-cephalin is the ambiguity of the reaction with native cephalin, where there is a claim of methylation of the amino group by diazomethane (Julian, Meyer and Iveson, 1945). When phosphatidyl serine is present, one may also expect methylation of the carboxyl group.

The work to be described began with the methylation of DMP-cephalin in the ox-brain preparations and continued with a study of the cephalin of egg yolk. Results with brain cephalin and model compounds are presented first, because they are more readily interpreted and form a reference basis for the appraisal of results with total phospholipid preparations. Throughout this work, the reaction with diazomethane was carried out according to a standard procedure. Only in concluding experiments was this procedure altered by the addition of certain catalysts to the diazomethane solution. Since these experiments were incomplete, they will be described in the concluding section.
(a) Studies with Brain Cephalin, Synthetic Cephalin and 2-Aminoethyl, Octadecylphosphoric Acid.

Experimental.

Materials.

Brain cephalin, fractions III and V, was the same preparation as described previously (Chapter 2).

Dimyristoyl-L-α-cephalin was a gift from Dr. E. Baer. The sample had 4.13% phosphorus (theory, 4.89%) and 2.26% nitrogen (theory, 2.20%). The proportion of amino-nitrogen to total nitrogen, by reaction with FDNB was 74%.

2-Aminoethyl, octadecylphosphoric acid was synthesised according to the method of Christensen (1940), with some modifications. The preparation was found to have 6.07% phosphorus (theory, 7.94%) and 2.40% nitrogen (theory, 3.56%). The proportion of amino-nitrogen to total nitrogen, by reaction with FDNB, was 100%.

DNP-octadecylamine was prepared by Dr. F.D. Collins from commercial octadecylamine.

N-nitrosomethylurea was prepared by diazotisation of acetyl methylurea (Arndt, 1943). The air-dried material was kept at -10°C.

The adsorbent most commonly used was celite ("Hyflo-supercel"). Eluants were similar to those employed in the chromatography of DNP-cephalin (Chapter 5), namely petroleum ether, chloroform and ethanol, or mixtures of these.

Methods.

Owing to the instability of diazomethane, it was prepared immediately
before use. Since the scale of experiments required 5 - 50 μ moles
DNP-cephalin, a suitable excess of diazomethane was prepared by decompos-
ing 20 - 50 mgs. (0.2 - 0.5 μ mole) of N-nitrosomethylurea added in
portions, with shaking, to 60% potassium hydroxide (about 2 mls.) covered
with ether (about 25 mls.), the reaction being carried out in an ice-bath.
The ether layer, after being poured off into a separate flask, was dried
by allowing to stand for at least an hour at -10°C. in contact with a few
pellets of potassium hydroxide. The yield of diazomethane, measured from
the extinction at 410 μ, where ε = 3.0 (Brinton and Volman, 1951) was
very nearly quantitative.

DNP derivatives were prepared as described in Chapter 5, the blank
being removed by means of the cold finger. After measurement of the ex-
tinction of the DNP derivative in petroleum ether, the solution was taken
to dryness and the DNP lipid dissolved in the ether solution of diazo-
methane. After standing for 0.5 - 1.0 hour at room temperature, the solu-
tion was taken to dryness at the water pump (removing both ether and diazo-
methane) and the residue again taken up in petroleum ether for measurement
of the absorption spectrum.

The chromatographic procedure varied a great deal, as did the results
of chromatography, so these details will be given in the Results section.

Results.

The reaction with diazomethane was first tested with the acid-washed
ox-brain cephalin fractions. A sample of fraction V, after reaction with
FDNB, gave curve A, figure 1, when measured in 125 mls. of cyclohexane.
After reaction overnight with diazomethane and re-solution in cyclohexane,
the colour of the solution was noticed to have faded. The reason for
Fig. 1.
Effect of reaction of diazomethane on the absorption spectrum, in cyclohexane, of DNP-lipid prepared from fraction V of the ox-brain cephalin. Curves A and B are the absorption spectra before and after reaction with diazomethane.
this was found upon measurement of the absorption curve (curve B, figure 1), which had been displaced into the ultra-violet with no loss of extinction at \( \lambda_{\text{max}} \). When the experiment was repeated, allowing only 5 mins. reaction time, the result was identical. Also, the change in \( \lambda_{\text{max}} \) was only evident when the absorption of the product was measured in cyclohexane or petroleum ether, but not in ethanol.

The change in \( \lambda_{\text{max}} \) was slightly more pronounced in the case of fraction III, where the product of methylation of the DNP derivative had \( \lambda_{\text{max}} \) in cyclohexane at 325 m\( \mu \). In this case also, there was no change in extinction if the product was dissolved in ethanol for measurement and overnight reaction with diazomethane gave a result no different from 30 mins. reaction.

The next step was to establish whether or not the product of methylation was homogeneous in composition. In early experiments, chromatography was carried out on a variety of adsorbents, including sucrose, kieselguhr, silica, talc, kieselguhr treated with silicone (Jutisz et. al., 1954) and powdered glass treated with silicone in the same way as kieselguhr. Approximately 1 gm. adsorbent was used per mgm. fat. There was extensive decomposition of methylated DNP-cephalin on sucrose but with the other materials, the greater part of the extinction could be recovered in weakly polar eluents such as benzene and chloroform. This is in contrast to DNP-lipids where the presence of ethanol in the eluant was generally necessary for elution (Chapter 5). Phosphorus analysis of the easily eluted portion of the methylated DNP-cephalin gave estimates of \( \xi_{\text{max}} \) (molecular extinction coefficient based upon phosphorus analysis, for one DNP group per phosphorus atom) of the expected order for a ratio of DNP
### TABLE 1.

**Analysis of Fraction III of the Brain Cephalin on Weakened Kieselguhr.**

<table>
<thead>
<tr>
<th>Eluant</th>
<th>( \lambda_{\text{max}} )</th>
<th>( \varepsilon_{\text{max}} ) (( \mu ))</th>
<th>( \varepsilon ) Total Extinction, a</th>
</tr>
</thead>
<tbody>
<tr>
<td>Petrol</td>
<td>328 ( \mu \mu )</td>
<td>20,500</td>
<td>36</td>
</tr>
<tr>
<td>Chloroform : petrol, 1:4 by vol.</td>
<td>325 ( \mu \mu )</td>
<td>20,300</td>
<td>44</td>
</tr>
<tr>
<td>Chloroform</td>
<td>325 ( \mu \mu )</td>
<td>15,600</td>
<td>14</td>
</tr>
<tr>
<td>Ethanol : Chloroform, 1:9 by vol.</td>
<td>328 ( \mu \mu )</td>
<td>...</td>
<td>6.5</td>
</tr>
</tbody>
</table>

a. Measured at 325 \( \mu \mu \).

Recovery of extinction after chromatography was 100%.

Recovery of lipid phosphorus after chromatography was 55%. 
### TABLE 2.

**Analysis of Fraction V of the Brain Sephalin on Weakened Kieselgel.**

<table>
<thead>
<tr>
<th>Eluant</th>
<th>$\lambda_{\text{max.}}$</th>
<th>$\varepsilon_{\text{max.}}$</th>
<th>% Total Extinction, a</th>
</tr>
</thead>
<tbody>
<tr>
<td>Petrol.</td>
<td>325 m $\mu$</td>
<td>21,000</td>
<td>8.3</td>
</tr>
<tr>
<td>Chloroform:Petrol, 1:4 by vol.</td>
<td>325 m $\mu$</td>
<td>19,200</td>
<td>39</td>
</tr>
<tr>
<td>Chloroform.</td>
<td>328 m $\mu$</td>
<td>8,700</td>
<td>35</td>
</tr>
<tr>
<td>Ethanol:Chloroform, 1:2 by vol.</td>
<td>338 m $\mu$</td>
<td>4,000</td>
<td>15</td>
</tr>
</tbody>
</table>

a. Measured at 330 m $\mu$.

Recovery of extinction after chromatography was 82%.

Recovery of phosphorus after chromatography was 51%.
groups to phosphorus of one. In the case of fraction III, 100% of the extinction was recovered from silica in one experiment, the main (acetone) eluate having $\lambda_{\text{max}}$ at 325 m $\mu$ and $\varepsilon_{\text{max}} = 17,200$ (cyclohexane); 81% of the extinction at $\lambda_{\text{max}}$ was recovered from talc in the chloroform and acetone eluates, $\varepsilon_{\text{max}}$ (P) being respectively 15,100 and 15,600 (cyclohexane).

Details of two chromatographies are given in Tables 1 and 2. In each case, 5 - 10 mgms. derivative were chromatographed on 5 - 10 gms. silicic acid kieselguhr. All spectra were measured in cyclohexane. Limitations of space make it impossible to reproduce all these spectra. The bulk of the material recovered from fraction III had good definition spectroscopically and the main (chloroform-petrol) eluate is shown in figure 2. Definition was not so good with fraction V. The petrol eluate resembled the material shown in figure 2; the subsequent eluate differed in that it had a subsidiary maximum at 350 m $\mu$ (figure 3). The other feature of fraction V is the low recovery of extinction. When the recovered absorption was subtracted from that before chromatography, the difference had a maximum at 345 m $\mu$, closely resembling unchanged DFP-cephalin; the final eluate in Table 2 is also predominantly of the character of DFP-cephalin, since the ratio $\frac{E_{360\mu}}{E_{330\mu}} = 0.83$ (for DFP-cephalin, this ratio is 0.97; for methylated DFP-cephalin, it is 0.565).

An attempt to effect further purification of fraction V was made in the following way: a quantity (about 1.5 m moles) of the methylated derivative from fraction V, having an extinction at 330 m $\mu$ of 0.54 in 40 mls. cyclohexane was placed on 5 gms. silica in chloroform and an acetone and an ethanol eluate collected. The acetone eluate was re-chromatographed
**TABLE 3.**

**Analysis of Silica Eluate on Glass.**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>$\lambda_{\text{max}}$</th>
<th>$\epsilon_{\text{max}}$ (P)</th>
<th>$\Delta$ Total Extinction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Petrol</td>
<td>325 m$\mu$</td>
<td>14,300</td>
<td>18</td>
</tr>
<tr>
<td>Chloroform: Petrol,</td>
<td>325 m$\mu$</td>
<td>19,400</td>
<td>52</td>
</tr>
<tr>
<td>1:4 by vol.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chloroform</td>
<td>330 m$\mu$</td>
<td>6,300</td>
<td>6.5</td>
</tr>
<tr>
<td>Ethanol: Chloroform,</td>
<td>340 m$\mu$</td>
<td>7,300</td>
<td>5.3</td>
</tr>
<tr>
<td>1:9 by vol.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ethanol, (from silica)</td>
<td>325 m$\mu$</td>
<td>14,200</td>
<td>19</td>
</tr>
</tbody>
</table>

a. Measured in cyclohexane.

b. Measured at $\lambda_{\text{max}}$.

Recovery of extinction, 73%; recovery of phosphorus, 50%.
**Fig. 2.**

Absorption spectrum in cyclohexane of the main product of the reaction of diazomethane with DNP-lipid prepared from fraction III of the ox-brain cephalin. The material shown was isolated as a chloroform-petroleum ether eluate from kieselguhr.
Fig. 3.

Absorption spectrum in cyclohexane of the main product of the reaction of diazomethane with DNP-lipid prepared from fraction V of the ox-brain cephalin. The material shown was isolated as a chloroform: petrol eluate from siliconed kieselguhr.
Fig. 4.
Absorption spectra in cyclohexane of the products of reaction of diazomethane with DNP-aminoethyloctadecyl phosphoric acid, separated by chromatography on kieselguhr.
on siliconed glass powder. The result of these two chromatographies is shown in Table 3. The overall recovery of extinction was 78%, which compares with that in Table 2, but the recovery of phosphorus was 50%, compared with 81% in Table 2. Re-chromatography has thus substantially effected further purification of the derivative. In Table 3, the chloroform-petrol eluate had a subsidiary maximum at 320 m., like the chloroform-petrol eluate from weakened kieselguhr.

Thus far, apart from the greater ease of elution, the only evidence that DNP-cephalin has, in fact, been esterified by diazomethane, lies in the alteration of the absorption maximum of the DNP substituent; provided, of course, that it can be demonstrated that this change is due to esterification of acid groups. It was hoped to accomplish a proof by reference to the absorption curves of suitable model compounds.

It has been seen that methylated DNP-cephalin is only distinguished spectroscopically from DNP-cephalin by measurement in a non-polar solvent, such as cyclohexane or petroleum ether. Hence it was desirable to have as model compounds, derivatives which are soluble in these solvents. The three petrol-soluble substances which were examined are octadecylamine, aminooethyl octadecylphosphoric acid and L-a-dimyrystoyl cephalin.

DNP-aminooethyl octadecylphosphoric acid was not readily soluble in ether and was reacted with diazomethane partly in the form of a fine suspension. Nevertheless, 90% of the extinction at 345 m.µ was recovered at 330 m.µ (cyclohexane) in the ether-soluble portion at the end of the reaction. When this was chromatographed on kieselguhr, it was found to consist of two components, both with \( \lambda_{\text{max}} \) in cyclohexane at 330 m.µ; one, however, had a subsidiary maximum at 390 m.µ, while the other was flat in
this region (figure 4). $\varepsilon_{\text{max.}}(\lambda)$ of the former (eluted by benzene) was 10,100 and of the latter (eluted by chloroform) was 16,700. Their relative preponderance (based upon extinction at 330 m\(\mu\)) was 2.5:1.0. Synthetic L-\(\alpha\)-dimyristoyl cephalin was analysed by Dr. F.D. Collins who found, upon chromatography of the products of methylation on celite, 92\% with $\lambda_{\text{max.}}$ (cyclohexane) at 328 m\(\mu\), 48\% with $\lambda_{\text{max.}}$ at 410 m\(\mu\) and 4\% with $\lambda_{\text{max.}}$ at 345 m\(\mu\). The "410" fraction moved ahead of the "328" on the column, and has since been found in traces in products of methylation of natural phospholipids. The main fraction had a subsidiary maximum at 330 m\(\mu\) and $\varepsilon_{\text{max.}}(\lambda)$ was 20,000.

DNP-octadecylamine, also prepared by Dr. F.D. Collins, had $\lambda_{\text{max.}}$ in petrolatum ether at 330 m\(\mu\) and $\varepsilon_{\text{max.}} = 18,600$ and in ethanol, $\lambda_{\text{max.}}$ at 348 m\(\mu\) and $\varepsilon_{\text{max.}} = 17,300$. In petrol, there was a subsidiary maximum at 390 m\(\mu\).

**Discussion.**

The salient facts of the diazomethane reaction are: (a) the reaction with DNP-cephalin is almost instantaneous at room temperature; (b) there is no loss of DNP groups on reaction with diazomethane; (c) the reaction involves a substituent which influences both the light absorption of the DNP group and the strength of adsorption of DNP-cephalin to talc and kieselguhr. In spite of complicating features such as apparently incomplete reaction with fraction V and the occurrence of two forms of methylated derivatives with $\lambda_{\text{max.}}$ at 325 m\(\mu\), the positive evidence of conversion to a new derivative gave promise of useful application.

At the commencement of these experiments, a method for the deter-
mination of the methyl ester was not envisaged; it was therefore fortunate that a demonstrable change took place on methylation, which might be interpreted as qualitative evidence of the formation of the methyl ester. The reader will already have anticipated the arguments of this case from the absorption curves in figures 2, 3 and 4. Aminooctyl octadecylphosphoric acid was regarded as a close approximation to cephalin for spectroscopic purposes, since only the diacylglyceryl portion is replaced by an alkyl chain. The formulations of DNP-octadecylamine and DNP-aminooctyl octadecylphosphoric acid implicate the phosphoric acid group as the substituent responsible for the difference in $\lambda_{\text{max}}$. in petroleum ether: the most plausible explanation is hydrogen bonding between the phosphoric acid group and the DNP chromophore. If this is true of DNP-aminooctyl octadecylphosphoric acid, it must also apply to DNP-cephalin. In methyl esters, the possibility of hydrogen bonding no longer exists. In this respect, they are comparable to DNP-octadecylamine, although in the latter compound any dipole effect may be different from that in the methyl ester of DNP-cephalin. The fact of intramolecular hydrogen bonding accounting for $\lambda_{\text{max}}$ at 345 - 350 m$\mu$ for the DNP derivatives of the free acids in petroleum ether is indicated by the absence of any marked difference in the absorption curve if it is measured in ethanol, a hydrogen bonding solvent. The manifest similarities between DNP-aminooctyl octadecylphosphoric acid and the DNP derivatives of both synthetic and natural cephalin suggests that these products are, in fact, the methyl esters: the absorption curves in a non-hydrogen bonding solvent are basically similar, all showing a displacement in $\lambda_{\text{max}}$. compared with that in ethanol, or with that of the free acid. The similar results obtained
with synthetic and natural cephalin are novel evidence that the latter, or that part of it which has reacted with DNP, conforms to the "classical" structure. This is exemplified best by fraction III, where the yield of ester or "325" product, was over 90%.

Some problematical features are (a) the apparent resistance to esterification of about 20% of the DNP derivative from fraction V, (b) the formation of a trace of derivative with $\lambda_{\text{max}}$ at 410 m$\mu$ in petrol, in the case of synthetic cephalin and (c) the occurrence of the main product, with $\lambda_{\text{max}}$ at 325 - 330 m$\mu$, in two forms, one having a weak maximum at 380 - 390 m$\mu$, while the other has only an inflection in this region.

A possible cause of incomplete reaction could be the association of part of the DNP-cephalin with cations which may have been incompletely removed by acid-washing. In studying the effect of acid-washing on the composition of brain cephalin (Chapter 2), ash determinations were not carried out. However from the data of Folch (1942), it is clear that fraction III had a much higher ash content than fraction V. Yet fraction III provided a higher yield of ester; therefore, some alternative explanation is necessary. As will be shown in later sections, the occurrence of an incomplete yield of ester on methylation is quite general for total phospholipid preparations and for this reason further discussion is reserved for later presentation.

Evidence of spectroscopic heterogeneity in the methylated DNP derivatives brings to mind a similar heterogeneity found on chromatography of DNP-cephalin (Chapter 5). This, and the marked difference between the products from fraction III as compared with fraction V, suggests a difference in composition of the starting material, rather than ambiguity in the
reaction with FDNP or diazomethane. For example it may be that the esterified carboxyl group of serine exerts a sufficient inductive effect on the DNP group of the methyl ester of DNP-phosphatidyl serine to abolish the subsidiary maximum at 380 - 390 m\(\mu\) which is found in the derivatives of ethanolamine.

(b) The Composition of Methylated DNP-Phospholipids.

Methylated DNP-cephalin prepared from total lipid extracts that have been prepared in this laboratory, has not as yet consisted entirely of material with \(\lambda_{\text{max.}}\) at 325 - 330 m\(\mu\) which, from the preceding section, is regarded as characteristic of the methyl ester of DNP-cephalin. A proportion of the total DNP-phospholipid has shown apparent resistance to esterification and this proportion was observed to vary from one tissue to another. The composition of methylated DNP-lipids of rat tissues has been studied in detail by Dr. F.D. Collins who found for example, 74\% of methylated DNP-cephalin prepared from liver to be of ester form and 43\% from brain. A much more unexpected result was obtained with the lipids of egg yolk where there appeared a fraction, readily separated by chromatography from the ester and apparently unchanged DNP derivative, which had \(\lambda_{\text{max.}}\) in petrol at 355 m\(\mu\). No trace of this material was detected in lipids from rat tissues. This experience supported the view that the "355" product was not an artefact, but derived from a complex form of aminophospholipid, a notion which could be extended to the "345" remaining after methylation. In order to test this hypothesis, native egg yolk phospholipids were fractionated into ethanol-insoluble and ethanol-soluble fractions in the belief that if three different forms of egg yolk cephalin existed, it should be possible to effect a separation of the native forms.
A different approach, taken by Dr. F.D. Collins, was to seek a differential rate of incorporation of $^{32}$P into the two forms of aminophospholipid recognisable in rat liver after reaction with FDNB and diazomethane. Because this work is closely related to my own, a brief description of it will be given also.

**Experimental.**

**Materials.**

The fractionation of egg yolk phospholipids by means of cadmium chloride, previously described in Chapter 6, was carried out primarily for the present purpose, but was of equal interest for the consideration of analytical data relating to amino-nitrogen and for this reason, was presented earlier. Each fractionation will be considered consecutively.

Diazomethane was prepared as described previously.

**Methods.**

The conditions of reaction with diazomethane were as previously described for brain cephalin, 2-aminoethyl octadecylphosphoric acid, etc.

Chromatography was an essential tool for the analysis of the products formed on methylation and in all cases was carried out on hyflo-supercel, with a ratio of approximately 1 - 2 mgm. fat per gm. of adsorbent. The columns were prepared in petroleum ether, the adsorbent being packed in sections under air pressure of 5 lbs/in.$^2$. Checks on the recovery of extinction were made by summing the extinction in all fractions isolated, at wavelengths from 300 - 420 m$\upmu$ and subtracting this total from that taken for chromatography. By this means, it was possible to obtain an accurate estimate of the proportions of the three components
Fig. 5.

Analysis of an alcohol-insoluble preparation of egg yolk phospholipid (from the first fractionation experiment). The absorption spectra, in petroleum ether, are as follows: A and B, the DNP derivative before and after methylation, measured in the same volume of solvent; C, the extinction recovered after chromatography at wave-lengths shown (open triangles); and D, the difference obtained on subtraction of C from B.
formed on methylation. The manner of development of the column varied according to content of DNP derivative in the sample for analysis, so full details of each analysis will be given in the Results section.

Results.

For the sake of brevity, the three components of methylated DNP-cerebrin of egg yolk will be designated from now on by reference to their respective $\lambda_{\text{max}}$, in petroleum ether, viz., "328", "345" and "355".

Analysis of the Ethanol-Insoluble Fraction.

Ethanol-insoluble phospholipid comparable to Polak's (1942) ox-brain cerebrin, was isolated in the first fractionation of egg yolk phospholipid, described in Chapter 6.

For analysis of the ethanol-insoluble fraction, 48.5 mg, were reacted with DME to give the absorption spectrum shown in Figure 5 (curve A), measured in 1 litre of petroleum ether. Methylation gave curve B, measured in the same volume. For chromatography, a relatively high proportion of adsorbent was used (40 g), since it was evident from curve B that "328" material was the main product and this is only weakly adsorbed. Development of the chromatogram with petrol resulted in the appearance of three distinct yellow bands, two moving down the column and one adhering at the top. Band formation in petrol has been observed with methylated DNP-lipids from a variety of sources, generally no more than two bands moving away from the top of the column in this solvent. Unfortunately, movement ceases shortly after their separation and with the change to more polar solvents, they tend to be eluted together. However, where a suitable ratio of adsorbent to fat was used, it was possible to
Fig. 6.
Composition of the products of methylation of DNP-lipid prepared from an alcohol-insoluble fraction of egg yolk phospholipid. The absorption curves are drawn to scale to show the relative amounts of "328" (curve A), "345" (curve B) and "355" (curve C). The latter component was not recovered on chromatography (see fig. 5).
### TABLE 4.

Analysis by Chromatography of the Products of
Reaction of JNB and Diazomethane with the
Ethanol-Insoluble Fraction of Egg Yolk Phospholipid.

<table>
<thead>
<tr>
<th>Eluant</th>
<th>( \lambda_{\text{max.}} ) (petrol)</th>
<th>( \varepsilon_{\text{max.}} ) (P)</th>
<th>% Total LP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Petrol:benezene.</td>
<td>328 m( \mu )</td>
<td>16,600</td>
<td>34</td>
</tr>
<tr>
<td>Benezene, Chloroform.</td>
<td>328 m( \mu )</td>
<td>12,000</td>
<td>47</td>
</tr>
<tr>
<td>Chloroform:Ethanol.</td>
<td>343 m( \mu )</td>
<td>11,900</td>
<td>8</td>
</tr>
<tr>
<td>Not recovered.</td>
<td>328 m( \mu )</td>
<td>....</td>
<td>10</td>
</tr>
<tr>
<td>&quot;</td>
<td>360 m( \mu )</td>
<td>....</td>
<td>4</td>
</tr>
</tbody>
</table>
separate the two components which move in petrol by employing mixtures of benzene and petrol. In the present case, it was possible to elute the two bands separately and both had $\lambda_{\text{max}}$ at 328 m$\mu$ and the subsidiary maximum at 385 m$\mu$, in petrol. However the subsidiary maximum was more pronounced in the faster fraction, where the ratio $\frac{385 \text{ m} \mu}{328 \text{ m} \mu}$ was clearly higher, viz. 0.372 as compared with the value of 0.323 found for the bulk of the "328" fraction. The second band was not completely eluted with benzene-petrol, owing to tailing; the remainder on the column was eluted in two portions by benzene and chloroform. The third band remaining at the top of the column was not affected by benzene, but spread in chloroform. Mixtures of chloroform with ethanol (10 - 20% by vol.) eluted it easily. When the absorption spectrum was measured in petrol, $\lambda_{\text{max}}$ was at 345 m$\mu$. Provided a large enough column was used, there was no overlapping of "345" and "328" in chloroform but on large columns, the recovery of "345" was low. The extinction recovered in the present experiment is shown in curve C, figure 5. Curve D is the difference, obtained after subtraction from the absorption curve of the material before chromatography. It can be seen that the recovery of "328" is 90%, while there is evidence of a trace of material with $\lambda_{\text{max}}$ at 360 m$\mu$ in petrol, which was not recovered at all. The relative proportions of the three components are shown in figure 6 and Table 4. The proportions calculated in Table 4 assume the same $\varepsilon_{\text{max}}$ for the three entities, which may be expected to be approximately correct. The "328" product in this preparation accounted for about 90% of the DNP derivative.
Fig. 7.
Analysis of an alcohol-soluble preparation of egg yolk phospholipid (from the first fractionation experiment). The absorption spectra, in petroleum ether, are as follows: A and B, the DNP derivative before and after methylation, measured in the same volume of solvent; C, the extinction recovered after chromatography at wave lengths shown (open triangles); and D, the difference obtained on subtraction of C from B.
Analysis of an Ethanol-Soluble Fraction.

The ethanol-soluble preparation referred to is that obtained in the first fractionation of egg yolk phospholipids, described in Chapter 6.

The ethanol-soluble preparation (65 mgms.) was reacted with FDNB to give curve A in figure 7, measured in 500 mls. petroleum ether. Reaction with diazomethane resulted in curve B, a response which is clearly different from that of the ethanol-insoluble preparation shown in figure 5. Here, the extinction at 330 mμ has decreased, with a shift in λₘₐₓ to only 340 mμ and an increase of extinction at 400 mμ. Chromatography on 30 gms. hyflo-supercel in this instance (2 mgms./gms. adsorbent) again gave two fast-running bands in petrol, one of which was eluted with petrol. Further fractions were eluted with petrol-benzene and benzene; all had λₘₐₓ at 328 mμ. Development with benzene-chloroform caused portion of the yellow band at the top to move down the column, a clear space separating it from the remainder, adsorbed at the top. Thus it was possible with this preparation to resolve the slower-moving component into two fractions. Development with chloroform completely eluted the faster part, which had λₘₐₓ in petrol at 345 mμ. The remainder appeared to be completely eluted with chloroform:ethanol (4:1 by vol.) and had λₘₐₓ in petrol at 355 mμ, with a subsidiary maximum at 410 mμ. The recovery curve (curve C, figure 7) showed that part of the "355" fraction was lost, while the "328" and "345" in this chromatography were completely recovered. The absorption curves of these constituents are reproduced in figure 8, drawn to scale in order to show their relative proportions, based upon the total extinction of each at its respective λₘₐₓ. Further data are given in Table 5.
Fig. 8.
Composition of the products of methylation of DNP-lipid prepared from an alcohol-soluble fraction of egg yolk phospholipid. The absorption curves are drawn to scale to show the relative amounts of "328" (curve A), "345" (curve B) and "355" (curve C). The proportions have been corrected for the low recovery of the "355" component on chromatography (see fig. 7).
TABLE 5.

Composition of the Ethanol-Soluble Fraction of Egg Yolk Phospholipid.

<table>
<thead>
<tr>
<th>Eluant</th>
<th>$\lambda_{max.}$</th>
<th>$\epsilon_{max.}$ (\text{(P)})</th>
<th>% Total DPP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Petrol</td>
<td>328 m$\mu$</td>
<td>16,000</td>
<td>10</td>
</tr>
<tr>
<td>Petrol-benzene, Benzone</td>
<td>328 m$\mu$</td>
<td>17,800</td>
<td>31</td>
</tr>
<tr>
<td>Chloroform-benzene</td>
<td>345 m$\mu$</td>
<td>7,100</td>
<td>5</td>
</tr>
<tr>
<td>Chloroform</td>
<td>345 m$\mu$</td>
<td>9,030</td>
<td>29</td>
</tr>
<tr>
<td>Ethanol-chloroform 1:1 by vol</td>
<td>355 m$\mu$</td>
<td>2,500</td>
<td>16</td>
</tr>
<tr>
<td>Not recovered</td>
<td>355 m$\mu$</td>
<td>....</td>
<td>6</td>
</tr>
</tbody>
</table>
Fig. 9.

Three forms of the "328" component isolated from the products of reaction of FDNB and diazomethane with egg yolk phospholipid. Each formed a separate band on chromatography. A was eluted by benzene; B and C were eluted by mixtures of benzene and chloroform. The absorption curves are drawn to scale to show the relative amounts of each form.
Analysis of the Petroleum Ether Extract of Egg Yolk.

In the first fractionation of egg yolk phospholipid, the petroleum ether extract following ethanol:ether extraction, was found to have a composition very similar to that of the ethanol-soluble fraction (Table 6, Chapter 6), although its manner of isolation was quite different. Likewise, the composition of the methylated DNP derivative was similar, as may be seen by comparison of Tables 5 and 6. Of particular interest in this chromatography was the separation of the "328" product into three distinct components.

The fat (50 mgw.) was reacted with EDHS and diazomethane and placed on 30 gms. hyflo-supercel in petroleum ether solution. Three bands moved away from the top in petrol. The first, eluted by benzene, had a subsidiary maximum at 399 m.μ and $E_{399} = 0.405$. Chloroform:benzene eluted two following bands in succession. The first was the typical "328" product, with $E_{380} = 0.31$; the second had no subsidiary maximum and $E_{328} = 0.405$. The absorption curves are shown in figure 9.

Second Fractionation of Egg Yolk Phospholipids.

It will be recalled that in the second fractionation of egg yolk phospholipids (Chapter 6), the portion precipitated by ethanol was discarded and the fractionation applied to the ethanol-soluble fraction, rich in lecithin. That is, the fractionation was simply a purification of lecithin. In the methylation studies, the products were chromatographed without prior measurement of the extinction of the methylated derivatives, so it has not been possible to calculate recoveries. Therefore, the proportions of "328" derivatives shown in Table 7 are probably somewhat high.
TABLE 6

Composition of the Petroleum Ether Extract of Egg Yolk

<table>
<thead>
<tr>
<th>Eluant</th>
<th>( \lambda_{\text{max.}} )</th>
<th>( \varepsilon_{\text{max.}}(P) )</th>
<th>% Total DNP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benzene</td>
<td>328 m( \mu )</td>
<td>15,100</td>
<td>17</td>
</tr>
<tr>
<td>Chloroform-benzene (1)</td>
<td>328 m( \mu )</td>
<td>17,500</td>
<td>22</td>
</tr>
<tr>
<td>(2)</td>
<td>328 m( \mu )</td>
<td>16,500</td>
<td>2</td>
</tr>
<tr>
<td>Chloroform</td>
<td>345 m( \mu )</td>
<td>10,600</td>
<td>30</td>
</tr>
<tr>
<td>Ethanol-chloroform 1:4 by vol.</td>
<td>355 m( \mu )</td>
<td>2,150</td>
<td>28</td>
</tr>
</tbody>
</table>
**TABLE 7.**

**Second Fractionation of Egg Yolk Phosholipid.**

**Composition of Methylated DNP Derivatives.**

**Fraction 1.**

<table>
<thead>
<tr>
<th>Eluant</th>
<th>( \lambda_{\text{max}} )</th>
<th>( \varepsilon_{\text{max}} ) ( (\text{P}) )</th>
<th>% Total DNP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benzene.</td>
<td>a. 328 m ( \mu )</td>
<td>17,300</td>
<td>68</td>
</tr>
<tr>
<td>Chloroform.</td>
<td>b. 328 m ( \mu )</td>
<td>14,600</td>
<td>4</td>
</tr>
<tr>
<td>Ethanol-chloroform, 1:4 by vol.</td>
<td>342 m ( \mu )</td>
<td>13,000</td>
<td>28</td>
</tr>
</tbody>
</table>

a. Subsidiary maximum at 390 m \( \mu \).

b. No subsidiary maximum.

**Fraction 2.**

<table>
<thead>
<tr>
<th>Eluant</th>
<th>( \lambda_{\text{max}} )</th>
<th>( \varepsilon_{\text{max}} ) ( (\text{P}) )</th>
<th>% Total DNP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benzene.</td>
<td>a. 328 m ( \mu )</td>
<td>15,700</td>
<td>59</td>
</tr>
<tr>
<td>Chloroform.</td>
<td>345 m ( \mu )</td>
<td>9,200</td>
<td>25</td>
</tr>
<tr>
<td>Ethanol:chloroform, 1:4 by vol.</td>
<td>355 m ( \mu )</td>
<td>6,400</td>
<td>16</td>
</tr>
</tbody>
</table>

a. Subsidiary maximum at 390 m \( \mu \).

**Fraction 3.**

<table>
<thead>
<tr>
<th>Eluant</th>
<th>( \lambda_{\text{max}} )</th>
<th>( \varepsilon_{\text{max}} ) ( (\text{P}) )</th>
<th>% Total DNP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benzene.</td>
<td>a. 328 m ( \mu )</td>
<td>13,600</td>
<td>25</td>
</tr>
<tr>
<td>Chloroform.</td>
<td>345 m ( \mu )</td>
<td>9,100</td>
<td>28</td>
</tr>
<tr>
<td>Ethanol:chloroform, 1:4 by vol.</td>
<td>355 m ( \mu )</td>
<td>8,070</td>
<td>47</td>
</tr>
</tbody>
</table>

a. Subsidiary maximum at 390 m \( \mu \).
This is not a serious short-coming, since the trend is quite marked; that portion of the cadmium chloride complex of cephalin which is least readily extracted into ether (like lecithin) has the most "355". Conversely, the most ether-soluble fraction has the most "328". Solubility in ether is a feature of the cadmium chloride complex of cephalin as prepared by the usual alcohol precipitation method. It may also be remarked that the "355"-rich fractions were found to have an unusual composition (Chapter 6, Table 7).

Third Fractionation of Egg Yolk Phospholipids.

The third fractionation, in which the ethanol-soluble phospholipids were split into a larger number of fractions than previously, was nevertheless disappointing inasmuch as "345" and "355" were not obtained free of "328". In fractions 2 - 9 (Chapter 6, Table 9), the products of methylation were similar in composition, the "328" fraction predominating. The fractions with the highest content of "355" were fractions 1 and 10. Fraction 1 was free of choline-containing phospholipids, while fraction 10 was in effect, a purified lecithin preparation. The peculiar composition of fraction 1 shows some resemblance to preparations 2 and 3 of the second fractionation experiment; the proportion "328":"345":"355" (without recovery correction) was 27:46:27.

The analysis of fraction 10 will be described in detail, since it demonstrates the sensitivity and wide application of the methylation procedure as an analytical method for aminophospholipid.

The lecithin (fraction 10) (13 mgas.) was reacted with FMB and diazomethane and the absorption curve measured in 25 ml. petroleum ether.
Fig. 10.
Analysis of an impure preparation of egg yolk lecithin (from the third fractionation experiment). The absorption spectra, in petroleum ether, are as follows: A, the products of methylation of the DNP derivative; B, the residue after subtraction from A of unidentified absorption in the petrol eluate obtained on chromatography; C, the extinction recovered after chromatography at wavelengths show (crosses); and D, the difference between B and C.
Fig. 11.

Composition of the products of methylation of the DNP derivative of an impure preparation of egg yolk lecithin. The absorption curves are drawn to scale to show the relative amounts of "328" (curve A), "345" (curve B) and "355" (curve C). The proportion of each component has been corrected for the low recovery of the "355" on chromatography (fig. 10).
Fig. 12.
Absorption spectrum in petroleum ether of the "410" component of the products of methylation of DNP-lipid prepared from hen's liver fat.
### TABLE 3.

Composition of Methylated DNP-Phospholipid in Purified Lecithin.

<table>
<thead>
<tr>
<th>Eluant</th>
<th>λ&lt;sub&gt;max&lt;/sub&gt;</th>
<th>ε&lt;sub&gt;max&lt;/sub&gt;(P)</th>
<th>% Total DNP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chloroform, (1)</td>
<td>323 mµ</td>
<td>17,200</td>
<td>19</td>
</tr>
<tr>
<td>Chloroform, (2)</td>
<td>545 mµ</td>
<td>9,100</td>
<td>12</td>
</tr>
<tr>
<td>Ethanol:chloroform, 1:4 by vol.</td>
<td>355 mµ</td>
<td>4,200</td>
<td>69</td>
</tr>
</tbody>
</table>
(figure 10, curve A). It was then transferred to a column of celite (hyflo-supercel, 10 gms.) in petrol. The material removed from the column by development with petrol had no maximum in the range of measurement and after subtraction from curve A, curve B resulted. Benzene did not elute any DNP material but chloroform brought about resolution into two bands, which were eluted separately. The first had \( \lambda_{\text{max}} \) at 328 m\( \mu \), with a subsidiary maximum at 335 m\( \mu \), while the second had \( \lambda_{\text{max}} \) at 345 m\( \mu \) in petrol. The remaining yellow band was eluted with ethanol:chloroform (1:4 by vol.) and had \( \lambda_{\text{max}} \) at 355 m\( \mu \) in petrol. The recovery curve (curve C, figure 10) showed that 73% of the "355" was recovered. The composition is summarised in Table 8 and figure 11.

Effect of Clostridium Welchii \( \alpha \)-Toxin on Egg Yolk Cephalin.

The possibility of effecting a separation of cephalin from lecithin by selective degradation of the lecithin, prior to further purification of the cephalin by chromatography etc., is presented by the results described in Chapter 6. It was of further interest to examine the specificity of the toxin, which reputedly contains only lecithinase \( D \), in the light of the present evidence of complex forms of DNP-cephalin. The same toxin-treated phospholipid, the preparation of which was described in Chapter 6, was analysed by reaction with diazomethane and chromatography.

The toxin-treated phospholipid (24.4 mgms.), after reaction with FENB and diazomethane, was chromatographed on 15 gms. hyflo-supercel to give the composition shown in Table 9. If these results are compared with those of the same preparation without toxin treatment (Table 7), it will be seen that the "328" component has increased from 25% to 72% of the total.
**Table 9.**

Composition of Toxin-treated Phospholipid after Reaction with FDNB and Diazomethane.

<table>
<thead>
<tr>
<th>Eluent</th>
<th>( \lambda_{\text{max}} )</th>
<th>( \varepsilon_{\text{max}} ) (P)</th>
<th>% Total D.F.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benzene</td>
<td>a. 328 m( \mu )</td>
<td>15,300</td>
<td>61</td>
</tr>
<tr>
<td></td>
<td>b. 328 m( \mu )</td>
<td>12,700</td>
<td>11</td>
</tr>
<tr>
<td>Chloroform</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ethanol:chloroform, 1:4 by vol.</td>
<td>345 m( \mu )</td>
<td>11,600</td>
<td>23</td>
</tr>
</tbody>
</table>

a. This eluate has a subsidiary maximum at 370 m\( \mu \).

b. No subsidiary maximum.
DNP derivative, while the "345" has remained unchanged. These figures are, of course, based upon the assumption of the same \( \Delta_{\text{MAX}} \) for all three \( \Delta \) derivatives. With this limitation in mind, there is a suggestion of quantitative conversion of "355" to "322" as a result of toxin treatment. A suitable control for the test system of MacFarlane and Knight could only be obtained by continual addition of alkali to the digest, in order to maintain it at constant pH. Under the conditions employed above, the pH would have dropped to about 1 - 2 in the intervals between addition of alkali to pH 8. In an experiment in which an emulsion of egg yolk phospholipid (registering pH 3) was incubated at 37°C, overnight, there was no loss of lipid phosphorus, but a considerable loss of titratability (Chapter 2). When analysed by reaction with FDNB and diazomethane, "355" material was still present. The lecithin preparation from the third fractionation experiment (fraction 10) was submitted to the action of the toxin in the bicarbonate buffer system of Zamecnik, Brewster and Lipmann (1947). It was not possible under these conditions to obtain complete hydrolysis of lecithin and at the same time, there was only partial loss of "355" precursor.

_Egg Yolk Cephalin prepared according to Lee, Rhodes and Stoll (1950)_

Experience with this method has been described in Chapter 6. The fraction isolated was free of choline but only 72% of total nitrogen was amino-nitrogen by direct reaction with FDNB. The quantity of cephalin recovered corresponded to about 70% of that originally present in the sample of total egg yolk phospholipid taken for chromatography.

A detailed analysis of the products of methylation of the DNP derivative by chromatography was not undertaken. However the absorption
curve of the total products in petrol, had $\lambda_{\text{max.}}$ at 328 m$\mu$ and the ratio $E_{328 \mu} / E_{328 \mu}$ was 0.37 as compared with the values 0.323 and 0.372, which are the values found for the two forms of "328" derivative so far described. In spite of having not carried out the chromatography of the methylated DTP derivative, it can nevertheless be concluded that this preparation of cephalin was substantially of the "classical" composition.

**Methylated DTP-lipid from Hen's Liver Fat.**

In order to determine whether the ability to furnish a "325" derivative was peculiar to egg yolk or to avian phospholipid in general, a sample of hen's liver lipid was analysed.

An acid-washed ethanol:ether extract of hen's liver was found to contain 0.226 $\mu$ mole amino-nitrogen/agm. total fat. After reaction with FDNB and diazomethane, the fat (35 mgm.) was chromatographed on 30 gms. hyflo-supercel. A greater number of bands appeared on the column on development with benzene than had been the experience with egg yolk phospholipid. Three chromatographically distinct components had $\lambda_{\text{max.}}$ at 328 m$\mu$, while a component previously not encountered had $\lambda_{\text{max.}}$ at 410 m$\mu$ in petrol. This moved between the first and second "328" bands. Careful development of the column with increasing amounts of chloroform in petrol enabled the separate elution of these bands, with a little overlapping: the last was eluted with equal parts of chloroform and benzene. There then remained one zone of adsorption, which was eluted with ethanol:chloroform (1:4 by vol.) and had $\lambda_{\text{max.}}$ in petrol at 345 m$\mu$. The recovery of extinction in this experiment was low, possibly owing to the high
ratio of adsorbent to DNP material. The difference curve showed that there had been loss of both "328" and "345", but showed no evidence of "355". A calculation of the proportion of "328" to "345" in the difference curve could be made by interpolation from theoretical mixtures, using the ratio of extinction at 330 m\(\mu\) to that at 360 m\(\mu\). This showed that there had been 35\% recovery of "328", but only 52\% recovery of "345", from which it was deduced that there were approximately equal amounts of "328" and "345" in the mixture before chromatography.

The absorption spectrum of the "410" component is shown in figure 12. This appeared as a bright yellow band on the column but actually made up only a very small part of the total extinction, viz. 2\%, based on the sum of extinctions at \(\lambda_{\text{max}}\). Since it is a fast-running component, its presence in small amounts might account for slight variations in the height of the subsidiary maximum in "328" fractions obtained from egg yolks. Its solubility in petroleum ether indicates that it is not a split product but must be a fatty molecule.

**Experiment with Radioactively Labelled Rat Liver Phospholipid.**

Rat liver lipids were studied independently by Dr. Collins who used the same procedure of reaction with FDNB and diazomethane as described for egg yolk phospholipid. The aim of Dr. Collins' experiments was to establish a biochemical difference between the "328" and "345" components of rat liver phospholipid, which would corroborate the spectroscopic differences.

The most interesting results were obtained with an in vitro system, consisting of a liver homogenate supplemented with glycerol, sodium suc-
cinate and adenylic acid, on the lines of Kennedy's (1953) system. \( P^{32} \) was present in the form of inorganic phosphate. After incubation at room temperature, the homogenate was freeze-dried and the lipid extracted in the usual manner (ethanol:ether).

Choline-containing phospholipids (isolated by the method of Taurog and others, 1944) had no radioactivity, while the "328" fraction of the cephalin had only low radioactivity (this varied from 43 - 200 cts./min./\( \mu \) mole P in six fractions obtained on re-chromatography). A "410" fraction was also found in rat liver lipid, moving ahead of the "328" on chromatography. This fraction was associated with high specific radioactivity in the first chromatography, but on re-chromatography, moved behind a high specific activity component (S.A. = 7580 cts./min./\( \mu \) mole) which was not associated with DNP groups; the specific activity of the "410" material then fell to 90 cts./min./\( \mu \) mole P.

The "345" fraction from rat liver lipid accounted for about 30% of the DNP-cephalin. The first fraction eluted had high specific radioactivity (S.A. = 2950 cts./min./\( \mu \) mole P) but there was a fall in subsequent fractions. When the "345" recovered from the first chromatography was reacted with diazomethane, it was seen that part of it was converted to "328"; on re-chromatography, there again appeared a fast-running fraction not associated with DNP groups, which had a high specific radioactivity (S.A. = 9590) while the "328" fraction was low (S.A. = 200 cts./min./\( \mu \) mole P). Again, the first fraction of the "345" to be eluted had high specific radioactivity (S.A. = 4520 cts./min./\( \mu \) mole P) and accounted for about 50% of that recovered from the first chromatography. The final fraction (eluted with ethanol) had a specific radioactivity of only 200 cts./min./\( \mu \) mole P.
Discussion.

The above account presents an unexpectedly complex picture of the composition of aminophospholipids. At least five different products, spectroscopically and chromatographically distinct, have resulted from the action of diazomethane on DNP-lipids. Only two of these, with $\lambda_{\text{max}}$ in petroleum ether at 328 m$\mu$, can possibly correspond to the methyl ester of DNP-cephalin. Of the remainder, that with $\lambda_{\text{max}}$ at 410 m$\mu$ occurs only in traces and that with $\lambda_{\text{max}}$ at 355 m$\mu$ has been found only in egg yolk phospholipid. The unexpected form of most general occurrence is the "345", i.e. that portion of the DNP-aminophospholipids which remains unaltered with regard to its absorption spectrum on treatment with diazomethane.

It will be appropriate to consider the available data on each of the above forms consecutively. From the discussion of the preceding section, it is reasonably certain that the "328" derivative corresponds to the "classical" formulation of cephalin, whether the base is ethanolamine or serine. A high yield of this derivative was obtained from the alcohol-insoluble brain cephalin, the yield approaching 100% with fraction III but less with fraction V, which is relatively more alcohol-soluble. Similarly, it was the alcohol-insoluble fractions of egg yolk phospholipid which gave the highest yield of "328" derivative. These findings are in accord with expectations since traditional methods for the preparation of cephalin are based upon its supposed alcohol-insolubility. However, there was a multiplicity of "328" forms which, considering the possibility of incomplete separation in some cases, probably reduces to two: one has a subsidiary maximum at 330 - 390 m$\mu$ and in spite of this, the ratio $\frac{328 \text{ m} \mu}{330 \text{ m} \mu}$
is higher than in the other form, where the subsidiary maximum is absent. These forms occurred as products both of synthetic cephalin and natural cephalin; hence there appears to be a certain degree of ambiguity in the methylation step, but since both forms are chromatographically very similar, this is probably not of serious proportions. It may be, for example, that the secondary amino-nitrogen group of the DNP derivative is subject to methylation by diazomethane. The tertiary amine so formed would probably not be very dissimilar spectroscopically, from the secondary amine. For practical purposes, the important properties are ease of elution and stability of the derivative. In this work, the only criterion of the isolation of an intact derivative was $\varepsilon_{\text{max}}$. (P). This figure was frequently about 20% higher than that taken for DNP-cephalin and compares with the increase in extinction at $\lambda_{\text{max}}$, which takes place on methylation, as may be seen in figure 1 for example. It may be recalled that $\lambda_{\text{max}}$ of DNP-octadecylamine in petroleum ether is 18,600 as compared with 17,300 in ethanol. $\varepsilon_{\text{max}}$. (P) of the "328" from rat liver was 18,000 after re-chromatography.

The formation of material with $\lambda_{\text{max}}$ at 410 m$\mu$ in petroleum ether has not been found generally, since it has only been detected in trace amounts and may not always have been separated from the "328". Its formation from synthetic cephalin suggests that it is a product of degradation of the DNP derivative, leading to some considerable alteration of the chromophore. While it is not quantitatively important, it presents a problem of interpretation which cannot be discussed adequately without suitable reference compounds. The nearest approximation to the "410" type of spectrum which has been encountered in the present work, is the product formed from DNP-ethanolamine and DNP-serine in the Janovský reaction (Chapter 4);
however this product is not only extremely unstable, but its light absorption was measured under conditions which bear no comparison with petroleum ether. Arising from this comparison though, is the suggestion of a high degree of resonance in the "410" derivative, which is inherent in the molecule and does not arise in response to a special environment.

The occurrence of methylation of a derivative with $\lambda_{\text{max}}$, at 355 m$\mu$ in petroleum ether presents a similar problem of interpretation but in contrast to the "410" fraction, the "355" is strongly adsorbed to celite, the strength of adsorption being of the same degree as that of DNP-cephalin without methylation. The fact of the "355" derivative having only been encountered in egg yolk phospholipid and demonstration of its abundance in the ethanol-soluble fraction, particularly as a contaminant of "purified" lecithin implies either (a) the existence of a native precursor of more complex composition than "classical" cephalin, or (b) the fractionation of a substance which interferes in the methylation reaction. Obviously more can be said about (a). The same considerations apply to the "345" derivative, although the fractionation of the "345" precursor was not so marked as with the "323" and "355".

A clue to the structure of the "355" and "345" fractions is found in their chromatographic behaviour. It is clear that since they are much more strongly adsorbed than the "323" they must contain polar groups incapable of methylation; the polar groups must be involved in various forms of hydrogen bonding with the chromophore. DNP-cephalin from egg yolk always showed $\lambda_{\text{max}}$, at 345 m$\mu$ in petrol, whether it gave rise predominantly to "323" or "355" on methylation, from which it would appear that hydrogen bonding between the phosphoric acid group and the chromophore supersedes
other forms which may be possible in a complex molecule, provided the acid group is free: once it is esterified, other types of hydrogen bonding with the chromophore are possible. For example, if sugar or inositol (both of which are known as constituents of phospholipids) were so disposed as to undergo hydrogen bonding with the chromophore, this would be of a different character to that made possible by the readily dissociated proton of the acid substituent. In the case of the "345", the residual hydrogen bonding after esterification of the phosphoric acid group must be different from that which occurs in the "355".

It will have been noticed that both the "345" and "355" fractions were associated with more phosphorus than is consistent with an atomic ratio of amino-nitrogen to phosphorus of unity, if one sets reasonable limits for $\epsilon_{\text{max}}$ of each of these chromophores. So far as the "355" is concerned, contamination with lecithin very probably accounts for the low values of $\epsilon_{\text{max}}$ (P). However, an examination of the last four tables reveals that a value of the order 9,000 for $\epsilon_{\text{max}}$ (P) occurs frequently for the "345" component. Out of six examples, where $\lambda_{\text{max}}$ is clearly at 345 m$\mu$, four are in the range 9,030 - 9,200, while one gave 10,600 and the other 7,100. In the remainder, $\lambda_{\text{max}}$ was nearer 343 m$\mu$, suggesting a slight degree of contamination with the "328" fraction and $\epsilon_{\text{max}}$ (P) was 11,900 and 13,000. This measure of replication of $\epsilon_{\text{max}}$ (P) is the more impressive because of the wide degree of variation in the overall composition of the preparations analysed. It suggests that the low $\epsilon_{\text{max}}$ (P) of the "345" component may not be due to contamination, but perhaps to a ratio of DNP groups to phosphorus of 0.5. The "fast-running" fractions earlier obtained by chromatography of DNP-cerebralin (Chapter 5) had a similar ratio of DNP groups
to phosphorus and also a ratio of fatty acids to phosphorus higher than 2. Thus, there is some analytical evidence to corroborate the indications of structural complexity arising from absorption spectra.

Supporting evidence for this theme was found in the effect of d-toxin of Clostridium welchii on the composition of a phospholipid preparation with a high proportion of "355", where there was no change in the proportion of "345" component after toxin treatment but a complete disappearance of the "355", accompanied by an equivalent increase in "325". However, to conclude that the "355" component contains a choline phosphoric acid residue is contrary to the finding of "355" in fraction 1 of the third fractionation experiment, where choline was absent. Alternatively there may exist in the toxin an enzyme which effects the removal of the unknown moiety, leaving "classical" cephalin as a breakdown product; although it must be admitted that the experiment was mainly exploratory and only lack of time prevented a thorough study of the toxin.

A like interpretation may be made of the apparent instability of the "345" fraction on chromatography, whereby on re-methylation it furnishes more "325". As will be shown in the next section, if the "345" fraction is isolated by counter-current distribution, it remains unchanged on re-methylation. Chromatography of the remethylated "345" fraction not only led to an increase in the specific radioactivity of the recovered "345" (which in the first chromatography, was already much higher than that of the "325") but also revealed the presence of a fast-running component of higher specific activity, which was not associated with DNP groups. Since it is most unlikely that this fast-running fraction would have been incompletely removed from the "345" fraction in the first chromatography, it
must be presumed to have been formed by decomposition of the "345" on chromatography at the same time as the "328" precursor was split off. In summary, this experiment has shown (a) that the "345" fraction is metabolically distinct from the "328" and (b) that the "345" fraction contains two metabolically distinct phosphorus atoms, only one of which is associated with DHP groups.

The advantages of the present procedure are firstly, a means for the identification of "classical" cephalin without degradation of the molecule; and secondly, the formation of a derivative which possesses none of the polar properties of native phospholipids and is therefore separated with considerable ease both from non-aminophospholipids and complex aminophospholipids. Granted the validity of the present claims of the existence of complex aminophospholipids, the first advantage is a novel advance in the chemistry of cephalin, inasmuch as it permits the recognition and assay of "classical" cephalin with much greater precision than hitherto. A good illustration of this is the analysis of the cephalin preparation obtained by chromatography of egg yolk phospholipid on silica gel, according to Lea, Rhodes and Stoll (1955). The recovery of cephalin by this method was low and it was evident from the absorption curve of the total products of methylation of the DHP derivative that "classical" cephalin was recovered preferentially.

The facility of chromatographic purification of the "328" derivative is well demonstrated by the analysis of a lecithin preparation (Table 3). In this preparation, amino-nitrogen was equivalent to only 2% of total phosphorus; the sample for analysis contained approximately 0.26 mgm. cephalin, of which only 20% of the amino-nitrogen was converted to the "328"
product. In spite of a 250-fold excess of lecithin, the "32P" fraction was isolated with the approximately correct proportion of phosphorus to DNP groups (ε_{max}, (P) = 17,200). However, the separation from phospholipids of minor occurrence may not be readily achieved and would require re-chromatography. For example, it may be expected that phosphatidic acid is converted by diazomethane to the dimethyl ester, which is in the same class as the "32P" derivative with regard to the absence of strongly polar substituents. The chromatographic studies of McIlhinney and Taylor (1952) and counter-current distribution studies of Ayres and Dooley (1948) have revealed the existence of a relatively non-polar phospholipid containing inositol. Although such phospholipids exist only in traces in total lipid extracts of tissues, for the purpose of turnover studies of cephalin, re-chromatography of the "32P" fraction to constant specific radioactivity or mass ratio would be the only reliable measure of purity.

The study of the chromatography of the "32P" fraction is admittedly far from being complete, the most serious omissions being analyses for fatty acids, glycerol and especially of DNP groups by chemical assay, which would provide a direct estimate of ε_{max}. Only in one instance was an analysis for fatty acids of a "32P" fraction carried out (by the method described in Appendix II) and a ratio of fatty acids to phosphorus of 2.0 was found. This particular chromatography was not otherwise of interest and has not been described in the Results section.

(c) A Study of Conditions in the Diazomethane Reaction.

Introduction.

In the previous section, the reaction of diazomethane with DNP-lipids has been studied with special reference to the source and treatment of the
lipid being analysed. The significance of these results warranted a closer investigation of the reaction with attention to factors affecting the reactivity of diazomethane; it was hoped that the results of this investigation might reinforce the interpretation presented previously. Unfortunately, the experiments had to be concluded at an untimely stage but the following two points were established: 1. The same minimal amount of "328" derivative is formed whether the reaction with diazomethane is allowed to proceed overnight at room temperature, or for a short period at -60 to -70°C; 2. increasing amounts of "328" may be obtained by the addition of water and methylamine to the ether solution of diazomethane. The second line of experimentation requires some introduction.

Independently of the present work, Baer and Maurukas (1955) found that solutions of diazomethane affect degradation of native cephalin, with the formation of the dimethyl ester of phosphatidic acid. This reaction they labelled "diazometholysis" but they suggested no mechanism or formulation. The reaction is therefore obscure, but may involve the amino group since there has been no evidence in the present study of DNP-lipids, of a dissociation of phosphorus from the DNP moiety. In any case, a much larger excess of diazomethane is employed by Baer and Maurukas (1955) and repeated treatment of the lipid sample was necessary to obtain 50 - 60% yield of phosphatidic acid dimethyl ester. A possible explanation of "diazometholysis" not considered by Baer and Maurukas (1955), may be found in the observations of O'Donnel and Swan (1953) on the degradation of wool by diazomethane. Diazomethane has long been known to contain methylamine as an impurity (v. Pechmann, 1890). Although there is apparently very little reaction between diazomethane and amines in water-free solution (Francis
and Katchalski, 1943), when water is present, methyldiene, but not higher
aliphatic amines, is slowly methylated ultimately to tetramethyl ammonium
hydroxide. Base-free preparations of diazomethane obtained by repeated
treatment with methyl iodide and p-toluene sulphonyl chloride, showed no
degradative attack on wool. The lengthy reaction time and scale of
operations of Daer and Matsubara (1955) were comparable to those found by
O'Donnel and Swan (1953) to effect degradation of wool. It seems possible
therefore, that "diazomethylolysis" is in effect a partial hydrolysis of the
phospholipid, followed by esterification of the liberated acid groups.

It is doubtful whether the above side-reactions take place under the
present conditions of application of diazomethane solutions. The solutions
of diazomethane were quite dilute, dried before use and the reaction time
was short. Nevertheless it was important on the one hand, to dispose
any ambiguity and on the other, to observe what effect conditions favou-
ring the formation of tetramethyl ammonium hydroxide would have on the pro-
ducts of reaction with EDP-lipids.

Experimental.

Materials.

The phospholipid preparations were ether or ethanol-ether extracts
of egg yolks, submitted to acetone separation and acid-washing. The EDP
derivative was prepared as described previously.

Methyldiene hydrochloride was E.D.H. laboratory reagent.

Methods.

In a series of experiments, samples of EDP-lipid were reacted with
diazomethane. The proportion of "328" in the reaction products was
determined by a simple counter-current distribution procedure devised by Dr. F.D. Collins, who made use of the solvent system of Lovern (1952), prepared by equilibrating equal parts by volume of petroleum ether and ethanol:water (85:15 by vol.). The distribution coefficients (upper phase as denominator) of "323" and "345" respectively in this system were 0.24 and 9.3. Four separating funnels of 50 ml. capacity were sufficient to carry out the separation. The mobile phase throughout was the lower phase; after the first three transfers, the upper phase of the terminal funnel was decanted into a reservoir for "323" fraction and the upper phase in this replenished. The upper phase was again decanted following two subsequent transfers, by which time the terminal lower phase had been extracted a sufficient number of times to be decanted into the reservoir for "345". The terminal lower phase was again removed after the following transfer, leaving four funnels with which the above process was repeated until an additional two lots of upper phase and one of lower phase had been collected, leaving three funnels. At this stage, about 93% of the IMP derivative has been recovered.

Conditions I. The Effects of Temperature, Reaction Time and Moisture on the Reaction Products.

In the following experiments, the diazomethane solution (in ether) was distilled in order to remove last traces of water. The amount of diazomethane in the distillate was determined from the extinction at 410 nm, taking $\varepsilon_{410 \text{ nm}} = 3.0$ (value given by Brinton and Volman, 1951, for diazomethane in the gaseous state). In each experiment, the lipid sample contained 6.45 μ moles of IMP groups.
Experiment A.

The solution of DNP-lipid in 5 mls. ether was cooled to -60 to
-70°C in a mixture of alcohol and solid carbon dioxide. Diazomethane
(100 μ moles) in 1.5 ml. ether was then added and the mixture allowed to
remain at the above temperature for 1 hour, after which the flask was
immediately attached to the water pump and the ether and diazomethane
removed without any application of heat apart from that derived from the
atmosphere.

Experiments B and C.

The components of the reaction mixture were the same as in experiment
A, but the reaction was carried out at room temperature for 1 hour (experi-
ment B) and overnight (experiment C).

Experiment D.

When the same diazomethane solution (experiment A) was shaken up
with a small quantity of water, the density at 410 mμ fell by a factor
of 5. 5.0 mls. of the wet ether solution containing approximately 100 μ
moles of diazomethane were applied to the DNP-lipid sample, which was
allowed to remain in solution overnight at room temperature.

Experiment E.

A very concentrated solution of diazomethane was prepared over 30%
potassium hydroxide and distilled. Upon adding water to the solution
there was effervescence at the water surface; after vigorous shaking,
the ether layer was poured off. The DNP-lipid was dissolved in 12 mls.
of this solution (5.3 μ moles diazomethane) and allowed to stand overnight
at room temperature.
Conditions 2. The Products of Reaction in the Absence and Presence of Methylamine.

Experiment A.

Nitrosomethyl urea (about 50 mgas.) was decomposed to diazomethane under ether and the ether solution allowed to stand 2 hours at room temperature with an equal volume of methyl iodide. The mixture was finally distilled, added to the lipid sample, containing 6.45 μ moles of DNP groups and the reaction mixture allowed to stand overnight at room temperature. The amount of diazomethane in the system would have been about 500 μ moles.

Experiment B.

An ether solution of diazomethane was dried over potassium hydroxide and distilled. It contained 870 μ moles of diazomethane.

An ether solution of methylamine was prepared by decomposing methylamine hydrochloride (600 μ moles) under ether, in the presence of moist pellets of potassium hydroxide. The ether solution was finally distilled.

After shaking the diazomethane solution with a few drops of water, both this and the methylamine solution were added to the fat, containing 6.45 μ moles of DNP- cephalin. After standing 2 hours at room temperature, a further 2.2 μ moles of diazomethane (dry ether) were added and the system allowed to stand overnight.

Tests for Bases in Diazomethane Solutions.

A distilled solution of diazomethane in ether, after shaking with water, was 0.166 M. To 2.0 mls. were added 2 drops of 1.0 N ethanolic hydrochloric acid (sufficient to discharge the yellow colour). After taking to dryness in vacuo, ethanol (1.0 ml.) and 0.1 M triethylamine in
ethanol (1.0 ml.), also FDNB (one drop) were added. A blank control, without diazomethane, was similarly prepared. Both were warmed 2 - 3 mins. at 50°C, made acid with 1.0 N hydrochloric acid, the volume adjusted to 10.0 mls. with ethanol and the extinction read at 420 m.μ. The reading in the experimental tube was 0.135; in the blank, 0.132. When 16.2 μ moles methylanine (solution in ether standardised by titration) were submitted to the same treatment as the diazomethane solution, the extinction at 420 m.μ was 0.425 in 100 mls. 0.1 N ethanolic hydrochloric acid.

A similar aliquot of the above diazomethane solution, after standing overnight, became colourless. To it were added 5.0 mls. of the boric acid - mixed indicator solution of Conway (1947) for ammonia determination. There was no change in the colour of the solution and the addition of one drop of 0.012 N hydrochloric acid immediately changed the colour to red.

Another solution of diazomethane in ether, 0.083 M, was not distilled, but dried over pellets of potassium hydroxide for 1 hour at -10°C. After standing overnight, the yellow colour of diazomethane had persisted. When 2.0 mls. of this solution were added to 5.0 mls. of boric acid - mixed indicator, a green colour resulted, but this was discharged by one drop of 0.012 N hydrochloric acid.

Results.

In the analysis of the reaction products by counter-current distribution, the upper phase gave a "328" absorption curve similar to those obtained by chromatography. λ max. of the lower phase was never at 345 m.μ, but nearer 350 m.μ (in petrolatum ether), suggestive of the presence of "355" material as well as of "345". The proportion of "355" was roughly estimated
by interpolation, using values of $E_{350 \text{ m} \mu}/E_{400 \text{ m} \mu}$ calculated for theoretical mixtures. The results of the experiments under "Conditions 1" are summarised in Table 10. A minimal value for the "328" derivative is clearly shown in experiments A, B and C. A 15-fold excess of diazomethane in wet ether results in a marked increase in "328" compared with dry ether (experiment D), without any significant loss of extinction. It is not clear whether there is any significant increase in "355". An 800-fold excess of diazomethane in wet ether resulted in about 50% loss of DNP-lipid (experiment E). In this case, a large part of the DNP material in the lower phase was insoluble in petroleum ether. The petroleum ether-soluble portion did not contain a well defined absorption maximum.

The results of experiments in "Conditions 2" are given in Table 11. By comparison with Table 10, it may be seen that the result in the presence and absence of methyl iodide is well replicated. The absorption curve of the total reaction products in experiment B is shown in figure 13. The product in experiment B has two interesting properties which distinguish it from the "328" derivative previously encountered. Firstly, the ratio $E_{400 \text{ m} \mu}/E_{328 \text{ m} \mu} = 0.354$; the "328" isolated in experiment A had $E_{400 \text{ m} \mu}/E_{328 \text{ m} \mu} = 0.324$, while for "328" isolated by chromatography (the main component without evidence of "410") this value is $0.295 - 0.300$. Secondly, upon counter-current distribution only 32% of the total extinction passed into the upper phase. The material in both phases had the same $E_{400 \text{ m} \mu}/E_{328 \text{ m} \mu}$. The DNP material in the lower phase was not only soluble in petroleum ether but formed a turbid emulsion in water, resembling a fatty derivative.

The final experiments described indicate that (a) no methyamine is
### Conditions (1) for the Reaction of Diazomethane

Details of each experiment are given under the appropriate heading in the Experimental section.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>( \lambda '320' )</th>
<th>( \lambda '345' )</th>
<th>( \lambda '355' )</th>
<th>Total Extinction ( a ) at ( \lambda_{\text{max}} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>53</td>
<td>40</td>
<td>7</td>
<td>1.99</td>
</tr>
<tr>
<td>B</td>
<td>52</td>
<td>47</td>
<td>1</td>
<td>1.99</td>
</tr>
<tr>
<td>C</td>
<td>51</td>
<td>45</td>
<td>4</td>
<td>1.96</td>
</tr>
<tr>
<td>D</td>
<td>63</td>
<td>28</td>
<td>9</td>
<td>1.92</td>
</tr>
<tr>
<td>E</td>
<td>45(^b)</td>
<td>( \ldots )</td>
<td>( \ldots )</td>
<td>0.89</td>
</tr>
</tbody>
</table>

\( a \). Measured in 50.0 mls. of petrol.

\( b \). Based on previous value for total extinction, viz. 1.99.
TABLE II.

Conditions (2) for the Reaction of Diazonmethane.

Details of each experiment are given under the appropriate heading in the Experimental section.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>$% 328$</th>
<th>$% 345$</th>
<th>$% 355$</th>
<th>Total Extinction</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>49</td>
<td>43</td>
<td>8</td>
<td>1.93</td>
</tr>
<tr>
<td>B</td>
<td>100</td>
<td>...</td>
<td>...</td>
<td>1.89</td>
</tr>
</tbody>
</table>

a. Measured at $\lambda_{max}$ in 50.0 mls. petrol.
Fig. 13.
The effect of the presence of methylamine and water on the products of methylation of DNP-lipid. Curve A: the absorption spectrum in petroleum ether of DNP-lipid prepared from total egg yolk phospholipid; curve B: the absorption spectrum in petroleum ether of the products of methylation of the DNP-derivative under the conditions described in the text.
detectable in a freshly prepared 0.116 M solution of diazomethane in water-saturated ether; and (b) such a solution, after standing long enough for most of the diazomethane to decompose, contains no titratable base; (c) diazomethane prepared in ether without subsequent distillation contains no more than traces of titratable base.

Discussion.

Experiments A, B and C of “Conditions 1” have shown that there is a component of DNP-lipid which reacts extremely readily with diazomethane to yield a derivative with \( \lambda_{max} \) in petroleum ether at 323 m\( \mu \), which for reasons discussed in previous sections, is believed to be evidence of a "classical" cephalin structure. This fraction has been all the more plainly distinguished from the remainder of the DNP derivative. Of particular interest however, is the ability of the remainder (the "345" fraction) to give rise to additional "329" under special conditions.

The effect of moisture in increasing the yield of "329" (experiment D, Table 10) may be (a) catalysis of the reaction of diazomethane with polar groups in the "345" component which are not methylated by diazomethane in dry ether, or (b) to render possible "diazometholysis", which causes splitting off of the "classical" cephalin moiety. It is evident from experiment E (Table 10) that high concentrations of diazomethane in wet ether can bring about extensive derangement of DNP-lipid; the conditions of this experiment approximate those employed by Baer and Mauritas (1955), who admitted the presence of moisture in their solvents, amounting to 2.2 to 2.6% of the weight of phospholipid.

The idea that a "345" \( \rightarrow "329" \) conversion may take place as a result of the partial degradation of a complex aminophospholipid would seem to be
a fruitful hypothesis, in view of the previously observed lability of the "345" fraction on chromatography. Because of the demonstrable absence of bases even in wet ether solutions of diazomethane at the concentrations employed in this work, a hydrolytic mechanism depending upon the formation of tetramethyl ammonium hydroxide does not seem to be an adequate explanation of the increase in "328" which occurs in a wet diazomethane solution. The alternative catalytic interpretation finds support in the reported catalysis of the methylation of aliphatic hydroxyl groups in carbohydrates (Mead, 1952). Nevertheless it has been shown that the addition of methylamine to the reaction mixture, which according to O'Donnell and Swan (1955) may be expected to bring about the formation of tetramethyl ammonium hydroxide, brings about a spectacular change in the composition of the reaction products (Table II). Again, catalysis may be an equally important factor, since amines are known to catalyse certain reactions of diazo methane (Gutsche, 1954).

Experiment II of Table II does not provide clear-cut evidence of "345" → "328" conversion, along the lines discussed above, because of the unusual distribution behaviour of the greater part of the "328" derivative. This indicates some additional effect of diazomethane, apart from esterification. If the "328" derivative has the structure of "classical" cephalin, the only likely sites for the introduction of an additional substituent are the double bonds. Any reaction of diazomethane with the aromatic portion would certainly affect light absorption, if it did not (as is most likely) cause degradation. Addition of diazomethane to ethylenic bonds however, is known to take place, pyrazole derivatives being formed (Mead, 1951):

\[
\text{CH}_2\text{CH}=\text{CH}\cdot\text{CO}_2\text{CH}_3 + \text{CH}_2\text{N}_2 \rightarrow \text{CH}_2\text{CH} = \text{CH} - \text{CO}_2\text{CH}_3
\]
If this reaction takes place with the unsaturated acyl residues of the phospholipid under the conditions of experiment 2, it may account for the observed distribution behaviour. This could be tested by studying the reaction of lipids previously submitted to catalytic reduction with hydrogen. Chromatography on seive may have provided additional information about the composition of this material.

The study of the methylation of DE-lipids ceased at this stage. In this section, a stage has been reached where not only have the distinguishing features of the "328" and "345" components been more clearly revealed, but a reaction has been found, which shows promise for the controlled degradation of the "345" fraction, thereby contributing to the ultimate elucidation of its structure.
186.

CHAPTER 7.

SUMMARY.

(a) The N-2,4-dinitrophenyl derivative of a fatty alkyl ester of ethanolamine phosphoric acid (2-aminoethyl octadecylphosphoric acid) has \( \lambda_{\text{max}} \) at 345 \( \mu \mu \) in petroleum ether and ethanol.

(b) Upon reaction with diazomethane, the above derivative undergoes a displacement in \( \lambda_{\text{max}} \) to 325 - 330 \( \mu \mu \), if the spectrum is measured in petroleum ether, but there is no displacement in ethanol.

(c) 2,4-dinitrophenyl octadecylamine has \( \lambda_{\text{max}} \) at 330 \( \mu \mu \) in petroleum ether, but at 345 \( \mu \mu \) in ethanol.

(d) N-2,4-dinitrophenyl lipids have \( \lambda_{\text{max}} \) at 340 - 345 \( \mu \mu \) both in petroleum ether and ethanol.

(e) When 2,4-dinitrophenyl lipid is treated with diazomethane, a portion reacts extremely readily to give a derivative with \( \lambda_{\text{max}} \) at 325 - 330 \( \mu \mu \) in petroleum ether, which may be isolated by chromatography or counter-current distribution. With egg yolk lipids, an additional component with \( \lambda_{\text{max}} \) at 355 \( \mu \mu \) in petroleum ether is formed, but with total lipid extracts in general, a substantial part of the 2,4-dinitrophenyl derivative remains unchanged with regard to \( \lambda_{\text{max}} \). This fraction may be converted to a "320" type of derivative if the reaction with diazomethane is carried out in the presence of water and methylamine.

(f) Lipid preparations which are insoluble in ethanol ("cephalin") furnish a high yield of the "320" derivative. Fraction III of ox-brain cephalin (Folch, 1942) was quantitatively converted to a derivative with \( \lambda_{\text{max}} \) at 325 \( \mu \mu \) in petroleum ether.
(g) Ethanol-soluble phospholipid preparations from egg yolk, which have a moderate lecithin content (30 - 60%) provide a low yield of "328" derivative, the substantial remainder have $\lambda_{\text{max}}$ at 345 m$\mu$, with a minor component having $\lambda_{\text{max}}$ at 355 m$\mu$ in petroleum ether. All three components may be separated by adsorption chromatography.

(h) An egg yolk lecithin preparation in which only 2% of total nitrogen was amino-nitrogen, gave the highest yield of "355" derivative and the lowest yield of "328" derivative. Although the "328" fraction was equivalent to only 0.4% of total phosphorus, it could be isolated by adsorption chromatography with an atomic ratio of nitrogen to phosphorus of unity.

(i) The ability of an egg yolk phospholipid preparation to furnish the "355" derivative was abolished by prior incubation with Clostridium welchii $\alpha$-toxin. At the same time, there was a compensating increase in the "328" fraction.

(j) Analysis of radioactively labelled rat liver phospholipids has shown that the phosphorus of the "345" fraction has a markedly higher turnover rate than the phosphorus of the "328" fraction.

(k) It has been concluded from the data in paragraphs a, b and c that the methyl ester of 2,4-dinitrophenyl cephalin should have $\lambda_{\text{max}}$ in petroleum ether at 328 m$\mu$. This conclusion was confirmed by an analysis of synthetic cephalin. On the basis of this and subsequent findings (e - j), a claim is made for the occurrence of complex aminophospholipids.
Determination of Fatty Acids.

A. Procedure employed in the analysis of ox-brain cephalin fractions (Chapter 2).

A sample of fat containing 2 to 10 μ moles of fatty acids was hydrolyzed by boiling for 4 to 5 minutes with 5 ml. of 0.1 N potassium hydroxide in ethanol. The hydrolysate was acidified with two drops of 10 N hydrochloric acid and taken to dryness in vacuo, the flask being allowed to remain attached to the pump for a few minutes after removal of ethanol in order to remove last traces of hydrochloric acid. The residue was then extracted three times with diethyl ether (10 to 15 ml.). The ether extracts are very easily decanted as a clear solution and were subsequently taken to dryness in vacuo. After taking the residue up in 5.0 ml. 95% ethanol (ethanol : water, 9 : 1 by vol.), an aliquot (1.0 ml.) was titrated under a stream of nitrogen with approximately 0.01 N aqueous potassium hydroxide, employing a micro burette (Somay, 1947). Standardisation was affected with oleic acid of at least 99% purity to a green end-point with triphenyl green.

B. Procedure employed in the analysis of DIP-coephalin (Chapters 5 and 7):

Fatty acids were isolated by saponification as described above. However, owing to the ether solubility of the DIP bases and the products of their decomposition by alkali (e.g., dinitrophenol, the indicator effect of which obscures the end-point), it was necessary to purify the fatty acids before they could be determined by titration. This was performed as follows:
A column (6 cm. x 1 cm.) of silica (R.D.H., "for chromatography") was prepared and the adsorbent washed first with ethanol and then with ether and a check made that the final ether wash contained no titratable material. The residue obtained upon evaporation of the ether extracts of the DNP-lipid hydrolysate was quantitatively transferred to the silica column in a few portions of ether and washed through with 15 to 20 ml. of ether. The eluate was finally taken to dryness, dissolved in 5.0 ml. 90% ethanol and an aliquot (1.0 ml.) titrated as described previously. Samples of oleic and stearic acid when submitted to this treatment, were recovered quantitatively.
APPENDIX II.

Preparation of DNP-Ethanolamine.

The present method for the preparation of DNP-ethanolamine has taken advantage of the solubility of the base in a solvent (diethyl ether) which is unreactive towards DNB, thereby avoiding a large amount of side-products, which is a feature of the system of Sanger (1945), intended for the water-soluble amino-acids. Also the removal of excess DNB, which is readily accomplished in the case of DNP-amino-acids by distribution between aqueous bicarbonate and ether, was best accomplished in the case of DNP-ethanolamine by volatilisation. The preparation was carried out as follows:

Ethanolamine (610 mgm. or 10 m moles) was dissolved in about 50 mls. of anhydrous diethyl ether and a solution of DNB (2 gms. or 11 m moles) in about 10 mls. of ether was added with stirring at room temperature. The reaction mixture was allowed to stand for half an hour to cool, when triethylamine (2 gms. or 19 m moles) was added. After standing overnight at room temperature the excess triethylamine was removed by evaporation to dryness in vacuo at the water pump. The residue was then distributed as evenly as possible over the interior of a large (3.5 litre) flask, which was fitted with a cold finger, placed in a boiling water bath and evacuated at about 0.05 mm. mercury pressure. The residue readily crystallised from benzene:diethyl ether and gave a m.p. of 90°C, which remained the same on re-crystallisation. The yield was 1.21 gms. (53% of theory); it was later observed that DNP-ethanolamine is somewhat volatile in a high vacuum.

Reflecting the diversity of their composition, the analytical chemistry of phospholipids requires the use not only of lipid techniques, but of a wide range of analytical methods more closely allied to other classes of biologically important substances. In fact, so far as one is concerned with the water-soluble fragments of phospholipids obtained on hydrolysis, it may be expected that more instruction would be gained from the experience of investigators concerned with quite different classes, of which these molecular "building stones" are more characteristic. This approach formed the starting point of the research described in this thesis and it was soon realised that a reagent which has particularly illuminated the structure of proteins (fluoro-d,4-dinitrobenzene) might be applied just as effectively and even more appropriately to the amino-phospholipids. Therefore a reagent which has been known for some years only in connection with proteins and their products of hydrolysis (peptides, amino-acids) has been shown to be pre-eminently a tool for the
study of a particular group of lipids, which possesses free amino-nitrogen. The analytical determination of aminophospholipids without disruption of the molecule was a new contribution to phospholipid analysis and the formation of a coloured derivative offered a new approach to the chromatographic isolation of cephalin, a task which had been conspicuously unrewarding in the past. This last objective was brought to a successful conclusion by esterification of the free acid groups of the derivative with diazomethane, another reagent eminently suited to the chemical modification of lipids.

The foregoing summarises the main trend of the research undertaken; integrating with this were studies of the purification and fractionation of phospholipids, the direct determination of lecithin and cephalin by titration and the analysis of lipid hydrolysates for choline, ethanolamine and serine. Although each of these topics was not developed quite fully, the groundwork was done and their possibilities demonstrated.

It remains to mention the most stimulating encounter, which was the incomplete yield of a characteristic derivative upon reaction of ED-lipid with diazomethane. This unexpected result was the culmination of two accidents: firstly, the choice of a dinitrophenyl derivative, the light absorption of which is influenced by hydrogen bonding, for the replacement of the amino group and secondly, the choice of a hydrocarbon and hence non-hydrogen bonding solvent, for the measurement of absorption spectra. While there are other claims of the occurrence of complex aminophospholipids, based upon fractionation and isolation (to be discussed below), this is the first direct evidence of their widespread occurrence in animal tissues.
2. The Determination of Hydrolytic Fragments.

In contrast to earlier work in this field, for example that of Levine and Chargaff (1951), my own experience of which is described in Chapter 4, recent research has aimed at the isolation of more complex fragments than the free base. The work of Dawson is perhaps the most outstanding advance in analytical technique during this period. Dawson has employed mercury-catalysed acid hydrolysis (1954a) and mild saponification (1954b) to remove fatty aldehydes and fatty acids from the phospholipid molecule and obtain water-soluble products containing combined glycerol, phosphoric acid and base, which are readily identified by paper chromatography. So long as there remains no satisfactory means of isolating pure samples of phospholipids on a small scale, this method of Dawson is invaluable as a tool for studying the turnover of the phosphorus moiety. Paper chromatography of all the possible fragments of dipalmitoyl-L-α-glycerylphosphorylcholine, including fatty acid containing fragments, has been studied by Hunekeins, Hanahan and Uziel (1953). Olley and Dawson (1956) have given a recent communication on the paper chromatography of phosphate esters which may be obtained as fragments of phospholipids or occur as such in tissues.

The work of Dawson and others mentioned, while it offers a better approach to metabolic studies than has previously been available, is subject to two important criticisms: (a) the methods of degradation are not quantitative, so that the behaviour of the phospholipid as a whole has to be inferred from fragments obtained in low (about 50%) yield; (b) evidence of the existence of complex phospholipids based upon glyceryl phosphoric acid, to be discussed under a later heading, points to the possibility of
the above fragments being derived from a multiplicity of parent substances and not just "lecithin" and "cephalin" as they are known at present.

Two ways of adapting the Edman method to the above purpose are possible. One may either react Dawson's fragments with Edman to obtain the DNP derivatives, which, being coloured, may be directly determined from their extinction after elution from a paper chromatogram, thus avoiding the uncertainties which are a feature of the quantitative ninhydrin method (Chapter 4). Alternatively, DNP-lipid may be hydrolysed under suitable conditions to obtain the phosphorylated DNP-base. In Chapter 5, mild alkaline hydrolysis was found to effect this. The latter method might be applied with particular interest to the "323" and "345" fractions of radioactively labelled rat liver phospholipids, whereby it may be possible to demonstrate a difference in turnover of the phosphorus moiety of the serine- and ethanalamine-containing phospholipids.

3. The Direct Determination of Phospholipids.

If it were possible to determine phospholipids directly, it would clearly be an advantage to do so, thereby avoiding a time-consuming operation accompanied by degradation of the phospholipid molecule. Nevertheless, this manner of determining phospholipids has not as yet been accepted as an analytical procedure, although Thanhauser, Benotti and Reinstein (1939) made an attempt to introduce direct precipitation of sphingomyelin with Reinecke salt, a procedure which has been shown by Hack (1946) to be nonspecific. Not many phospholipids lend themselves to direct determination by a reaction as specific as that between amino-nitrogen and Edman, which has been described in this thesis; for example, there are no comparable
reactions of the quaternary ammonium group or of inositol, or of bound sugar in cerebrosides. However, it should be possible broadly to determine classes of phospholipid by estimation of equivalents of acid and base. In Chapter 3, it was shown that titration with perchloric acid in dioxane gave an estimate of total phospholipid agreeing exactly with total nitrogen, while by carrying the titration out in glacial acetic acid, there was evidence of the separate titration of amino-nitrogen and quaternary ammonium nitrogen. Undoubtedly in the later case, a better inflection in the titration curve would result if the titration were performed on a sample of lipid after reaction with FDNB, whereby the amino group would be converted to a much weaker basic substituent. Determination of acid groups in a total phospholipid preparation by titration in an aqueous system (Chapter 2) gave an estimate of cephalin only slightly in excess of amino-nitrogen by direct reaction with FDNB, although fractionated phospholipids frequently contained acid groups greatly in excess of amino-nitrogen (Chapter 6). The occurrence of complex acidic phospholipids as suggested by Olley (1956) may well explain the excess of acid equivalents over amino-nitrogen; if this is so, then it is of particular importance to determine acid groups as well as amino-nitrogen in any fractionation study. However, so far as total phospholipids are concerned, no great error would be introduced by unknown acidic lipids which are of fairly minor occurrence.

Following the discovery of the heterogeneity of aminophospholipids by reaction of the DNP derivative with diazomethane, a method for the direct determination of "classical" cephalin is presented by determination of the amount of "328" derivative formed. This may be ascertained from
the total light absorption by interpolation from theoretical mixtures of "328" and "345" derivatives, or more accurately, after chromatography or counter-current distribution. This result emphasizes the importance of the direct determination of the functional groups of a molecule, whether by formation of a characteristic derivative or titration or other means, since it is not always possible to infer unambiguously the structure of a substance from its products of hydrolysis.

4. The Complexity of Phospholipids.

(a) With Regard to the Partition of Lipid Nitrogen.

The consistently low value for amino-nitrogen as compared with non-choline-nitrogen may be attributed to two possible causes: (a) instability of lipid amine groups, or (b) the occurrence of unknown nitrogenous lipids. This assumes of course, that non-lipid nitrogen is not present.

This incomplete account of lipid nitrogen was particularly noteworthy in "purified" cephalin preparations, using procedures described by Folch (1942) and Lea, Rhodes and Stoll (1955). Since both of these preparations could be obtained with atomic ratios of nitrogen to phosphorus of unity and in the former case, with two moles of fatty acid per atom of phosphorus, there is no question of contamination with cerebroside which is free of phosphorus and contains one fatty acid per atom of nitrogen. Moreover, the authors found all of the nitrogen present in their preparations to be amino-nitrogen; accepting their analytical results without criticism, the most probably explanation of low results with the Nessler procedure (also by the ninhydrin method in the case of the brain cephalin) is loss of amino-nitrogen during isolation. Instability of amino-nitrogen on storage of
fats has been observed by Chargaff, Ziff and Rittenberg (1942), Polch (1943), and Lea and Rhodes (1955), although I have not found loss of amino-nitrogen on heating lipids in ethanol-ether.

In view of the possible instability of lipid amino groups, there may be fine points of precaution in isolation procedures, which are not made explicit in published accounts. Following the discovery of cephalin by Thudichum (1884), almost fifty years elapsed before it was prepared by Rudy and Page (1930) with total nitrogen in the form of amino groups. Very likely, the lack of success of earlier workers was to a large extent due to the severe treatments employed in its isolation. Even to-day, it is only in the hands of experienced workers that cephalin with the "classical" composition has been prepared. For example, Klark and Bohmen (1955) recently described a "classical" cephalin preparation with 26% of its nitrogen as amino-nitrogen. However, a preparation of Scholfield and Dutton (1955) had only 8% amino-nitrogen.

A different explanation of the apparently low values for amino-nitrogen lies in the possible occurrence of bound forms of nitrogen in phospholipid species as yet unrecognized. In view of the substantial agreement between direct and indirect estimates with FDNB, it is unlikely that this consists of "bound" ethanolamine (the amino group in combination with sugar, for example), because such a structure would liberate amino groups on hydrolysis. It is of interest that a phospholipid containing such a structure has been isolated from ground nut "lecithin" (commercial product) by alcohol precipitation (Waltin and Poole, 1953). The purified material had a nitrogen to phosphorus ratio of 0.5 and gave a ninhydrin reaction only after hydrolysis. The products of hydrolysis were consistent with
formulation as the N-glycosyl derivative of the ethanolamine ester of phosphatidyl inositol phosphate:

\[
\text{R} = \text{mainly palmitoyl, stearoyl, oleyl, linoleyl.}
\]

\[
\text{S} = \text{trisaccharide or disaccharides of arabinose and galactose.}
\]

If Folch's (1942) cephalin were contaminated with such material, it would give a higher result for amino-nitrogen after hydrolysis than before. A possible alternative is the occurrence of a bound fatty amine in a form (other than sphingomyelin or cerebroside) which is not readily separated from the alcohol-insoluble fraction. Such a fatty amine, if liberated upon acid hydrolysis, would be removed by ether extraction of a neutralised hydrolysate. Determination of nitrogen recovery in hydrolysates of the phospholipid samples analysed would have provided a valuable pointer to this, but has been regretfully neglected in the present work. Comments such as those which follow, on the method of Schmidt, Benotti, Harshman and Thannhauser (1946) for the determination of sphingomyelin, based upon the resistance to hydrolysis of the linkage between sphingosine and phosphoric acid, provide some support for this hypothesis. For example, Brante (1949) found a proportion of cephalin to be equally resistant to hydrolysis, an observation which has also been made by
It is conceivable that this fraction of cephalin, which Brante (1949) terms "cephalin D", may be related to the unaccountable nitrogen. A criticism in this connection may be made upon the procedure of Burmaster (1946b) in calculating free amino-nitrogen in his analysis of cephalin fractions obtained by the method of Polch (1942). Burmaster (1946b) determined nitrogen in the hydrolysate, the recovery of lipid-nitrogen ranging from 99% for fraction III to 64% for fraction V. His estimate of amino-nitrogen as a percentage of total nitrogen is calculated on a _per cent_ basis, which is hardly justifiable in view of possible contamination with fatty amines, including sphingosine, which may have been removed upon ether extraction of the hydrolysate. If one makes no recovery correction then the proportion of amino-nitrogen to total nitrogen in his preparation of fraction V is 61%, which compares with the result obtained with my own preparation (59%, Chapter 6). It is not clear whether Polch (1942) made the same calculation as Burmaster (1946b), who worked in the same laboratory.

Recently, a method has been described for the determination of sphingosine as the N-succinyl derivative (Wittenberg, 1955). The application of this procedure to lipids, together with determination of choline and amino-nitrogen, may give a clearer account of total lipid nitrogen than would be achieved using the method of differential saponification, referred to above.

It has been pointed out in Chapter 6 that the excess of non-choline-nitrogen over amino-nitrogen in a large number of egg yolk phospholipid fractions obtained by the cadmium chloride method (for example, Table 9) agreed with the excess of acid equivalents over amino-nitrogen, although
acid equivalents might be greater than non-lecithin phosphorus. The additional acid is therefore not a derivative of phosphoric acid. McKibbin and Taylor (1952), analysing phospholipid fractions readily eluted from silica gel, found evidence of non-fatty carboxylic acid in hydrolysates and some fractions were also accompanied by an unknown sphingolipid. The anomalous acidity of cephalin in aqueous dispersion (Chapter 2) is a property which may be explained by the occurrence of unknown acidic phospholipids. Additional inflections about the equivalence point indicated the presence of an acid substituent considerably weaker than the phosphoric of phosphatidyl ethanolamine.

A consideration which has to be borne in mind whenever claims are made for the occurrence of new bases in phospholipids is the adequacy of the methods of purification of the lipid extract which have been employed. Hecht and Link (1952) for example, made a claim for the occurrence of a glutamic acid containing phospholipid in brain cephalin which had been submitted to dialysis as a purification step. This claim was based largely upon qualitative data obtained by means of paper chromatography. In Chapter 2, I have described the occurrence of a relatively large amount of a substance in dialysed ox-brain cephalin, which resembles glutamic acid but is readily removed from the lipid by acid-washing. A more recent claim of the existence of bound glutamic acid in the phospholipid fraction of liver (Pilgeram and Greenberg, 1955) is equally doubtful in view of the lack of any purification procedure beyond suspension of a crude extract in petroleum ether, a procedure which I have also shown to be inadequate for the removal of non-lipids (Chapter 2).
(b) The Incomplete Yield of a Characteristic Derivative of "Classical" Cephalin.

Contrasting in principle with evidence of complexity considered above, are the results of methylation of DNP-lipids with diazomethane (Chapter 7). The evidence here concerns the configuration of the molecule as a whole rather than its elements and thus is independent of the presence of major contaminants such as lecithin, containing the same elements which introduce a margin of error in the interpretation of analytical data. Therefore the method can be applied to preparations of which cephalin is a very minor component and has a wide application. The derivative of "classical" cephalin was obtained in highest yield from alcohol-insoluble preparations, in agreement with established procedures for the isolation of cephalin. Alcohol-soluble preparations however, contained a high proportion of apparently complex forms of aminophospholipids. The magnitude of the difference in spectroscopic properties between these unexpected derivatives and the derivative of "classical" cephalin is of an order which suggests some such effect on the chromophore as intramolecular hydrogen bonding, although it is not at present known what substituents are responsible for this, since the derivatives have not been isolated in a pure state. A similar interpretation of absorption spectra has been made with the 2,4-dinitrophenyldydrasones of pyruvic acid, where the cis-isomer is said to be stabilised by hydrogen bonding between the 2-nitro group and the amino and carboxyl groups, resulting in a displacement in $\lambda_{\text{max}}$ in sodium bicarbonate solution of 10 m$\mu$ compared with the trans-isomer (Isherwood and Cruickshank, 1954). Stereoisomerism is made possible in these derivatives by the existence of double bonded carbon and nitrogen. Of the known constituents of
lignids which are capable of hydrogen bonding and might therefore form part
of a complex aminophospholipid, there are inositol, glycerol, sugars and
possibly the amino-nitrogen of the ceramide bond (which is formed between
sphingosine and a fatty acid). Such a structure has been described by
Malkin and Poole (1953) as mentioned above, but their material was alcohol-
insoluble, and did not contain a free amino group. While my own work was
in progress, evidence was presented from several quarters for the existence
of complex phospholipids based upon polymers of glycerophosphoric acid,
which evidence, while it does not immediately explain my own results, de-
serves a brief description.

Diether structures were claimed by Klenk and Deuch (1954) to account
for the low phosphorus content of most cephalin preparations, compared with
synthetic cephalin. This claim is based upon the formation of chymyl- (I)
and batyl- (II) phosphoric acid on mild acid hydrolysis of the product of
catalytic reduction of a plasmal-rich phosphatidyl ethanolamine fraction of
brain lipid.

\[
\begin{align*}
\text{I} & : \text{CH}_2-O-\text{CH}_2(\text{CH}_2)_{14}-\text{CH}_3 \\
& + \text{CH}_2-O-\text{CH}_2(\text{CH}_2)_{16}-\text{CH}_3 \\
& \text{CHOH} \\
& \text{CHOH} \\
& \text{CH}_2-O-\text{PO(OH)}_2 \\
\end{align*}
\]

If the precursor of the hydroxyl group is an ether bond then a struc-
ture of the type III, based upon diglycerophosphoric acid, may be envisaged:

\[
\begin{align*}
\text{III} & : \text{R} \\
& \text{CH}_2-O-\text{CH}-O-\text{CH}_2-O-\text{CH}-O-\text{CH}_2 \\
& \text{CHO-CO-R} \\
& \text{CHO-CO-R} \\
& \text{CH}_2-O-P \rightarrow O \\
& \text{HO} \quad \text{O(CH}_2)_2\text{NH}_2 \quad \text{OH} \quad \text{O(CH}_2)_2\text{NH}_2 \quad \text{OH} \quad \text{O(CH}_2)_2\text{NH}_2
\end{align*}
\]
There may exist a relationship between the findings of Klenk and Debuch (1954) and the "polyglycerol phosphatide" of McKibbin and Taylor (1952). The latter material has been described in Chapter 5, where its possible bearing on the composition of the "fast-running" fraction of DNP-cephalin was discussed. The "polyglycerol phosphatide" was not obtained pure but a higher proportion of fatty acid radical, determined by chronic acid oxidation, than could be accounted for on the basis of two fatty acid residues per molecule of glycerol. The fatty acid might be combined in the manner proposed by Klenk and Debuch (1954). However, an important difference is that the latter workers' preparations gave high atomic ratios of nitrogen to phosphorus, ranging from 1.3 to 1.5, while the "polyglycerol phosphatide" was almost free of nitrogen. It is possible that the "polyglycerol phosphatide" at least represents a fragment of the structure envisaged by Klenk and Debuch.

Contributing further interest to the structures proposed by Klenk and Debuch (1954), Olley (1956) found considerable complexity in the glycerol phosphates formed on alkaline hydrolysis of phospholipid fractions obtained by counter-current distribution. These fell into five classes, dependent upon the solubility of the potassium salts in ethanol, paper chromatography and stability to hydrolysis at pH 4. Phosphate esters with more than one glycerol residue per atom of phosphorus were especially abundant in the ethanol-soluble fraction.

Evidence of the complexity of phospholipids presented in the three papers referred to above is admittedly fragmentary, involving a variety of starting materials and isolation procedures, but none the less it compels a revision of the traditional simple concept of the composition of phospholipids, thereby falling in line with the results obtained from a study of
the DNP derivatives. The suggestion of polymer phospholipids possibly based upon diesters of phosphoric acid, introduces an analogy with nucleotides (having the same basic linkage), so that further work may be suggested by research which has been carried out in this field. Unfortunately, the variety of isolation procedures which are available for water-soluble substances such as nucleotides (ion-exchange, electrophoresis, etc.) cannot be employed without serious disadvantages in the case of phospholipids. Hence the main problem in the phospholipid field is that of isolation of pure material.

**Chromatography of Phospholipids.**

Advances in the chromatography of phospholipids up to the time of commencement of my own work have been considered in the Introduction. The only conspicuous success has been achieved with lecithin which can be eluted from relatively strong basic adsorbents such as alumina and magnesium oxide, leaving native cephalin so strongly adsorbed that it cannot be recovered. However, it was found by Bergström (1952) that both lecithin and cephalin can be eluted from silica, an acidic adsorbent, and Lea, Rhodes and Stoll (1955) subsequently developed a method for the isolation of egg yolk cephalin by this means. Thus it would appear that the free acidic group of cephalin is mainly responsible for its strength of attachment to adsorbents, while in lecithin the acidic group is internally neutralised and cannot bear an equal effect. This fundamental difference between lecithin and cephalin does not seem to have been widely appreciated and apparently it did not guide the above authors in their search for a suitable adsorbent. The present approach attacked the problem from the other side: since all phospholipids contain relatively strong polar substituents, if the "polarity" of one species is greatly reduced, it should be readily separated from the remainder. This has been accomplished by
dinitrophenylation and methylation of cephalin, whereby the "classical" derivative with $\lambda_{\text{max}}$ at 328 m$\mu$ more closely resembles the glyceride fraction than the phospholipid fraction, with regard to strength of adsorption, solubility, etc. and is therefore easily separated from unreacted phospholipids. There are no reasons to suspect that lecithin is affected by the reactions concerned, although a phospholipid like phosphatic acid which is of minor occurrence, would undergo esterification and thus may be expected as a contaminant of the cephalin derivative.

The principles discussed above refer of course, to the derivative with $\lambda_{\text{max}}$ at 328 m$\mu$; these advantages do not exist in the more complex derivatives with $\lambda_{\text{max}}$ at 345 m$\mu$ and 355 m$\mu$, which presumably contain additional polar substituents not affected by diazomethane. However, two ways of increasing the yield of "328" derivative have been presented in Chapter 7: (a) treatment of the fat with Clostridium welchii $\alpha$-toxin prior to reaction with DMF, whereby an increase in "328" takes place, which is equivalent to the amount of "355" found in the untreated fat, there being no change in the amount of "345" component; and (b) reaction of the DNP derivative with diazomethane in the presence of water and methylaniline, which results in a quantitative yield of "328". This behaviour has been attributed to the splitting off of the complex fragments under the above conditions leaving behind the "classical" cephalin structure. Whether or not there is an alternative explanation, the outlines of a procedure for the isolation of associated amino-nitrogen phosphorus and fatty acids have been presented. It is of interest that in repeating the work of Lea, Rhodes and Stoll (1955), I found a low recovery of cephalin from silica, wherein the precursor of the "328" derivative was recovered preferentially.
A serious difficulty in the isolation of a pure phospholipid species whether by chromatography or other means, is the complex composition of native phospholipid mixtures, not to mention the predominantly random distribution of fatty acids. This feature of phospholipids has been discussed fully in Chapter 6 in connection with problems of analysis. The sufficient appraisal of a method for the quantitative isolation of a phospholipid would require application to a variety of preparations showing a broad enough spectrum in their overall composition to provide assurance that when one component is present as a minor quantity, its recovery as a contaminant is not overlooked due to inadequacy of the analytical methods employed. For example, if the method of Lea, Rhodes and Stoll (1955) were applied to an alcohol-insoluble preparation of phospholipids, then the presence of inositol-containing phospholipids as well as sphingolipids as contaminants of "cephalin" is bound to be noticed in view of the experience of McKibbin and Taylor (1952) with the same adsorbent. This would not have been noticed by Lea, Rhodes and Stoll (1955) with their preparation of total egg yolk phospholipid, where inositol- and sphingosine-containing phospholipids occur only in traces. On the other hand, Martinetti and Stots (1955) found that paper chromatographic methods which have been described for the separation of "purified" lecithin and cephalin were ineffective when applied to total lipid extracts.

In the description of the present method, it has been applied to numerous fractions of egg yolk phospholipid of varying composition. $\varepsilon_{\text{max}}(\nu)$ of the "32:1" fraction varied over quite a narrow range from alcohol-insoluble to alcohol-soluble preparations and even when amino-nitrogen was equivalent to only 0.24% of total nitrogen, $\varepsilon_{\text{max}}(\nu)$ was
of the expected order. (Chapter 7, Table 9), showing that lecithin is easily separated. The one occasion when $E_{\text{max}} (F)$ of a "323" fraction was conspicuously low was after treatment with Clostridium welchii toxin (Table 9, Chapter 7) in contrast to the much higher $E_{\text{max}} (\lambda)$ of the same fraction isolated from the untreated fat (Table 7, Chapter 7). Since toxin treatment was accompanied by the disappearance of the "355" precursor, a possible explanation is the splitting off of a phosphorus-containing fragment of a fatty nature (otherwise it would have been removed on acid-washing) which on treatment with diazomethane, was converted to a "non-polar" derivative of similar ease of elution as the "323" fraction.

Probably the best test of the homogeneity of material obtained on chromatography is secured by differential labelling of the components with a readily determined isotope. A "323" fraction obtained by Dr. F.D. Collins from radioactively labelled ($D^{32}$) rat liver lipid was found to be contaminated with a component of higher specific radioactivity on rechromatography but the middle fractions containing the greater part of the "323" fraction, were of uniform specific radioactivity.

What may turn out to be a disadvantage in the present technique is the absence in the methylated derivatives, of sufficient difference in polarity between derivatives of serine and ethanolamine to enable the ready separation of phospholipids based upon these respective units. This particular problem has not been studied in detail, but Dr. F.D. Collins has observed a greater ease of elution of the ethanolamine-containing fraction of the "323" derivative in rat liver lipid when this is chromatographed on celite. A further interesting observation of Dr. Collins, is the occurrence of virtually all of the serine of rat brain lipid in the "323" fraction,
there being only traces in the "345" portion, which accounts for somewhat more than half of the total DNP-lipid.

Scope of Application of the Present Technique.

A most notable feature of 2,4-dinitrophenyl derivatives is the variety of absorption spectra which they display according to the environment of the DNP substituent, both intra- and extra-molecular. Derivatives with \( \lambda_{\text{max}} \) ranging from 325 m\( \mu \) (Chapter 7, figure 2) to 570 m\( \mu \) (Chapter 4, figure 10) have been described in this thesis, not to mention variants with regard to subsidiary maxima. This uncommon property has not only led to the discovery of complex aminophospholipids, but promises further useful advantages for both the analysis and characterization of aminophospholipids. Such advantages as have already been dealt with are (a) the simultaneous determination of DNP-serine and DNP-ethanolamine by a differential colour reaction (Chapter 4) and (b) the determination of the "classical" cephalin content of tissues by methylation of the DNP lipid (Chapter 7).

Of such interest would be the application of the DNP - diazomethane reaction to some recent preparations of complex aminophospholipids, such as that of Klenk and Debusch (1954) referred to above. The determination and isolation of sphingosine should be readily accomplished by its conversion to the DNP derivative.

The scope of the DNP procedure could not be better illustrated than in the study of lipopeptides. These ether-soluble substances were first observed by Polich and Lees (1951) and recent work (Schrade and others, 1954) has led to the isolation of thirteen individual lipopeptides from blood.
plasma, employing two-dimensional paper chromatography. It may be expected
that both the EP and methylated EP derivatives of lipopeptides would be
soluble in light petroleum and a study of absorption spectra in this sol-
vent together with degradation studies, may be a valuable approach to
knowledge of the structure of protein-lipid complexes.
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