

A STUDY OF PHOSPHOLIPIDS OF MARINE ORIGIN

A. J. de Koning

(Ir. Delft)

Fishing Industry Research Institute,
University of Cape Town,
Rondebosch, Cape Town, South Africa.

Thesis submitted for the degree of Doctor of
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CHAPTER IV. BIOCHEMISTRY OF THE PHOSPHOLIPIDS

Function and occurrence	42
Biosynthesis	43

PART II : INVESTIGATIONS

<u>SUMMARY</u>	47
----------------	----

<u>CHAPTER V. IDENTIFICATION OF AN ARTIFACT IN THE HYDROLYSIS OF CHOLINE-CONTAINING PHOSPHOLIPIDS</u>	50
---	----

<u>CHAPTER VI. A SENSITIVE METHOD FOR THE MICRO- DETERMINATION OF CHOLINE</u>	55
---	----

Calibration Curves	55 - 60
Determination of Choline in a phospholipid	58, 62
Interference by compounds forming insoluble phosphomolybdates	61
Interference by ammonia	62

<u>CHAPTER VII. THE PRESENCE OF AMINO ACIDS IN PHOSPHOLIPIDS</u>	64
--	----

Evidence for polypeptide impurity	65 and Fig. 1
-----------------------------------	---------------

<u>CHAPTER VIII. PHOSPHOLIPIDS OF THE HAKE</u>	68
--	----

Extraction and purification of the phospholipids	68
Determination of the phospholipid composition	
1. by hydrolysis	71 - 78
2. by chromatography on silicic acid impregnated paper	79 - 82
3. by column chromatography on silicic acid	79, 83, 84
Results and Discussion	80 - 85
Stabilisation of a supersaturated cholesterol solution	78, 86

<u>CHAPTER IX. PHOSPHOLIPIDS OF THE ROCK LOBSTER</u>	90
--	----

Total lipids and phospholipids in various tissues	Table I, 91
Phospholipid composition	93 - 95
Hemolytic effect of lysophosphatidyl choline on human blood	95

**CHAPTER X. PHOSPHOLIPIDS OF THE PILCHARD WITH PARTICULAR
REFERENCE TO THEIR OXIDATION IN PILCHARD MEAL
MANUFACTURE**

98

Experimental

99

Oxidation of the lipids in pilchard meal

101 - 104

Phospholipid composition of the pilchard

104 - 108

Phospholipid composition of marine products

Table V, 106

CHAPTER XI. ESTIMATION OF SPHINGOMYELIN

109

**CHAPTER XII. HYDROLYSIS OF PHOSPHOLIPIDS WITH
HYDROCHLORIC ACID**

The investigation of the progress of the hydrolysis
of DL- α -lecithin, DL- α -cephalin and sphingo-
myelin with 2 N HCl at 120°.

Method

113

Results

117

CHAPTER XIII. STRUCTURE OF COMPLEX PHOSPHOLIPIDS

124

ACKNOWLEDGEMENTS

127

PUBLICATIONS

128

INTRODUCTION

SCOPE

This thesis deals with phospholipids of marine origin. Non-phosphorylated lipids will only be discussed in a few instances, mainly for reasons of comparison. The phospholipids are of marine origin since the work was carried out while the writer was employed at the Fishing Industry Research Institute, University of Cape Town, Rondebosch, Cape Town, South Africa.

The phospholipids of the following marine products were investigated:

1. Hake (*Merluccius capensis*, Castelnau)
2. Rock Lobster (*Jasus lalandii*)
3. Pilchard (*Sardina ocellata*, Jenyns)

The qualitative and quantitative composition of these phospholipids was determined by a hydrolytic cleavage procedure and by chromatographic techniques. Particular emphasis will be placed on the detection and the determination of the hydrolytic cleavage products and a reaction mechanism will be suggested for the hydrolysis of phospholipids with hydrochloric acid. Evidence is produced that the presence of all amino acids - except serine - found in phospholipid hydrolysates was due to lipo-soluble polypeptide impurities. The oxidation of phospholipids and non-phosphorylated lipids in the manufacture of fish meal has been examined.

LAYOUT

The thesis will be divided into short chapters with literature references at the end of each chapter. Repetition of certain references is therefore unavoidable, but the advantage is that each chapter may be read as an independent section.

NOMENCLATURE

Wherever possible the modern method of naming phospholipids will be followed. In certain instances the use of the older nomenclature could not be avoided, e.g. the word lecithin-choline is to be preferred to phosphatidyl choline-choline. In the following table the names and synonyms which are used in this thesis are listed.

TABLE I

NAMES AND SYNONYMS USED IN THIS THESIS

Phospholipids	phosphatides
phosphatidyl choline	lecithin
phosphatidyl ethanolamine)	cephalin
phosphatidyl serine	
phosphatidyl inositol	mono phosphoinositide
phosphatidyl glycerol	
diphosphoinositide	
triphosphoinositide	
sphingomyelin	
lyso phosphatidyl choline	lyso lecithin
lyso phosphatidyl ethanolamine	
plasmalogens	acetal phospholipids
phosphatidic acids	monoglycerophosphatidic acid
bis-glycerophosphatidic acids	acylated phosphatidyl glycerol
cardiolipin	
polyglycerophosphatide)	diphosphatidyl glycerol
non-phosphorylated lipids	neutral lipids
triglycerides	
cholesterol esters	cholesteryl esters
cerebrosides	
phosphorylcholine	phosphocholine
phosphorylethanolamine	O-phosphoethanolamine
glycerophosphate	glycerophosphoric acid
inositol phosphate	
glycerylphosphorylcholine	glycerophosphorylcholine
glycerylphosphorylethanolamine	glycerophosphorylethanolamine
glycerylphosphorylserine	glycerophosphorylserine
glycerylphosphorylinositol	glycerophosphorylinositol
sphingosylphosphorylcholine	

SUMMARY

A STUDY OF PHOSPHOLIPIDS OF MARINE ORIGIN

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PART I - LITERATURE STUDY

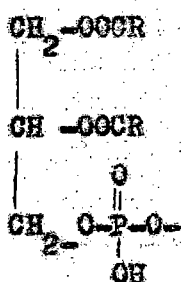
1. The chemistry of phosphatidyl choline, phosphatidyl ethanolamine, phosphatidyl serine, phosphatidyl inositol, lyso-phosphatides, plasmalogens and sphingomyelins is discussed.
2. Procedures for the extraction, purification and separation of phospholipids are described.
3. Methods for the analysis of the following phospholipid constituents are discussed: phosphorus, nitrogen, choline, ethanolamine, serine, myo-inositol, sphingosine, glycerol, glycerophosphate and fatty acids.
4. A brief description of the function, occurrence and biosynthesis of the phospholipids is presented.

PART II - INVESTIGATIONS

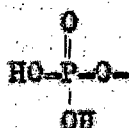
1. 2-Chlorocholine was identified as an artifact in the hydrolysis of choline-containing phospholipids with HCl. This substance is in all probability identical to an unknown nitrogenous base described by other workers.
2. A sensitive method for the microdetermination of choline (0.1 - 3 μ moles) was developed. Choline is precipitated as the insoluble phosphomolybdate, the excess phosphomolybdic acid is carefully removed and phosphorus determined spectrophotometrically in the precipitate by reduction to molybdenum blue. Compounds capable of forming insoluble phosphomolybdates interfere. Interference of ammonia can be prevented by the addition of formaldehyde.
3. It is shown that the presence of all amino acids - except serine - in a phospholipid hydrolysate was due to the presence of polypeptide impurities. The presence of many amino acids detected by other workers in phospholipid hydrolysates can probably be explained in the same way.
4. The composition of the hake flesh and hake liver phospholipids was determined by an hydrolytic procedure and by chromatography on silicic acid. The hydrolytic procedure involved determination of lecithin-choline, sphingomyelin-choline, lyso lecithin-choline, ethanolamine, serine, myo-inositol and the average equivalent weight of the liberated fatty acids. The composition of the flesh and liver phospholipids was very similar, the latter contained more sphingomyelins and cardiolipins at the expense of phosphatidyl choline. Phospholipid fatty acids had a markedly higher equivalent weight and unsaturation than the corresponding non-phosphorylated lipid fatty acids. The cephalin fractions contained more long chain polyunsaturated (C_{22} and higher) fatty acids, more stearic acid ($C_{18:0}$) and less palmitic acid ($C_{16:0}$) than the phosphatidyl choline fractions. Sphingomyelins were particularly rich in lignoceric ($C_{24:0}$) and nervonic ($C_{24:1}$) acid.

7. The progress of the oxidation of phospholipids and non-phosphorylated lipids in the manufacturing process of pilchard meal was examined. It was shown that pilchard phospholipids were oxidized to a certain extent in the drying process, but a severe destruction of phospholipid and non-phosphorylated lipid fatty acids took place on storage of the meal at room temperature. Phospholipid oxidation could be determined by the drop in average unsaturation of the fatty acids, but equally well by the drop in free amino nitrogen.
8. Small quantities (± 3 mg) of phosphatidyl choline fractions were capable of stabilizing a supersaturated solution of cholesterol in a triglyceride oil.
9. A new procedure for the estimation of sphingomyelin was developed by determining sphingomyelin-choline.
10. The progress of the hydrolysis of DL- α -lecithin, DL- α -cephalin and sphingomyelin with 2 N HCl at 120° was investigated. The hydrolytic products: glycerophosphate, phosphorylcholine, phosphorylethanolamine and inorganic phosphate were determined at intervals. Hydrolysis of DL- α -lecithin and DL- α -cephalin proceeds similarly. Initially no inorganic phosphate is liberated and bond fission takes place in such a manner that 84% of the phosphorus remains esterified to the glycerol moiety (glycerophosphate) and 16% to the choline moiety (phosphorylcholine). In the hydrolysis of sphingomyelin inorganic phosphate is also not formed initially, but phosphorus remains esterified predominantly, if not exclusively, to the choline moiety. From the data obtained a reaction mechanism is suggested for the acid hydrolysis of the abovementioned substances.
11. The structure of "Complex" phospholipids as described in the literature is challenged. A pyrophosphate linkage instead of a phosphate triester group is postulated to be present in these compounds.

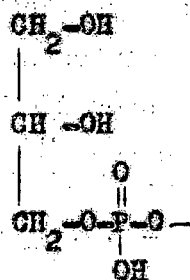
It should be noted that the name phosphatidyl has a specific chemical meaning, and should not be confused with phosphoryl. Structures are shown below.



phosphatidyl



phosphoryl



glycerylphosphoryl

HISTORY

Early work on the lipids of South African marine products was carried out at the University of Cape Town by Rapson, Schwartz and others. This work, which is published in 29 papers of the Journal of the Society of Chemical Industry (see e.g. Karnovsky, Lategan, Rapson and Schwartz¹) and 4 papers in the Journal of The Science of Food and Agriculture (see e.g. Ligthelm, Novellie, Schwartz and von Holdt²), deals mainly with the oil yields and seasonal variations in the different kinds of fish. Interest was focussed particularly on the Vitamin A and D content of the liver oils. No analyses of the phospholipids were reported. The phosphorus content of snook (*Thyrsites atun*, Euphr.) liver and intestinal oils³ and rock lobster (*Jasus lalandii*) gonad oils² were found to be extremely high, indicating a rich source of phospholipids. The fatty acid composition of the lipids was determined by the Hilditch methyl ester fractionation technique⁴.

Subsequent work on the analysis of South African fish oils was carried out in the laboratories of the Council for Scientific and Industrial Research, Pretoria, by Sutton, Silk, Sephton and others. These investigations were of a more fundamental nature and precise fractionation of pilchard (*Sardina ocellata*, Jernyns) oil fatty acids was accomplished by a sequence of lithium soap - acetone separation, urea complex fractionation, molecular distillation and reversed phase partition chromatography. A summary of this work appeared in "Essential fatty acids"⁵.

In 1962 investigations on the phospholipids of marine origin were started at the Fishing Industry Research Institute as a result of the studies by Wilkens and Krut⁶ which show that human serum phospholipids, in particular the lecithin fraction, are capable of stabilising a supersaturated solution of cholesterol in a triglyceride oil. This characteristic of lecithins may be of importance in the study of ischaemic heart disease.

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PART I

LITERATURE STUDY.

University of Cape Town

CHAPTER I

PHOSPHOLIPID CHEMISTRY

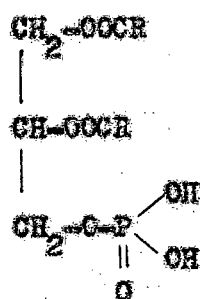
Phospholipids can be broadly divided into glycerophosphatides and sphingolipids. The first class of compounds contains glycerol, the second class contains sphingosine as the common unit.

1. GLYCEROPHOSPHATIDES

In contrast to the triglycerides, which contain three fatty acids esterified with glycerol, the glycerophosphatides possess two fatty acids and one phosphoric acid molecule esterified with glycerol.

1.1 GLYCEROPHOSPHATIDIC ACIDS

The MONO-PHOSPHATIDIC ACIDS (I) have been extracted from



I

MONO- α -GLYCEROPHOSPHATIDIC ACID

animal and plant tissues:

e.g. Hübcher and Clark¹

isolated these lipids from

animal livers and Hanahan

and Chaikoff² from cabbage

leaves. The last mentioned

authors have suggested that

these compounds may be arti-

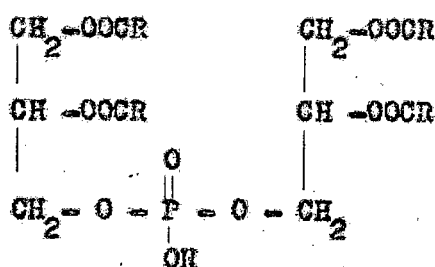
facts due to the presence of

an enzyme which is capable of

splitting choline from phosphatidyl choline. This enzyme, which is phospholipase D (phosphatidyl-choline phosphatido hydrolase), is also capable of splitting ethanolamine and serine from phosphatidyl ethanolamine and phosphatidyl serine respectively³.

Destroying the enzyme by treatment of the fresh plant tissues with steam before extraction of the phosphatides greatly reduced the amount of phosphatidic acid². All naturally occurring phosphatidic acids probably possess the L- α -structure. This statement will be exemplified in the discussion of phosphatidyl choline. The synthesis of α and β phosphatidic acids has been accomplished by treatment of the appropriate α , β and α , β -diglyceride with diphenyl chlorophosphate $((\text{PhO})_2\text{POCl})$ in the presence of pyridine. Catalytic hydrogenation over a Pt/active C catalyst removes the phenyl groups in the form of cyclohexane. This type of synthesis has been carried out by Daer⁴ and independently by Uhlenbroek and Verkade⁵.

BIS- α -GLYCEROPHOSPHATIDIC ACIDS (II) have been prepared



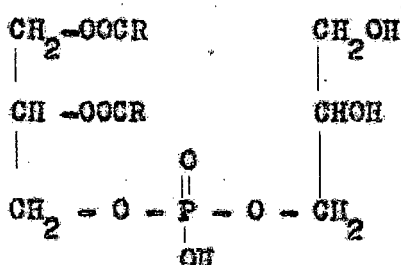
II

BIS- α -GLYCEROPHOSPHATIDIC ACID

by Baer⁶ and bis- β -glycerophosphatidic acids by van Gyzen, Hoefnagel and Verkade⁷ in essentially the same way, i.e. by treatment of the appropriate diglyceride with phenyl dichlorophosphate (PhO.POCl_2) in the presence of pyridine followed by catalytic removal of the phenyl group. These com-

pounds have, at least to our knowledge, not been isolated from natural products.

Bis- α -glycerophosphatidic acids can be considered as

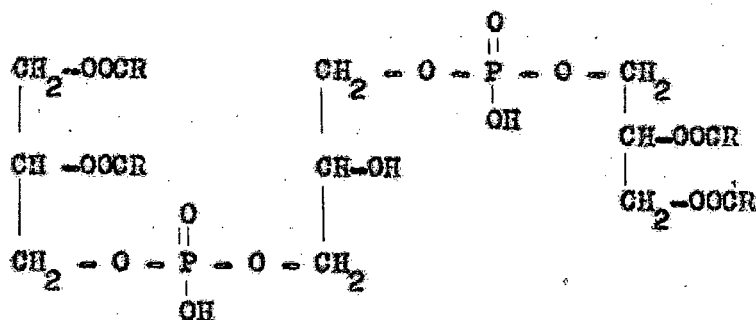


acylated derivatives of phosphatidyl glycerol (III). The latter substance has been isolated from bacteria and spinach leaves⁸.

III

α -PHOSPHATIDYL GLYCEROL

CARDIOLIPIN (IV) - a complex phosphatidic acid which is probably a diphosphatidyl glycerol - was isolated by Pangborn⁹ from ox-heart muscle and shown to be the active constituent in the Wassermann reaction of syphilitic serum. Different kinds of structures have been suggested for this substance by Pangborn¹⁰, Macfarlane and Gray¹¹ and also Faure and Morelec-Coulon¹². The latest structure proposed by the last two teams of investigators is shown below (IV).

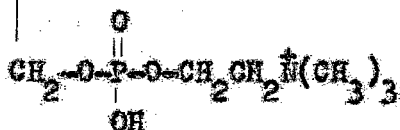


IV

CARDIOLIPIN

Phosphatidyl inositol and phosphatidyl serine are also acidic but are not classified under phosphatidic acid

1.2 PHOSPHATIDYL CHOLINE



V

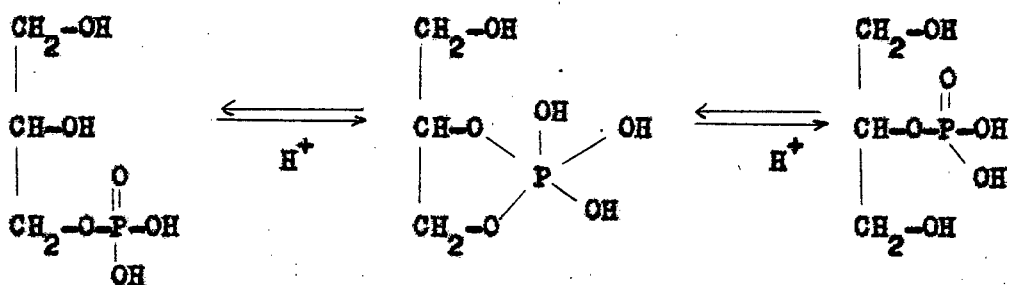
VI

α -PHOSPHATIDYL CHOLINE

CHOLINE

PHOSPHATIDYL CHOLINE or lecithin (V) can be considered as the choline ester of a mono-phosphatidic acid. Choline is an alcohol and a quaternary ammonium compound (VI). It should be noted that choline is linked in ester formation to the phosphatidic acid; reaction in vitro of a phosphatidic acid with choline results in the formation of a salt instead of an ester. Phosphatidyl choline, having an acid and a basic centre is - for all purposes - neutral. In solution phosphatidyl choline adopts a "Zwitter" ion structure, similar to the amino acids.

Several isomers of phosphatidyl choline may be envisaged. In the first place α - and β -compounds depending on whether the phosphorylcholine group is linked to the α - or the β -carbon atom of glycerol. In the second place two optical isomers in the α -series due to the presence of the central asymmetric carbon atom. (In order to facilitate the discussion the two fatty acid moieties are considered to be identical.) The early literature contains many references to the presence of α - and β -phosphatidyl choline¹³. This evidence is largely based on the finding of both α - and β -glycerophosphate (VII and VIII) in acid and alkaline hydrolysates of naturally occurring phosphatides. It was, however, shown by Bailly¹⁴ and by Verkade, Stoppelenburg and Cohen¹⁵ that α - and β -glycerophosphate are interconvertible in acid medium. In hot acid medium an equilibrium mixture of 85% α - and 15% β -glycerophosphate is obtained.



VII

α -GLYCEROPHOSPHATE

VIII

β -GLYCEROPHOSPHATE

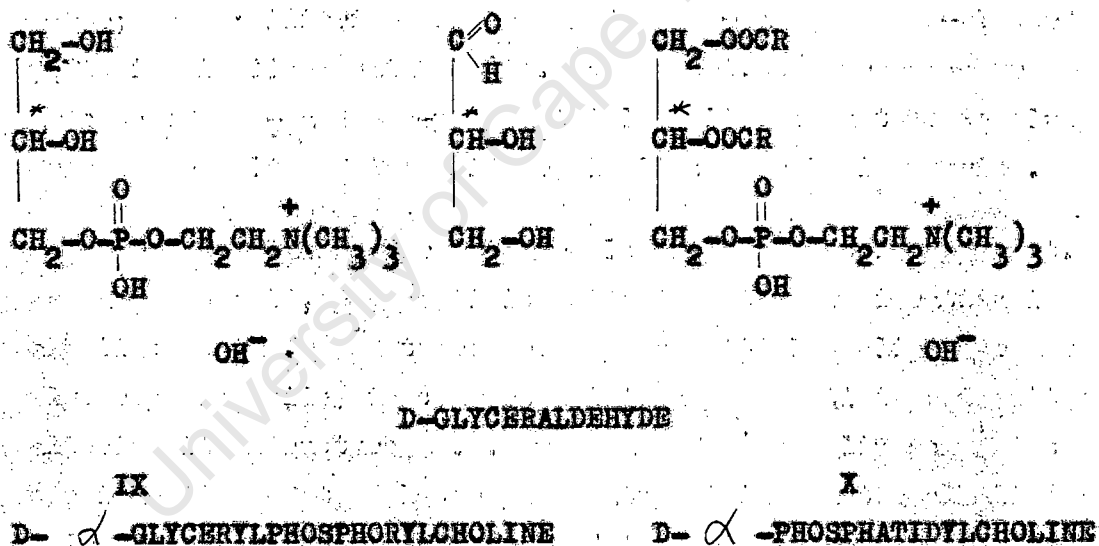
The conversion takes place with a cyclic phosphate diester as an intermediate substance.

In alkaline medium the situation is different, in that the monoesters of phosphoric acid, e.g. α - and β -glycerophosphate¹⁵ and α - and β -glycerophosphatidic acid⁴ do not show any migration. However, when subjecting diesters of phosphoric acid, e.g. phosphatidyl choline, phosphatidyl ethanolamine or glycerylphosphorylcholine to alkaline hydrolysis migration does occur; an equilibrium mixture of 45% α - and 55% β -glycerophosphate is finally obtained^{16,17}. In short, the presence or absence of α - and β -phosphatidyl choline cannot be concluded from the results of the acid or alkaline hydrolysis studies.

A solution for this problem is immediately apparent. As has been said, phosphatidic acids do not show migration in alkaline medium. If therefore a phosphatidyl choline (or any glycerophosphatide) is converted into a phosphatidic acid without altering its structure (e.g. by means of phospholipase D) the configuration of the glycerophosphate obtained after alkaline hydrolysis is identical to that of the original phosphatidyl choline. This principle has been applied by Long and Maguire¹⁸ to egg phosphatidyl choline, which was shown to possess the L- α -configuration. Similarly, Haverkate and van Deenen⁸⁰ demonstrated that naturally occurring phosphatidyl glycerol (III) belongs to the L- α -series.

A natural phosphatidyl choline examined by Baer and Kates¹⁹ was identical (elementary composition, melting point, solubility, X-ray diffraction pattern and optical rotation) to a synthetic L- α -phosphatidyl choline. In addition, Long and Maguire¹⁶ have shown the L- α -configuration of ox-heart and soyabean lecithin and ox-brain cephalin. It appears therefore that natural phosphatides belong predominantly to the L- α -series. Conclusive evidence for the absence of D- α or β -phosphatides is however still lacking (cf. Verkade²⁰).

It seems desirable to add a short discussion to the stereochemistry of the phosphatides. Baer and Kates¹⁹ have suggested as a convention to use D- α -glycerylphosphorylcholine as the reference compound for the stereochemical classification of the phosphatides. D- α -glycerylphosphorylcholine is related to D-glyceraldehyde in the manner shown below; that is the carbon atom not linked to the phosphorylcholine moiety serves as the potential carbonyl group (see also Fisher and Baer²¹).

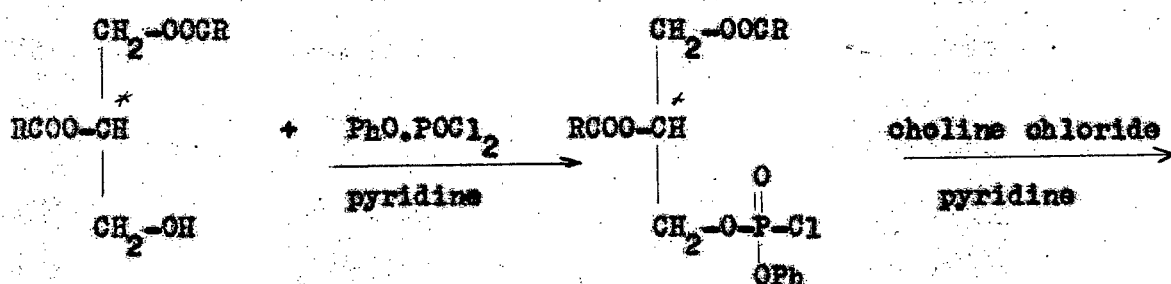


The opposite classification is reached if the corresponding D- α , β -diglyceride is taken as the reference compound, since in the classification of diglycerides the carbon atom with the free hydroxyl group - i.e. corresponding to the carbon atom linked to the phosphorylcholine moiety - serves as the potential carbonyl group²¹. This is clearly demonstrated in the synthesis of L- α -phosphatidyl choline by Baer and Kates¹⁹, where the starting material is D- α , β -diglyceride (XI) (see below).

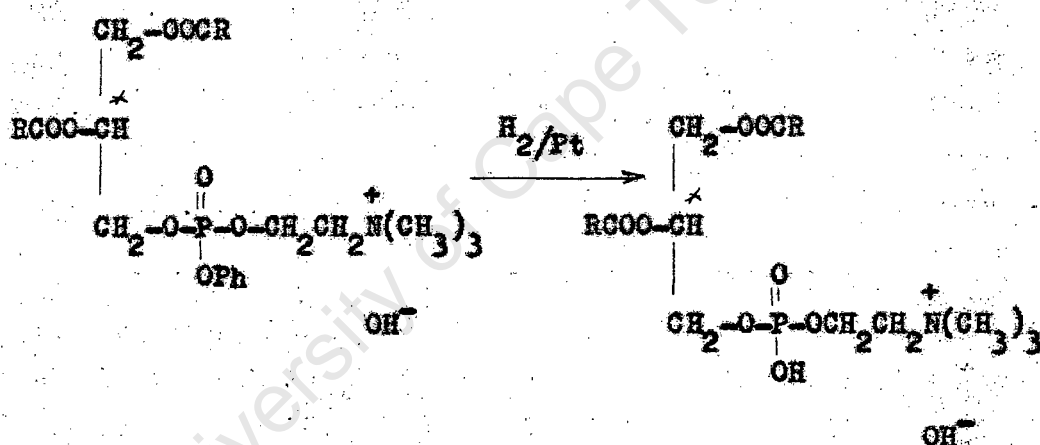
SYNTHESIS

Only two typical methods of lecithin synthesis will be given, for an exhaustive literature survey of phosphatide synthesis see Verkade²⁰.

Beer and Kates¹⁹ synthesized an L- α -phosphatidyl choline in the following manner:



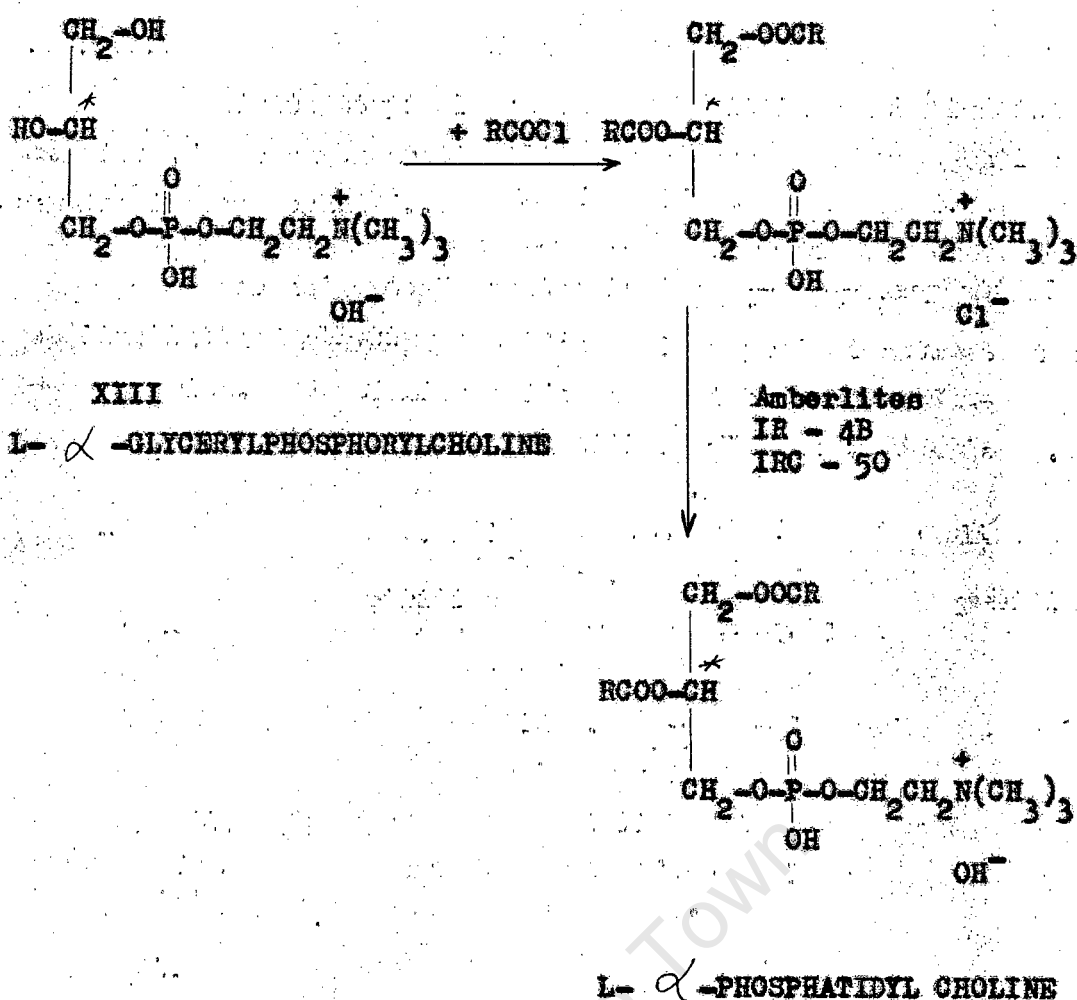
XI
D- α , β -DIGLYCERIDE



XII

L- α -PHOSPHATIDYL CHOLINE

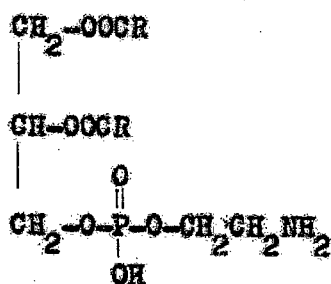
A D- α , β -diglyceride was treated with one equivalent of phenyl dichlorophosphate in the presence of pyridine. The reaction product was treated with choline chloride again in the presence of pyridine and the phenyl group removed by catalytic hydrogenation. (The laborious purification procedure with ammonium reineckate etc. has been omitted for the sake of clarity.) This synthesis has the disadvantage that inevitably a certain amount of bis- α -phosphatidic acid is formed as by-product (see under phosphatidic acids) and also the method is not applicable to the preparation of compounds containing unsaturated fatty acids. Kogl, de Haas and van Deenen²² used L- α -glycerylphosphoryl-choline (XIII) as the starting compound for the synthesis of



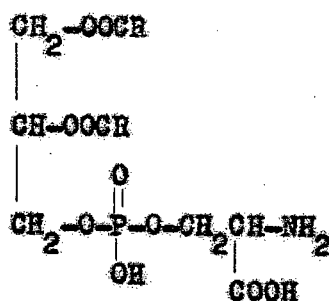
The first step, which is obvious, involves the reaction with an acyl chloride. The second step, i.e. treatment with a mixed cation-anion exchange resin is necessary in order to remove ionic by-products. Lyso phosphatidyl choline, which is also formed in the reaction, is removed by chromatography on silicic acid.

1.3 PHOSPHATIDYL ETHANOLAMINE AND PHOSPHATIDYL SERINE

PHOSPHATIDYL ETHANOLAMINE (XIV) contains the amino alcohol ethanolamine (colamine), and PHOSPHATIDYL SERINE (XV) the amino acid serine as the nitrogenous component. These two compounds are closely related.



XIV
PHOSPHATIDYL ETHANOLAMINE



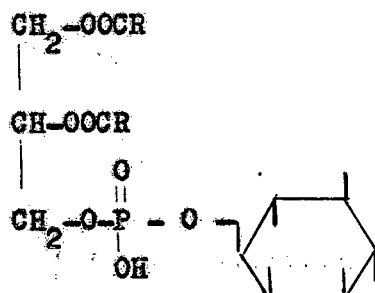
XV
PHOSPHATIDYL SERINE

In the early phospholipid work the cephalins were defined as the alcohol insoluble fraction of the phospholipids in contrast to the lecithins which are soluble in alcohol. Originally ethanolamine was considered to be the only base of the cephalins; Folch and Schneider²³, however, demonstrated the presence of L-serine in the cephalins. Recently, many workers have described the presence of a large variety of amino acids in phospholipids (not the cephalin fraction in particular) but these compounds are probably lipo-soluble impurities in many instances²⁴. The biological relationship between ethanolamine and choline has been demonstrated by Stetten²⁵. Methylation by for instance methionine converts ethanolamine into choline. L-serine acts as a precursor of ethanolamine and can be converted into the latter by decarboxylation²⁶.

The stereochemistry of phosphatidyl ethanolamine and phosphatidyl serine is similar to that of phosphatidyl choline. The presence of two asymmetric carbon atoms in phosphatidyl serine leads to the possibility of diastereoisomerism (two sets of optical isomers). Long and Maguire¹⁶ proved the L- α -configuration of an ox-brain cephalin, consisting mainly of phosphatidyl ethanolamine and phosphatidyl serine. Several methods for the synthesis of phosphatidyl ethanolamine and phosphatidyl serine are known in the literature (see e.g. Verkade²⁰).

1.4 PHOSPHATIDYL INOSITOL

PHOSPHATIDYL INOSITOL contains myo-inositol esterified with the phosphoric acid molecule.



XVI

PHOSPHATIDYL INOSITOL

Myo-inositol was first isolated from bacterial phospholipids by Anderson²⁷. Subsequently myo-inositol was discovered in phospholipids from many other sources. Inositol (hexahydroxycyclohexane) occurs in 8 isomeric forms, one of which is asymmetric and consists therefore of two optical isomers. The abbreviated structures of all possible isomers are shown below. The planar carbon ring is usually drawn for the sake of convenience, but it should be realised that the structure of inositol is better represented by a non-planar ring, consisting of 2 conformations - chair and boat - which are readily interconvertible (cf. Angyal and Anderson²⁸).

Structures of inositol

Each number denotes an hydroxyl group, above or below the planar carbon ring.

<u>123456</u>	<u>12345</u> 6	<u>1234</u> 56	<u>123</u> 456	
cis	epi	allo	neo	
<u>1235</u> 46	<u>1245</u> 36	<u>123</u> 456	<u>124</u> 356	<u>134</u> 256
myo	muco	scyllo	dextro(+)	levo(-)

Myo-inositol is the only isomer which has so far been found naturally in combination with phosphate²⁸. In the present work on fish phospholipids only myo-inositol was isolated. The brilliant studies of Ballou and collaborators²⁹ have shown that in phosphatidyl inositol of natural origin the phosphate group is linked to carbon atom 1 of myo-inositol. This has subsequently been confirmed by Hawthorne, Kemp and Ellis³⁰, although these authors had originally suggested that inositol was esterified in position 2 to the phosphate group³⁰.

A brief discussion of the work of Ballou follows. For a complete understanding of this work the following must be mentioned. Firstly, myo-inositol 1-phosphate and 3-phosphate are enantiomers, the same applies for the 4- and 6-phosphate. Myo-inositol 2- and 5-phosphate are optically inactive. Secondly, hydrolysis in acid or alkaline medium of inositol phosphates may result in phosphate migration. This migration takes place via a 5-numbered ring phosphate diester, and only proceeds with *cis*-hydroxyl groups. Thus myo-inositol 2-phosphate is converted to a mixture of (+)-myo-inositol 1-phosphate and myo-inositol 2-phosphate²⁹. Ballou et al²⁹ obtained by weak alkaline hydrolysis of phosphatidyl inositol an optically active myo-inositol phosphate and a small quantity of myo-inositol 2-phosphate. The latter substance was known from a synthesis by Iselin³¹. The optically active substance could not have originated from 2-phosphatidyl myo-inositol or 5-phosphatidyl myo-inositol even migration during hydrolysis can not produce optical activity. Therefore, the inositol phosphate obtained originated either from a 1 (3) or a 4 (6) - linkage. There were two reasons for preferring the 1 (3) - position. In the first place a small quantity of myo-inositol 2-phosphate was identified in the hydrolysate besides the optically active phosphate. In the second place the periodate oxidation rate of the optically active phosphate indicated the absence of three adjacent *cis*-hydroxyl groups. Ballou and Fizer²⁹ subsequently synthesized the optically active myo-inositol 3-phosphate - details of this synthesis are irrelevant here - which showed equal but opposite rotation to the unknown myo-inositol phosphate. The unknown substance was therefore myo-inositol 1-phosphate. The last authors suggested - as a convention - to name this last substance 1-myo-inositol 1-phosphate. The configuration on the asymmetric carbon atom of glycerol is by analogy L, the complete name of natural phosphatidyl inositol is therefore (1- α -phosphatidyl)-1-1-myo-inositol.

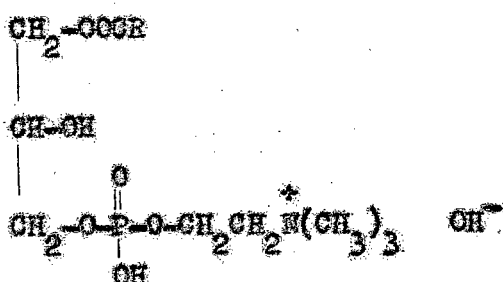
Phosphoinositides are lipids which are still inadequately characterised. Apart from the simple phosphatidyl inositol containing one phosphorus atom there are others containing 2 or even 3 phosphorus atoms. These compounds possess extra phosphoric acid moieties esterified with myo-inositol. Diphospho- and triphospho-inositides have been isolated from ox-brain³²; the acidic nature of these substances makes their separation from protein very difficult. The structures have been elucidated by Brockerhof and Ballou³³ i.e. 1-phosphatidyl L-myo-inositol 4-phosphate and 1-phosphatidyl L-myo-inositol 4,5-diphosphate.

Mycobacterial phospholipids, which have been examined by many workers (e.g. Lederer³⁴), consist largely of the phosphatidyl inositol type. In these compounds mannose, linked glycosidically to the myo-inositol, is also present. For instance, phosphatidyl-myo-inositol mannoside, -dimannoside and -pentamannoside have been isolated from *M. tuberculosis*^{34b}. Recently amino acid-containing derivatives of phosphatidyl glycerol have been isolated from several bacteria⁷. Phospholipids containing inositol as well as galactose, glucose, mannose and arabinose have been isolated from soyabeans³⁵. Davies and Malkin³⁶ have synthesized a 2-phosphatidyl myo-inositol by reaction of diacetoxy- α -iodohydrin with the silver salt of 2-(1,3,4,5,6-penta-O-acetyl) myo-inositol phenyl phosphate. The protective groups were removed by hydrogenation and ethanolic hydrazine.

Finally, it should be mentioned that the inositol phospholipids have been reviewed by Hawthorne³⁷.

1.5 LYSO-PHOSPHATIDES

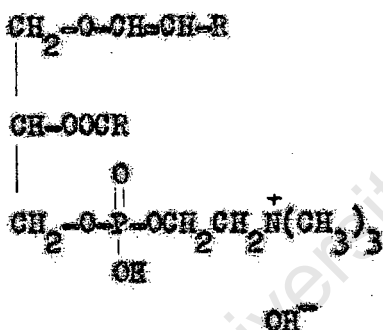
These compounds are phospholipids minus one fatty acid and are produced by the action of an enzyme on the



phospholipid. This particular enzyme, phospholipase A (phosphatide acyl-hydrolase), which is present in snake venom, selectively removes a fatty acid from the β - position of phosphatidyl choline and phosphatidyl

Unsaturated fatty acids show a clear preference for the β -position and therefore an unsaturated fatty acid is frequently liberated with the simultaneous formation of a saturated α -acyl lyso phosphatide^{38,39}. Phospholipase A is also capable of removing a saturated fatty acid from fully saturated phosphatidyl choline⁴⁰. (Some confusion exists in the older literature as to the site of attack of phospholipase A, since Hanahan⁴¹ has shown that this enzyme specifically hydrolyses the α -ester linkage. Acyl migration during his experiments probably caused this error.) Brockerhof, Ackman and Hoyle³⁹ have shown with the aid of phospholipase A that the polyunsaturated fatty acids in the phosphatidyl choline fraction of certain marine phospholipids are located in the β -position. Lyso phosphatidyl choline and lyso phosphatidyl ethanolamine have a strong hemolytic effect on blood (they destroy the cell structure). The effect of a lyso phosphatidyl choline extracted from rock lobster on human blood will be demonstrated in this thesis.

1.6 PLASMALOGENS (ACETAL PHOSPHATIDES)



XVIII

CHOLINE-PLASMALOGEN

These compounds possess a long chain aldehyde in place of a fatty acid. The plasmalogens first examined contained ethanolamine as nitrogenous constituent⁴², but plasmalogens containing choline⁴³ and serine⁴⁴ have also been detected. Originally it was suggested that the long chain aldehyde was attached

in acetal linkage to the α - and β -hydroxyl group of glycerol (i.e. a 5 membered ring and no fatty acid present)^{42,45}.

Recent work has shown that this acetal structure is an artifact produced during the isolation, which involved treatment with alkali. Two long aliphatic chains are present, one a fatty acid in ester formation, the other a "fatty" aldehyde which is probably linked as a vinyl ether to glycerol⁴³. A hemi acetal structure (i.e. hydrated structure XVIII) has also been suggested, but evidence for the vinyl ether is very strong⁴³.

The position of the aldehyde moiety is a matter of controversy. Marinetti, Erbland and Stotz⁴³ have shown that the aldehyde is predominantly linked in the α -position in pig heart and beef heart plasmalogens. These authors isolated in excellent yields long chain α -glyceryl ethers after hydrogenation of the vinyl ether linkage and removal of the fatty acid and phosphorylcholine moiety. As pointed out by these workers migration of an alkoxy group in a saturated glyceryl ether is extremely unlikely, in fact no migration was observed when an α -glyceryl ether was subjected to identical treatment. Their work is in accordance with that of Franzl and Rapport⁴⁶ who showed that phospholipase A hydrolyses natural choline plasmalogen, indicating that the ester linkage is situated on the β -carbon atom. (The conclusion reached by Franzl and Rapport was, however, the opposite. These investigators were of the opinion that phospholipase A removed fatty acids specifically from the α -position, which was directly due to the error committed by Hanahan⁴¹ as described in the previous section.) The investigations of Klenk and Debusch⁴⁷ also indicate that the aldehyde group is linked in the α -position to the glycerol moiety.

Gray⁴⁸ removed the fatty aldehyde from beef heart plasmalogens by treatment with 90% acetic acid and identified the resulting lyso phosphatide as the α -isomer. He concluded, therefore, that the fatty aldehyde in beef heart plasmalogen is linked to the β -carbon atom of the glycerol moiety, which is in contrast to the results of Marinetti, Erbland and Stotz⁴³. The conclusion of Gray⁴⁸ is undoubtedly incorrect since it is well established that in acid medium an acyl group migrates from the β - to the α -position in glycerol derivatives (see van Lohuizen and Verkade⁴⁹). The viewpoint that a species difference may explain this discrepancy has been discarded⁴³.

The other linkage in plasmalogens decomposes rapidly in acid solution to form the free aldehyde. The ester linkage is easily hydrolysed in alkaline medium and the artifact acetal phosphatide is formed, which was originally mistaken for the plasmalogen structure. A rich source of plasmalogens is ox and pig heart muscle⁴³. The marine products so far investigated contained very little plasmalogen^{50,51} (2-3% of total phospholipids). The fatty aldehydes derived from pig,

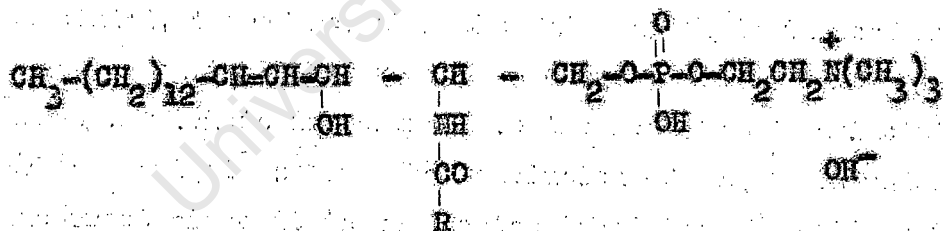
pigeon and trout flesh plasmalogens have been investigated by Gray and Macfarlane⁵¹. They consisted largely of palmitaldehyde, stearaldehyde and small quantities of unsaturated and branched chain aldehydes.

Plasmalogens are closely related to the glyceryl ether phospholipids which have only recently been discovered (cf. Hanahan and Thompson⁵²). The ether linkage in these substances is not of the vinyl-type and treatment with weak acid does not liberate an aldehyde. The ether radical is mostly derived from C₁₆ and C₁₈ alcohols; the glyceryl ethers have special names e.g. batyl alcohol which is D-glyceryl-1-octadecyl ether. Phosphatidic acids containing a batyl alcohol unit have been synthesized by Stegerhook and Verkade⁵³. The corresponding glyceryl ether diglycerides (alkoxydiglycerides) occur abundantly in the depot lipids of some fish species (Lovern⁵⁴).

2. SPHINGOLIPIDS

2.1 SPHINGOMYELIN

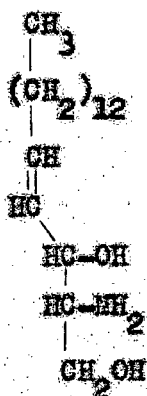
SPHINGOMYELIN contains sphingosine as the basic unit in the place of glycerol. The structure of sphingomyelin is shown below (XIX)



XIX

SPHINGOMYELIN

Sphingosine - 1,3-dihydroxy-2-amino-4-octadecene - has two asymmetric carbon atoms and a double bond, which implies the possibility of 8 isomers. Stereochemical studies have shown that natural sphingosine has the trans-D-erythro configuration (see e.g. Fujino and Carter⁵⁵). Shapiro and Segal⁵⁶ and also Grob⁵⁷ have synthesized trans-D,L-erythro-sphingosine, which was identical to the natural substance (apart from its rotation).



Dihydrosphingosine has also been found in small quantities in animal tissues⁵⁸. Prostenik and Majhofer-Drescanin⁵⁹ have recently shown the presence of a C₂₀-sphingosine (i.e. 1,3-dihydroxy-2-amino-4-oicosene) in horse and beef brain.

XX SPHINGOSINE

Sphingomyelin possesses like lecithin the phosphorylcholine moiety. A significant difference, however, between the phosphorylcholine group in sphingomyelin and lecithin is its behaviour towards hydrolysis in acid and alkaline medium. This subject will be fully amplified in Chapter XII, and it is sufficient to mention here that lecithin readily liberates choline, whereas sphingomyelin forms the extremely stable substance phosphorylcholine as an intermediate. The fact that sphingomyelin is much more resistant towards weak alkaline hydrolysis than lecithin has been utilised by e.g. Brante⁶⁰ to distinguish between lecithin-choline and sphingomyelin-choline.

Recently, the presence of the ethanolamine analogue of sphingomyelin (3.5% of the total phospholipids) has been indicated in the phospholipids of the housefly (*Musca domestica*⁶¹). Phospholipids of the housefly contain a large proportion of phosphatidyl ethanolamine (65%), and a smaller quantity of phosphatidyl choline (17%) and no (choline) sphingomyelin. The marine phospholipids investigated by us showed about 20% phosphatidyl ethanolamine, 60% phosphatidyl choline and 5% sphingomyelin. It seems therefore that ethanolamine takes the place of choline in the phospholipids of the housefly, when compared with the marine phospholipids. It is highly probable that small quantities of the ethanolamine sphingomyelin are also present in other phospholipids.

Rouser et al⁶² have recently isolated an unusual sphingolipid from the sea anemone (*Anthopleura elegantissima*) which contains aminoethylphosphonate ($\begin{array}{c} \text{H} \\ \parallel \\ -\text{O}-\text{P}-\text{CH}_2\text{CH}_2\text{NH}_2 \\ | \\ \text{OH} \end{array}$) instead of phosphorylcholine esterified to sphingosine.* Another unusual sphingolipid has been described by Landome and Bergmann⁶³ who isolated from the sponge (*Lissodendoryx isodactylalis*) an "acyl sphingomyelin phosphatidic acid". This is a sphingomyelin without the choline radical and acylated on carbon atom 3 of sphingosine.

The fatty acids in sphingomyelin are very rich in lignoceric ($\text{C}_{24}:0$) and nervonic ($\text{C}_{24}:1$) acid; these acids are almost completely absent in the glycerol phosphatides⁶⁴. This fatty acid pattern has also been found in the present investigation.

Finally, the total synthesis of sphingomyelin (D and L erythro) has recently been accomplished by Shapiro and Flowers⁶⁵.

2.2 CEREBROSIDES

CEREBROSIDES do not, strictly speaking, belong to the phospholipids, since they contain no phosphorus. They are, however, important sphingolipids and will therefore be briefly mentioned here. Cerebrosides are similar to sphingomyelin; instead of the phosphorylcholine moiety galactose (or sometimes glucose) is present combined as a glycoside to carbon atom 1.



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CHAPTER II

EXTRACTION, PURIFICATION AND SEPARATION OF PHOSPHOLIPIDS

1. EXTRACTION

Many solvents are available for the extraction of lipids from tissues. There is considerable evidence for the fact that phospholipids occur to a certain extent in the form of lipid-protein complexes in animal tissues and in the form of lipid-carbohydrate complexes in plant tissues^{1,2}. In order to ensure complete liberation of the phospholipids it is necessary to disrupt these complexes with a polar solvent^{1,2}. Non-polar solvents (e.g. light petroleum) are only suitable for the extraction of the neutral lipids. The most commonly used solvent for phospholipid extraction is a mixture of chloroform:methanol (2:1, by vol.). The extraction is done at room temperature (or lower if desired) and it is claimed that substantially all the lipids (apart from a certain amount of diphosphoinositide and triphosphoinositide) are extracted^{3,4}. This solvent mixture has the additional advantage that non-lipid contaminants may be easily removed by the gentle washing procedure of Folch et al³. A mixture of ethanol:ether (3:1 or 2:1, by vol. Bloer's mixture) is still used in some laboratories for lipid extraction⁵.

In our investigations all lipid extractions were done with chloroform:methanol (2:1, by vol.) at room temperature and in order to prevent oxidation of the highly unsaturated fatty acids solid carbon dioxide was added during the extraction.

2. PURIFICATION

All lipid extracts contain non-lipid impurities, which have to be removed. These impurities include polypeptides, amino acids, salts, sugars, etc.^{1,6}. The washing procedure of Folch et al³ for the removal of non-lipid impurities is at present used in almost all laboratories working in the phospholipid field and was also adopted by us. In this procedure the container with the chloroform:methanol extract is submerged in a large volume of water overnight at 0°. The methanol, together with the water soluble contaminants diffuses into the water and at equilibrium a clear lower phase of chloroform-lipid and an upper phase of water methanol is obtained.

The upper phase is removed by suction, the container again filled up with methanol and the washing process repeated. A particular problem with phospholipids is their tendency to solubilize non-lipid material in organic solvents, which this washing procedure is unable to remove^{1,6,7}. Wheeldon and Collins⁸ removed tenaciously held ninhydrin-reacting material by washing the phospholipid mixture in ethereal solution with 0.1 N HCl. In pilchard and rock lobster phospholipids we also encountered this difficulty, in these instances washing with 0.1 N HCl completely removed the polypeptide impurities. Removal of non-lipid impurities has also been effected by other methods, e.g. column chromatography on cellulose⁹, paper chromatography¹⁰ and dialysis¹¹. Some authors remove non-lipid contaminants by evaporating the solvent of the original extract and re-extracting the residue with chloroform^{2,5}.

3. SEPARATION

3.1 SEPARATION OF PHOSPHOLIPIDS FROM NEUTRAL LIPIDS

The older literature (and some recent e.g. Westley, Wren and Mitchell (1957)¹² contains abundant references to the separation of phospholipids from neutral lipids by means of tedious precipitations with acetone¹. Phospholipids are insoluble in cold acetone, but it is well known that in the presence of neutral lipids they become to a great extent soluble.

The separation of the two lipid classes can be successfully accomplished by chromatography on silica. This subject has been excellently reviewed by Wren¹³. Lovern, Olley and Watson¹⁴ describe the separation of phospholipids from the non-phosphorylated lipids by chromatography on activated silica gel (B.D.H., for chromatographic adsorption) in light petroleum. These authors found that phospholipids remain unadsorbed on silica gel in this solvent in contrast to the neutral lipids. On the other hand, phospholipids are strongly adsorbed on activated silicic acid (Mallinckrodt, A.R., 100 mesh) and are eluted after the neutral lipids from this adsorbent¹³.

The following interesting explanation for these observations has been given by Wren¹³. Phospholipids exist in light petroleum solution as large aggregates of molecules (micelles, molecular weight higher than 8000, see also Faure and Legault-Démare¹⁵) with a hydrocarbon surface and the polar groups inside. These micelles are readily broken up by the (more active) silicic acid but not by the silica gel. In contact with the silica gel therefore the phospholipids appear hydrophobic, whereas in contact with the silicic acid they are unimolecular and thus hydrophilic. Both adsorbents are used in the literature to separate the two lipid classes.

Dialysis in light petroleum through a rubber membrane has been used by van Beers, de Jongh and Boldingh¹⁶ to separate phospholipids and non-phosphorylated lipids. The phospholipids, dissolved in light petroleum, do not pass through the rubber membrane, whereas the neutral lipids do. This phenomenon can also be explained on the basis of micelles formation in light petroleum.

In our investigations the separation in light petroleum solution on silica gel was used. A complete separation of the phospholipids from the neutral lipids was achieved.

3.2 SEPARATION OF THE PHOSPHOLIPIDS INTO CLASSES

This separation is at present usually achieved by chromatography on activated silicic acid. Various techniques for the separation have been developed e.g. column chromatography, paper chromatography on silicic acid impregnated paper and thin layer chromatography. For a review of this subject see Wren¹³ and Marinetti¹⁷.

The column chromatographic technique of Hanahan, Dittmer and Harashina¹⁸ and the silicic acid impregnated paper procedure of Marinetti, Erbland and Kochen¹⁹ were used in our investigations. Thin layer chromatography was not employed as a routine method, since excellent results were obtained with silicic acid impregnated paper.

In the column chromatographic procedure of Hanahan et al¹⁸, the phospholipid mixture is placed on a column of activated silicic acid and the different components are eluted with a series of chloroform-methanol mixtures of increasing methanol concentration (i.e. increasing polarity).

In general it can be said that a 7:1 (by vol.) mixture elutes the phosphatidic acids including cardiolipin; a 4:1 (by vol.) mixture elutes phosphatidyl ethanolamine, phosphatidyl serine and phosphatidyl inositol; a 3:2 (by vol.) mixture elutes phosphatidyl choline; a 1:4 (by vol.) mixture elutes sphingomyelin and a 1:16 (by vol.) mixture elutes lyso phosphatidyl choline. The composition of these solvent mixtures is of course flexible, it is for instance possible to elute phosphatidyl ethanolamine satisfactorily with chloroform:methanol 7:1 (by vol.). An adequate separation of the "cephalin" components is not achieved. Rechromatography of the impure "cephalin" fraction in the presence of ammonia appears to separate phosphatidyl ethanolamine and phosphatidyl serine²⁰. Phosphatidyl inositol has also been obtained in a pure condition by rechromatography of the material originally isolated from silicic acid columns²¹. Plasmalogens are eluted with the corresponding diester fractions¹³. Large quantities of material in a reasonably pure condition may be obtained by the column chromatographic method. The separation is also used for the determination of the quantitative composition of a phospholipid mixture.

The silicic acid impregnated paper technique of Marinetti et al¹⁹ uses an ascending solvent system of diisobutyl ketone:acetic acid:water (8:5:1, by vol.). Other solvent systems have also been suggested²². Complete separation of the "cephalin", phosphatidyl choline, sphingomyelin and lyso phosphatidyl choline fractions is achieved. Phosphatidyl inositol is not separated from lyso phosphatidyl choline. Fractions are shown up by staining with a Rhodamine 6G solution or - more elegantly - with the staining solution of Hooghwinkel and van Niekirk²³ i.e. Edicol Supra Poncetu 4RS - Uranyl Nitrate. The ninhydrin and phosphomolybdic acid reagents of Lea, Rhodes and Stoll²⁴ are used to distinguish between amino-phospholipids and compounds containing choline. Plasmalogens are best detected with fuchsin-sulphurous acid (Schiff reaction) according to Hack and Ferrans²⁵. Although the paper chromatographic method is primarily a qualitative procedure, quantitative analyses have been carried out on phosphatide mixtures. Some workers elute the spots and determine lipid-phosphorus after digestion¹⁹. The following convenient semi-quantitative method has been developed by Hooghwinkel and van Niekirk²³.

The chromatogram is stained with a solution of Edicol Supra Ponceau 4RS-- Uranyl Nitrate, the red spots eluted from the paper and the optical density of the eluate is measured at 510 m μ . The last mentioned method has been used in our investigations and will be discussed there.

Thin layer chromatography of phosphatides on silica is at present used in many laboratories^{26,27}. The details of this procedure are essentially the same as those of silicic acid impregnated paper chromatography.

Recently, diethylaminoethyl (DEAE) cellulose has been recommended to separate phospholipid classes²⁸. This adsorbent is an anion exchange cellulose, and separations are therefore based on differences in isoelectric point of the phospholipids. Phosphatidyl serine and phosphatidyl ethanolamine for instance appear to be readily separated.

Finally, laborious precipitation procedures in different solvents and counter current distribution have also been applied to the separation of phospholipids¹.

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CHAPTER III

PHOSPHOLIPID ANALYSIS

1. ANALYSIS OF THE INTACT MATERIAL

The qualitative and quantitative analysis of the intact phosphatide can be obtained by fractional precipitations in different solvent systems, or by chromatographic separations. This subject has been discussed in the previous chapter.

2. ANALYSIS BY MEANS OF HYDROLYTIC CLEAVAGE

A phospholipid may be characterized and quantitatively estimated by analysis of the products which are formed on hydrolysis. Numerous hydrolytic procedures are known in the literature, each having its own merits and disadvantages. This statement will become clear in this chapter and the chapter on the hydrolysis of phospholipids. Hydrolysis in acid medium is usually employed for the liberation of the water soluble compounds choline, ethanolamine, serine and inositol and acid plus alkaline hydrolysis for the liberation of the fatty acids and sphingosine¹. Dawson² has introduced a mild alkaline hydrolytic procedure with methanolic sodium hydroxide at 37° for 15 min. This method preferentially liberates the fatty acids; the water soluble compounds - e.g. glycerylphosphorylcholine - which are formed, are resolved by two dimensional paper chromatography. This reaction must be carried out under carefully controlled conditions otherwise side-reactions occur such as breakdown of e.g. glycerylphosphorylcholine into methyl glycerophosphate or formation of cyclic phosphate esters^{3,4}.

2.1 PHOSPHORUS

Digestion of the phosphatide with perchloric acid⁵ or sulphuric acid-hydrogen peroxide⁶ is necessary to convert the lipid-phosphorus to inorganic phosphorus. Almost all phosphorus determinations are based on the curious fact that under certain conditions a phosphomolybdate complex is more easily reduced to molybdenum blue than molybdate itself. The optical density of the blue colour is directly proportional to the phosphorus concentration.

Fiske and Subbarow⁷ developed in 1925 a method which employs 1-amino-2-naphthol-4-sulphonic acid plus sulphite as the reducing agent. Since that time many improvements have been suggested, the most important being the increase in temperature during colour development from room temperature to 100°⁶. Other reducing agents include vitamin C⁸ and hydroquinone⁹. In our laboratory many thousands of phosphorus determinations have been successfully carried out according to Bartlett⁶, which is a modified Fiske and Subbarow method.

2.2 NITROGEN

Total nitrogen can be determined according to the Dumas method - details are found in chemistry text books - or by a Kjeldahl procedure¹⁰. Amino nitrogen of the intact and the hydrolysed phospholipid may be determined according to Wheeldon and Collins¹¹.

2.3 CHOLINE

CHOLINE is the hydrolysis product of lecithins, choline plasmalogens, lyso lecithins and sphingomyelins. Choline is liberated in acid or alkaline medium, depending on the preference of the investigator, although certain factors are important. Strong alkaline hydrolysis leads to partial decomposition of choline^{12,13}. Vigorous hydrolysis with HCl leads to the formation of 2-chlorocholine and probably to further decomposition¹⁴. Complete liberation of choline from sphingomyelin in acid or alkaline medium is time consuming¹ (see also Chapter XII). Typical methods of hydrolysis follow. Levine and Chargaff¹⁵ hydrolyse with 6 N HCl at 100° for 48 h, Lovern, Olley and Watson¹⁶ hydrolyse with 2 N HCl at 120° for 24 h and Bränte¹⁷ hydrolyses with saturated baryta water at 100° for 2 h.

Choline is identified chiefly by paper chromatography. It can be detected on paper chromatograms with iodine vapour¹⁸ or with phosphomolybdic acid followed by stannous chloride¹⁵. Identification as the picrate of m.p. 250-251° is also possible (see our experimental work).

Choline may be determined quantitatively in numerous ways. It is frequently precipitated as choline reineckate and determined spectrophotometrically by dissolving the reineckate in acetone¹⁹. This method has proved unreliable with fish phospholipids in Lovern's laboratory as well as in ours^{1,12}. A sensitive method for the determination of choline (0.1 - 3 μ moles) was developed in our laboratory¹². Choline is precipitated as the insoluble phosphomolybdate, the excess phosphomolybdic acid is carefully removed and phosphorus determined spectrophotometrically in the precipitate by reduction to molybdenum blue.

2.4 ETHANOLAMINE AND SERINE

ETHANOLAMINE and SERINE - originating from "cephalins" - are determined in an acid hydrolysate, since alkaline hydrolysis decomposes these substances to a great extent^{1,20}. The hydrolytic procedures with HCl mentioned for choline are also applicable here. It should, however, be borne in mind that the formation of 2-chloroethanolamine - similar to 2-chlorocholine - is possible. This substance has never been detected but this is possibly due to its instability e.g. it is known that 2-bromoethanolamine readily splits off HBr with the simultaneous formation of ethyleneimine which probably decomposes into ammonia and acetaldehyde²¹. Ammonia has been recognized as a decomposition product of ethanolamine in acid medium²². A favourable method for liberating ethanolamine and serine is the hydrolysis with anhydrous 6N methanolic-HCl, but the disadvantage is that serine is converted into its methyl ester^{20,22}. In our investigations hydrolysis according to Lovern et al¹⁶ i.e. 2 N HCl at 120° for 24 h was adopted for the liberation of choline, ethanolamine, serine and inositol.

Ethanolamine and serine are characterized on paper chromatograms sprayed with ninhydrin^{15,23}.

The quantitative estimation of ethanolamine and serine is complicated by the fact that phospholipid hydrolysates often contain small amounts of amino acid impurities. Either rigorous purification of the phosphatide prior to hydrolysis is necessary, or ethanolamine and serine must be completely separated from other amino acids in the hydrolysate. Methods for the separation of ethanolamine and serine include fractional distillation¹⁷ and fractionation on cation exchange resins²².

Determination of ethanolamine after separation from serine is achieved by estimation of amino-nitrogen¹⁷, periodate oxidation followed by determination of ammonia²² or colorimetric estimation after reaction with ninhydrin^{24,25}. Determination of serine is accomplished by volumetric determination of carbon dioxide which is formed on treatment with ninhydrin¹⁷, periodate oxidation²² or colorimetrically after reaction with ninhydrin^{24,25}.

Collins and Wheeldon²⁶ have described a method for the determination of ethanolamine and serine, which does not involve their separation. The intact phospholipid is treated with 1-fluoro-2,4-dinitrobenzene, the resulting dinitrophenyl-lipid is hydrolysed and 2,4-dinitrophenyl-ethanolamine and 2,4-dinitrophenyl-serine are determined by a differential colour reaction. In our investigations ethanolamine and serine were determined according to a modified Moore and Stein procedure²⁵.

2.5 MYO-INOSITOL

MYO-INOSITOL may be liberated from phosphoinositides by acid or alkaline hydrolysis^{27,28}. Acid hydrolysis is frequently used, probably because myo-inositol is extremely stable in acid medium²⁸. Vigorous conditions for the liberation of inositol are necessary e.g. 2 N HCl at 120° for 24 h¹⁶ in order to hydrolyse the initially formed inositol phosphate.

Myo-inositol can be detected on paper chromatograms with alkaline silver nitrate according to Anet and Reynolds²⁹. It is sometimes characterized as a crystalline material of m.p. 225 - 227° obtained after complete hydrolysis of the phospholipids followed by removal of ionic substances by passage over a mixed cation-anion exchange resin²⁷ (see our experimental work).

Chemical determinations of myo-inositol in a phospholipid hydrolysate are inaccurate. The method sometimes used consists of separating inositol from other reducing substances (e.g. glycerol) by paper chromatography, eluting the section containing the inositol followed by a determination with periodate³⁰. Area estimations of the black silver spots on paper chromatograms are also employed¹⁶. Gas chromatographic estimation of all inositols as their hexa-acetates has recently been developed by Krzeminski and Angyal; myo- and muco-inositol are however not separated³¹.

Myo-inositol is a growth factor (vitamin) and based on this many reliable microbiological procedures for its determination have been introduced. For instance the yeast strains *Saccharomyces Cerevisiae*^{32,33}, *S. Carlsbergensis*³⁴ and *Schizosaccharomyces pombe*³⁵ are used. These methods are specific for myo-inositol, the other eight isomers do not exhibit this vitamin activity^{32,33,36}.

In our work myo-inositol was detected on paper chromatograms, isolated as a crystalline material of m.p. 225 - 227° and determined with *S. Carlsbergensis*.

2.6 SPHINGOSINE

SPHINGOSINE is traditionally liberated by hydrolysis with saturated baryta water followed by a short hydrolysis ($\pm$ 2 h) with 1 N HCl, the latter in order to split the galactose linkage in cerebrosides¹⁰. Recently, hydrolysis with 2 N methanolic HCl³⁷ and 6 N HCl:butanol (1:1, by vol.), have been recommended³⁸.

Sphingosine can be detected on paper chromatograms with ninhydrin^{38,39} but the colour reaction of Saito⁴⁰ with fluorescein is preferable (e.g. Grone and Bridges⁴¹). Paper chromatography of the yellow 2,4-dinitrophenyl sphingosine has been developed by Karlsson⁴².

Sphingosine is frequently estimated according to the method of McKibbin and Taylor¹⁰. This method consists of a nitrogen determination in a chloroform extract of a phospholipid hydrolysate. Fatty acids and sphingosine (as the hydrochloride) are extracted into the chloroform phase whereas other nitrogenous bases remain in the aqueous phase. This procedure has been criticised by many workers (e.g. Lovern¹) on the grounds that not all the chloroform soluble-nitrogen is in fact sphingosine-nitrogen. The method proved unreliable in our laboratory, this will be shown in our investigations. Sweeley and Moscatelli³⁷ have described an unusual estimation of sphingosine. Sphingosine is oxidized with periodate and the fatty aldehyde reaction product (trans-2-hexadecenal) analysed by gas chromatography.

In our investigations sphingosine was estimated indirectly by determining sphingomyelin-choline (Chapter XI). This was found by determining the total choline in a phospholipid hydrolysate¹², and the relative proportions of lecithin-choline, sphingomyelin-choline and lyso lecithin-choline.

The latter were readily obtained by chromatography of the phospholipid mixture on silicic acid impregnated paper followed by staining according to Hooghuinkel and van Nierkerk⁴³. This method is of course not applicable to cerebroside-sphingosine.

2.7 GLYCEROL AND GLYCEROPHOSPHATE

The standard procedure for the determination of glycerol in a phosphatide is the method of Blix⁴⁴. The phosphatide is treated with HI which converts glycerol into isopropyl iodide; the latter is distilled off and determined titrimetrically. A correction must be applied for the presence of any sugars¹⁷. Hanahan and Olley²⁷ determine glycerol in a phospholipid hydrolysate - after removal of glycerophosphate by means of an anion exchange resin - by oxidation to formaldehyde with periodate (5 min. at room temperature). Formaldehyde is estimated colorimetrically with chromotropic acid. Under these mild oxidation conditions little interference is shown by glucose, inositol, ethanolamine and serine.

Glycerol may be detected on paper chromatograms by means of alkaline silver nitrate²⁹.

In our investigations glycerol was not determined.

Glycerophosphate is the initial reaction product in the hydrolysis of glycerophosphatides. It is very stable in acid but markedly so in alkaline medium. Glycerophosphate can be detected on paper chromatograms by the molybdenum-blue reaction of Burrows, Grylls and Harrison⁴⁵ with U.V. light development according to Bandurski and Axelrod⁴⁵ (cf. Lit. 27). Hawthorne⁴⁶ and Wade and Morgan⁴⁷ have indicated the separation of α - and β -glycerophosphate by paper chromatography, and by a combination of paper electrophoresis and chromatography respectively.

Total glycerophosphate (D- α , L- α and β) is determined by oxidation in hot acid medium with periodic acid to glycolic aldehyde phosphate, which is hydrolyzed to inorganic phosphate and determined. Under these reaction conditions β -glycerophosphate is converted to α -glycerophosphate which is oxidized by periodic acid^{48,49}. By employing milder oxidation conditions α -glycerophosphate may be selectively determined in this way⁴⁸. Essentially the same procedure has been described by Verkade, Stoppelenburg and Cohen⁵⁰ for the determination of α -glycerophosphate in the presence of the β -isomer; those authors do not analyse for the liberated phosphate but estimate the quantity of periodate consumed. L- α -glycerophosphate may be determined manometrically by measuring the oxygen uptake in the presence of the enzyme L- α -glycerophosphate dehydrogenase (cf. Long and Maguire⁵¹).

In our studies on the hydrolysis of phospholipids with 2 N HCl total glycerophosphate was determined.

2.8 FATTY ACIDS

The liberation of the fatty acid moieties in lipids is frequently accomplished in alkaline medium¹. Bütcher, Woodford, Boelens van Heute and van Gent⁵² however obtained incomplete saponification of artery phospholipids in alkaline medium and prefer transesterification with a saturated solution of hydrogen chloride in anhydrous methanol followed by saponification of the "impure" methyl esters with 0.5 N ethanolic potassium hydroxide. Dimethyl acetals of the fatty aldehydes derived from plasmalogens form part of the impurities, which are ^{removed with petroleum ether} ~~separated~~ by saponification in alkaline medium. The neutral lipids are directly saponified with 0.5 N ethanolic potassium hydroxide. Addition of an antioxidant (e.g. hydroquinone) is essential in order to prevent decomposition of the highly unsaturated fatty acids during saponification.

Older methods for the quantitative analysis of a fatty acid mixture include the paper chromatographic procedures of Kaufmann and his School⁵³ and the reversed phase chromatography of Belding⁵⁴. The last mentioned procedure was adopted by Silk and Hahn⁵⁵ for the resolution of pilchard oil fatty acids; these workers used paraffin oil on non-wetting kieselguhr as stationary phase and acetone as developing solvent. At present the analysis of a fatty acid mixture is usually achieved by gas-liquid chromatography of the methyl esters as developed by James and Martin⁵⁶, Farquhar et al⁵⁷ and recently Ackman and Burgher⁵⁸. Methyl esters are prepared by treatment with diazomethane, methanol-hydrochloric acid and methanol-boron trifluoride (cf. Lit. 59).

It is not our intention to describe in detail gas-liquid chromatography. In short it may be said that in our investigations the methyl esters were separated with a Barber Colman gas chromatograph on a polyethyleneglycol glutarate stationary phase with Argon as carrier gas at a temperature of approximately 200°. The fatty acids were identified by comparison with a standard mixture, by means of literature data of relative retention times⁵⁷ and by the straight line relationship existing between log retention time and carbon number⁵⁷.

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CHAPTER IV

BIOCHEMISTRY OF THE PHOSPHOLIPIDS

This chapter describes briefly some of the biochemical aspects of the phospholipids.

Function and occurrence

In 1914 Terroine¹ already clearly recognized two important lipid classifications; the "variable" and the "constant" element. Neutral lipids belong to the "variable" element and phospholipids to the "constant" element. The latter is an essential factor to support life.

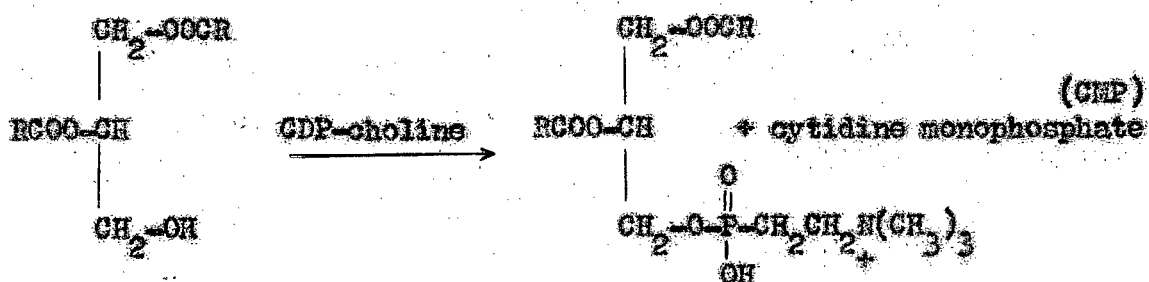
Different organs contain different quantities of phospholipids, the richest source being the brain². Phospholipids are present in all cell membranes. Isolation of pure membranes for study is difficult and only the membranes of red and white blood cells appear to have been adequately studied^{3,4}. The present theory is that cell membranes consist of a bimolecular leaflet of lipids (largely phospholipids) the polar ends of which are covered with a monomolecular layer of protein⁵. Phospholipids in human serum are associated with protein in the form of lipoprotein complexes. Plasma lipoproteins consist of tri-glycerides, phospholipids, cholesterol and protein; the phospholipids containing polar and non-polar groups are the stabilizing factors in these complexes⁵. Phospholipids in the tissues are also present as lipoprotein units; rupture of the protein-lipid bond can only be accomplished with polar solvents such as methanol⁶. Felch and Lees⁷ have isolated a protein-lipid complex from brain which they called proteolipid since it showed solubility characteristics of a lipid, e.g. solubility in chloroform:methanol. A proteolipid can be denatured by evaporating a chloroform:methanol solution containing water to dryness. Acidic phospholipids, in particular phosphatidyl inositol, diphosphoinositides and triphosphoinositides appear to be bound to basic peptides by electrovalent bonds. The lipids of these so-called phosphatido-peptides can only be liberated by means of a chloroform:methanol:HCl mixture^{8,9}. Recently Collins¹⁰ has shown the presence of "complex" phospholipids in animal tissues, the

molecular weight of these compounds is about double that of the normal phospholipids. The structures suggested by Collins are in all probability incorrect; this is further exemplified in Chapter XIII.

Sinclair¹¹ has demonstrated that the fatty acid composition of muscle and liver phospholipids of the rat was hardly affected by saturated fatty acids in a diet; polyunsaturated acids, however, were readily incorporated into the phospholipids. The latter has also been shown by Rhodes¹², who found highly unsaturated fatty acids in hen egg phospholipids after feeding the hens cod liver oil. There may be an important connection here between phospholipids and ischaemic heart disease, since some workers e.g. Sinclair¹³ and also Bronte-Stewart¹⁴ stress the importance of the polyunsaturated fatty acids for the prevention of this disease. Hokin and Hokin¹⁵ have assigned a function to phospholipids which involves a transport of cations across membranes. In particular the acidic phospholipids such as phosphatidyl serine, phosphatidic acid and phosphatidyl inositol seem suitable for this purpose. Phosphatidyl ethanolamine also plays an important role in the blood clotting mechanism¹⁶.

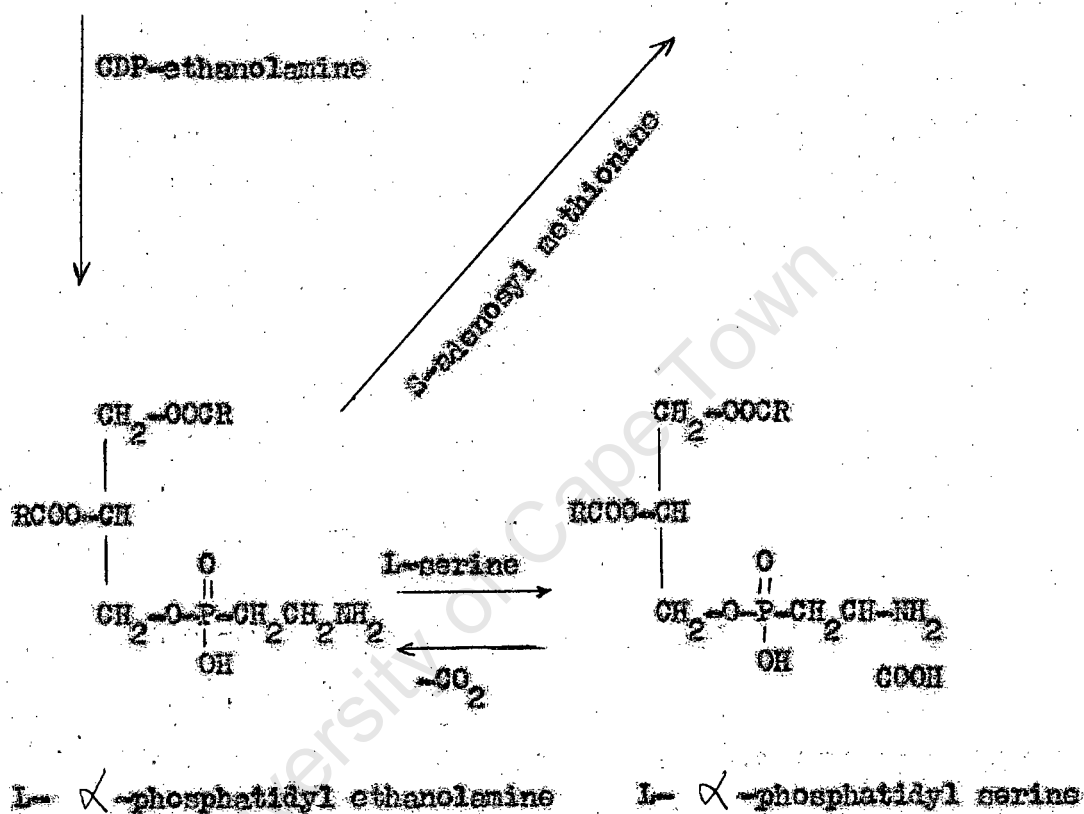
Biosynthesis

The biosynthesis of phospholipids has been reviewed by Kennedy¹⁷. L- α -phosphatidyl choline is synthesized from a D- α, β -diglyceride and cytidine diphosphocholine (CDP-choline); it may also be formed by methylation of L- α -phosphatidyl ethanolamine with the enzyme S-adenosyl methionine as methyl donor. L- α -phosphatidyl ethanolamine is synthesized in an entirely analogous manner. L- α -phosphatidyl ethanolamine and L- α -phosphatidyl serine are interconvertible, decarboxylation of the latter gives rise to the former and L- α -phosphatidyl serine can be synthesized by reaction of L-serine with L- α -phosphatidyl ethanolamine.

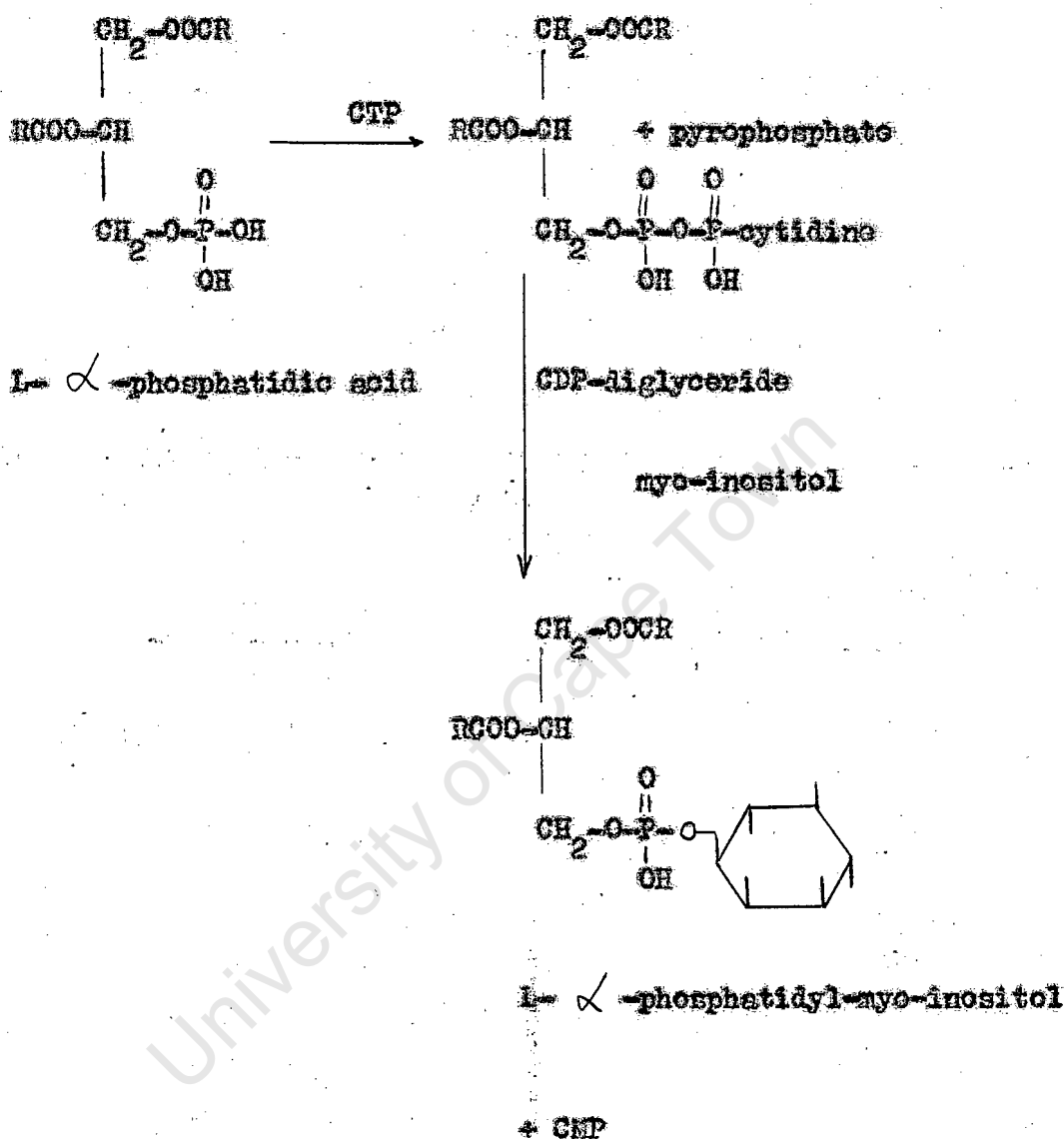


D- α, β -diglyceride

L- α -phosphatidyl choline



The biosynthesis of L- α -phosphatidyl inositol is different from the above-mentioned syntheses. In this instance L- α -phosphatidic acid is converted with cytidine triphosphate (CTP) into cytidine diphosphodiglyceride, which in turn reacts with myo-inositol to give L- α -phosphatidyl inositol.



The biosynthesis of sphingomyelin will not be discussed since it has not yet been completely clarified.

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PART II

INVESTIGATIONS.

SUMMARY

1. 2-Chlorocholine was identified as an artifact in the hydrolysis of choline-containing phospholipids with HCl. This substance is in all probability identical to an unknown nitrogenous base described by other workers.
2. A sensitive method for the microdetermination of choline (0.1 - 3 μ moles) was developed. Choline is precipitated as the insoluble phosphomolybdate, the excess phosphomolybdic acid is carefully removed and phosphorus determined spectrophotometrically in the precipitate by reduction to molybdenum blue. Compounds capable of forming insoluble phosphomolybdates interfere. Interference of ammonia can be prevented by the addition of formaldehyde.
3. It is shown that the presence of all amino acids - except serine - in a phospholipid hydrolysate was due to the presence of polypeptide impurities. The presence of many amino acids detected by other workers in phospholipid hydrolysates can probably be explained in the same way.
4. The composition of the hake flesh and hake liver phospholipids was determined by an hydrolytic procedure and by chromatography on silicic acid. The hydrolytic procedure involved determination of lecithin-choline, sphingomyelin-choline, lyso lecithin-choline, ethanolamine, serine, myo-inositol and the average equivalent weight of the liberated fatty acids. The composition of the flesh and liver phospholipids was very similar, the latter contained more sphingomyelins and cardiolipins at the expense of phosphatidyl choline. Phospholipid fatty acids had a markedly higher equivalent weight and unsaturation than the corresponding non-phosphorylated lipid fatty acids. The cephalin fractions contained more long chain polyunsaturated (C_{22} and higher) fatty acids, more stearic acid ($C_{18:0}$) and less palmitic acid ($C_{16:0}$) than the phosphatidyl choline fractions. Sphingomyelins were particularly rich in lignoceric ($C_{24:0}$) and nervonic ($C_{24:1}$) acid.

5. The complete analysis of rock lobster roe and hepatopancreas phospholipids was obtained in the same way as described for the hake. Rock lobster roe phospholipids were remarkable for their high content of phosphatidyl choline (71%) and low proportions of phosphatidyl serine (1%), phosphatidyl inositol (1%), cardiolipins (1%) and lyso phosphatidyl choline (trace). Rock lobster hepatopancreas phospholipids were similar in composition to hake flesh phospholipids. Fatty acids from the phospholipids and the non-phosphorylated lipids of both roe and hepatopancreas had the same average equivalent weight and unsaturation; the fatty acid composition showed a close resemblance. Large differences, however, were observed in the fatty acid composition of the phosphatidyl choline and cephalin fractions, the latter containing more of the polyunsaturated fatty acids. In general the rock lobster lipids contained more C_{20} and C_{18} and less C_{22} and C_{16} acids than the corresponding hake lipids.
6. Phospholipids of the pilchard were analysed as described for the hake. The following table records the composition of all phospholipids investigated in this work.

TABLE I
PHOSPHOLIPID COMPOSITION OF MARINE PRODUCTS
(expressed as per cent of total phospholipid)

SOURCE	PHOSPHOLIPID CLASS ^x						
	PC	PE	PS	PI	SP	PA	LP
whole pilchard	53	25	2	4	6	9	2
hake flesh	63	21	3	6	4	2	1
hake liver	53	23	3	5	9	7	2
rock lobster hepatopancreas	60	26	3	5	1	3	3
rock lobster roe	71	23	1	1	3	1	trace

- ^x PC phosphatidyl choline
 PE phosphatidyl ethanolamine
 PS phosphatidyl serine
 PI phosphatidyl inositol
 SP sphingomyelin
 PA phosphatidic acids (cardiolipins)
 LP lyso phosphatides

This table clearly illustrates the great similarity that exists in the different phospholipids, only those extracted from the rock lobster are to some extent different.

7. The progress of the oxidation of phospholipids and non-phosphorylated lipids in the manufacturing process of pilchard meal was examined. It was shown that pilchard phospholipids were oxidized to a certain extent in the drying process, but a severe destruction of phospholipid and non-phosphorylated lipid fatty acids took place on storage of the meal at room temperature. Phospholipid oxidation could be determined by the drop in average unsaturation of the fatty acids, but equally well by the drop in free amino nitrogen.
8. Small quantities (≈ 3 mg) of phosphatidyl choline fractions were capable of stabilizing a supersaturated solution of cholesterol in a triglyceride oil.
9. A new procedure for the estimation of sphingomyelin was developed by determining sphingomyelin-choline.
10. The progress of the hydrolysis of DL- α -lecithin, DL- α -cephalin and sphingomyelin with 2 N HCl at 120° was investigated. The hydrolytic products: glycerophosphate, phosphorylcholine, phosphorylethanolamine and inorganic phosphate were determined at intervals. Hydrolysis of DL- α -lecithin and DL- α -cephalin proceeds similarly. Initially no inorganic phosphate is liberated and bond fission takes place in such a manner that 84% of the phosphorus remains esterified to the glycerol moiety (glycerophosphate) and 16% to the choline moiety (phosphorylcholine). In the hydrolysis of sphingomyelin inorganic phosphate is also not formed initially, but phosphorus remains esterified predominantly, if not exclusively, to the choline moiety. From the data obtained a reaction mechanism is suggested for the acid hydrolysis of the above-mentioned substances.
11. The structure of "Complex" phospholipids as described in the literature is challenged. A pyrophosphate linkage instead of a phosphate triester group is postulated to be present in these compounds.

CHAPTER VIDENTIFICATION OF AN ARTIFACT IN THE HYDROLYSIS
OF CHOLINE-CONTAINING PHOSPHOLIPIDS.

In the course of a study of the phospholipids of the South African pilchard (*Sardina ocellata* Jernyns), choline was liberated by hydrolysis of the material for 48 h in 6 N HCl at a temperature of 115° in a sealed tube. Choline was identified by chromatography on filter paper according to the method of Levine and Chargaff^{1,2}, i.e. conversion to the insoluble phosphomolybdate followed by reduction to molybdenum blue. Two blue spots were invariably obtained, one with the mobility of choline and the other a hitherto unidentified faster moving compound.

Levine and Chargaff¹ hydrolysed lecithin preparations and beef brain phosphatides with 6 N HCl at 100° for 48 h. These authors also described the appearance of two spots on paper chromatograms, when testing for choline with phosphomolybdic acid. They quoted an R_F value of 0.43 in a solvent system of *n*-butanol-diethylene glycol-water (4:1:1, by vol.) for choline. However, for the unidentified substance in the above-mentioned solvent system, two R_F values were quoted: R_F = about 0.59 (purified lecithins) and R_F = 0.53 (beef brain phosphatides).

Phillips³ reported two compounds, which stained with phosphomolybdic acid, after hydrolysis of the lecithin and sphingomyelin fractions of human serum phospholipids with 6 N HCl at a temperature of 110° for 18 h. One compound, with an R_F value of 0.33 in the solvent system *n*-butanol-acetic acid-water (4:1:4, by vol., upper layer), was identified as choline; the other, with an R_F value of 0.41, remained unidentified.

It has been suggested that the unknown substance might be an unidentified nitrogenous base present in phospholipids^{1,2}. In this chapter it is shown that choline chloride when heated with aqueous HCl, gives rise to the formation of (2-chloroethyl)-trimethylammonium chloride (2-chlorocholine chloride). This compound is identical to the unidentified base obtained by hydrolysis of pilchard phospholipids with aqueous HCl.

EXPERIMENTAL

ISOLATION OF PILCHARD PHOSPHOLIPIDS

The isolation of phospholipids from fish will be fully discussed in Chapter VIII.

It is sufficient to state here that the phospholipid sample which was hydrolysed had P and N contents of 3.3% and 1.7% respectively.

IDENTIFICATION OF THE ARTIFACT

It was found in our laboratory that a solution of choline chloride in 6 N HCl heated for 48 h at 115° in a sealed tube and chromatographed on filter paper according to Levine and Chergaff¹ produced two spots - one corresponding to choline and the other to the unidentified compound arising from the hydrolysis of pilchard phospholipids. Similarly, the hydrolytic procedures of Levine and Chergaff¹, and of Phillips³ applied to choline chloride gave rise to the formation of the unknown substance. This substance, therefore, appeared to be an artifact produced in the hydrolysis with HCl, and was subsequently identified as 2-chlorocholine chloride. 2-Chlorocholine chloride was synthesized according to Taketomo⁴ and Tolbert⁵ (see below).

Chromatograms with choline chloride and 2-chlorocholine chloride as marker compounds were run in different solvent systems, as shown in Table I, and no difference between the artifact produced on heating choline chloride with HCl, 2-chlorocholine chloride and the unidentified compound in hydrolysates of pilchard phospholipids was observed. Mixtures of these compounds did not show any separation when chromatographed on filter paper.

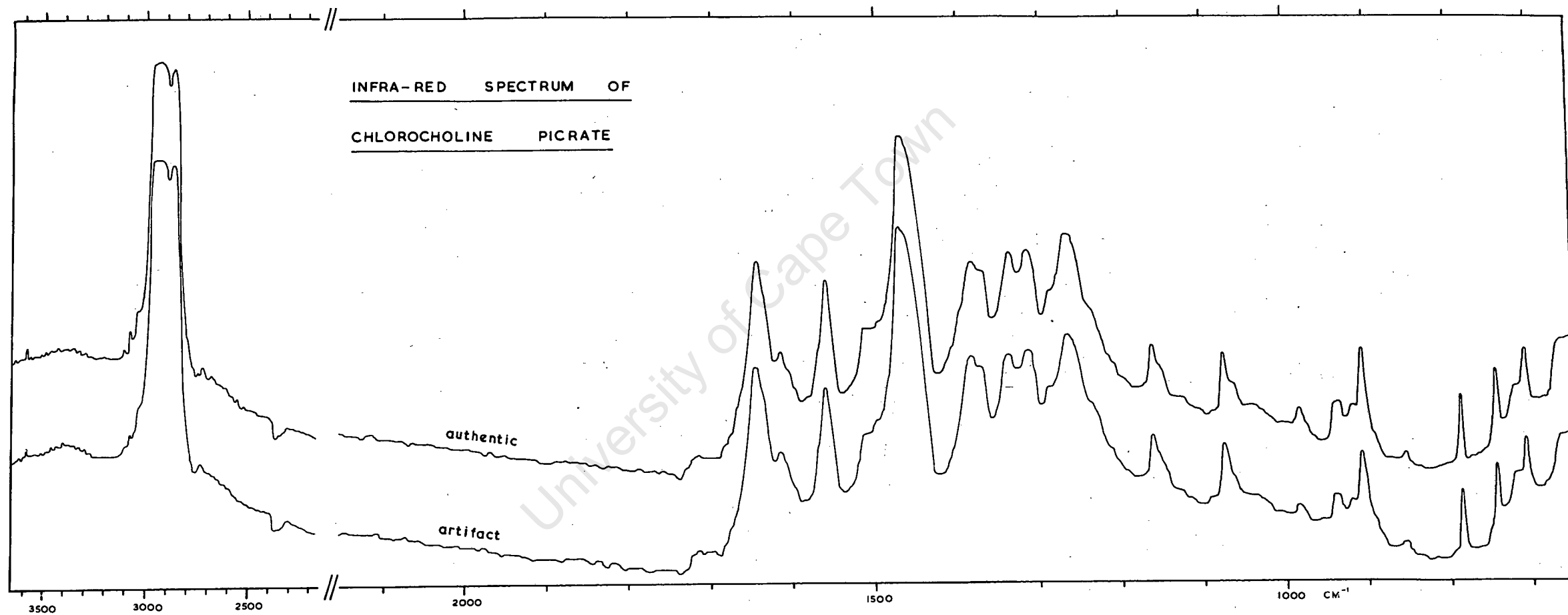
TABLE I

R_F VALUES OF CHOLINE CHLORIDE AND 2-CHLOROCHOLINE CHLORIDE
IN DIFFERENT SOLVENT SYSTEMS ON WHATMAN NO. 1 PAPER

SOLVENT SYSTEM (by vol.)	R_F (20 - 24°)				REMARKS
	Choline Chloride	2-chloro- choline chloride	Artifact from choline chloride and HCl	Compound in hydrolysate of phospho- lipids	
n-Butanol-diethylene glycol water (4:1:1)	0.45 0.40	0.58 0.55	0.60 0.54	0.59 -	Descending Ascending
n-Butanol-acetic acid-water (4:1:4, upper layer)	0.31	0.39	-	0.39	Ascending
Ethanol-ammonia (95:5) [*]	0.48	0.56	0.58	-	Ascending
n-Butanol-pyridine (4:1) saturated with water	0.14	0.21	0.21	-	Ascending
n-propanol:water (4:1)	0.35	0.45	0.45	-	Descending

* According to Dregoff, Roberts and Delwiche⁶.

FIG. 1.



An additional proof of the identity of the unknown substance was obtained by heating choline chloride in 6 N HCl at 115° for 168 h. Approximately 200 mg of the mixture was chromatographed on four sheets of Whatman No. 2 filter paper (20 x 40 cm) with a descending solvent system of n-propanol: water (4:1, by vol.). The papers were dried in air and for 0.5 h at 100°. A guide strip was cut off the papers to locate the position of the faster moving compound, and the corresponding area containing this compound was cut out. The pieces of filter paper were extracted with 2 x 50 ml hot ethyl alcohol. After filtration the ethyl alcohol was removed in vacuo, and the remaining material treated with an alcoholic solution of picric acid. The crystals obtained were filtered, washed with ethyl alcohol and dried over P_2O_5 in vacuo. The melting point of the crystals was 215 - 217°, and on mixing the material with the picrate of 2-chlorocholine no depression of melting point was observed. The infra-red spectra of the two compounds were identical (see fig. I).

Lovern, Olley and Watson⁷ in a study of the phospholipids of cod, hydrolysed the phospholipids in a sealed tube with 2 N HCl at 120° for 24 h. This hydrolytic procedure, applied to choline chloride, produced only very small quantities of 2-chlorocholine chloride, as judged by the intensity of the second spot on chromatograms. It did not produce 2-chlorocholine chloride when applied to a pilchard phospholipid fraction, and was subsequently adopted in this laboratory. The hydrolysis according to Levine and Chargaff¹ did not give as intense a second spot as the method of Phillips³. Preference was, however, given to the method of Lovern et al.⁷.

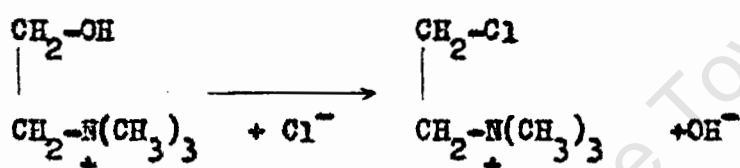
PREPARATION OF 2-CHLOROCHOLINE CHLORIDE

This substance was prepared according to Taketomo⁴ and Tolbert⁵. 20 g anhydrous trimethylamine was dissolved in 132 g 1,2-dichloroethane and heated in a waterbath under reflux at 80° for 3 h. The white precipitate was filtered off and washed with dry ether; weight 6.6 g of 2-chlorocholine chloride. The material was recrystallized from a mixture of 30 ml ether and 50 ml ethanol. The 2-chlorocholine chloride had no specific m.p., it decomposed at 249°. Taketomo⁴ states decomposition at 243° and Tolbert⁵ reports a m.p. of 300° with decomposition at 240 - 245°. The picrate of 2-chlorocholine had a m.p. of 214 - 215° after one recrystallization from ethanol. Tolbert⁵ found this to be 207°.

Analysis of 2-chlorocholine chloride; titratable chloride (argentometric): 22.36%; calculated for $C_5H_{13}Cl_2$ (158.07): 22.43%.

DISCUSSION

In the hydrolysis of lecithins¹, beef brain phosphatides^{1,2}, and human serum phospholipids³ with aqueous HCl, an unidentified nitrogenous base staining with phosphomolybdic acid has been found. This compound is in all probability identical to the artifact found in this work, i.e. 2-chlorocholine chloride, and is formed by reaction of HCl with choline. The formation of 2-chlorocholine undoubtedly takes place via a nucleophilic substitution reaction, which is accelerated by the presence of the strongly positive quaternary ammonium group.



Choline

2-chlorocholine

Hydrolysis of phospholipids with strong aqueous HCl therefore causes a loss of choline. The magnitude of the error introduced in quantitative choline determinations (in particular Levine and Chargaff's planimetric estimation of the molybdenum blue spot area¹) was not investigated, but it appears from the intensity of the two spots produced that hydrolysis according to Lovern et al.⁷ and Levine and Chargaff¹ only gives rise to the formation of small quantities of 2-chlorocholine chloride.

Frequently novel nitrogenous bases are reported to be present in phospholipids. Some of these claims are withdrawn shortly afterwards, e.g. ornithine and hydroxylysine by Lederer^{8,9} or are never confirmed by other workers, e.g. glutamic acid by Hecht and Mink¹⁰. Recently, β -methylcholine ($CH_3-CHOH-CH_2-N(CH_3)_3^+$) has been reported in the phospholipids of the blowfly (*Phormia regina*) especially if the larvae are fed carnitine ($HCOC-CH_2-CHOH-CH_2-N(CH_3)_3^+$)¹¹. Lovern et al.⁷ in a study of the phospholipids of cod describe the presence of an unidentified nitrogenous base occurring with choline in only one instance. This substance is probably an oxidation product of choline and its nature remains to be identified¹².

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CHAPTER VI

A SENSITIVE METHOD FOR THE MICRODETERMINATION
OF CHOLINE

A quantitative study of the composition of the phospholipids of the South African pilchard (*Sardina ocellata* Jernyns) necessitated the determination of small quantities of choline. Of the published methods for the determination of choline, one will be briefly mentioned here; a second will be discussed in more detail. Choline is frequently precipitated as choline reineckate ($C_5H_{14}ON.Cr(NH_3)_2(SCN)_4$) and determined spectrophotometrically by dissolving the reineckate in acetone^{1,2}. This method has proved unreliable with fractions of fish lipids in Lovern's laboratory³ as well as in ours.

Levine and Chargaff⁴ determine between 20 - 100 μ g of choline by chromatography on filter paper, followed by conversion of choline to the insoluble choline phosphomolybdate and reduction of the latter to molybdenum blue by means of stannous chloride. The area of the blue spot thus produced is measured with a planimeter and the amount of choline read from a suitable calibration curve. Two factors are important in this method. Firstly, choline phosphomolybdate is by no means completely insoluble in water (under the conditions described, less than 20 μ g of choline fails to form choline phosphomolybdate), thus the removal of excess phosphomolybdic acid from the paper by washing with running water has to be strictly standardized for temperature and time, in order to prevent loss of choline. Secondly, several workers have observed the presence of two blue spots when testing for choline according to Levine and Chargaff^{4,5}. Recently, it has been shown that this second spot is due to (2-chloroethyl)-trimethylammonium chloride (2-chlorocholine), an artifact formed from choline during the hydrolysis with aqueous hydrochloric acid⁶. This second spot, therefore, ought to be included in the calculation. This factor is, of course, of no consequence when alkaline hydrolysis is applied.

This chapter describes the determination of 0.1-3 μ moles (12-350 μ g) of choline by precipitation of choline with phosphomolybdic acid in a small volume (less than 0.3 ml). The precipitate, after centrifuging and careful removal of excess phosphomolybdic acid, is dissolved in 1 N sulphuric acid and assayed spectrophotometrically for phosphorus. Phosphorus is determined by Bartlett's modification of the Fiske and Subbarow method⁷. The method determines the total quantity of choline plus 2-chlorocholine and is extremely sensitive and accurate. It can be conveniently adopted in laboratories working in the phospholipid field, since it is essentially a phosphorus determination. In addition, the application of the method in the analysis of pilchard phospholipids is demonstrated.

EXPERIMENTAL

Reagents

Choline chloride. This reagent (BDH Laboratory Reagent; at least 99% pure) was recrystallized from ethanol and dried over P_2O_5 in vacuo. Two solutions containing respectively 22.92 μ mole and 4.57 μ mole of choline/ml were prepared by dissolving the appropriate amount of choline chloride in 0.1 N hydrochloric acid.

2-Chlorocholine chloride. This compound was prepared as described by Taketomo⁸. Titratable chloride (argentometric) 22.36%; calculated for $C_5H_{13}Cl_2$ (158.07) 22.43%. Two solutions containing 17.99 μ mole and 3.59 μ mole of 2-chlorocholine/ml were prepared in 0.1 N hydrochloric acid.

Phosphomolybdic acid ($H_3PO_4 \cdot 12MoO_3 \cdot 24H_2O$, A.R.). A 10% solution in water was prepared. This solution was filtered before use.

All other chemicals were of A.R. grade.

Calibration curves

Choline. Quantities of 0.1 to 4 μ moles of choline were pipetted with an Agla micrometer syringe (Burroughs Wellcome & Co., London) into graduated 10-ml centrifuge tubes. The volume was kept as small as possible (less than 0.2 ml). If a micrometer syringe is not available, an ordinary pipette may be used and the volume subsequently reduced in a boiling water bath. To the solution was added 0.1 ml of 10% phosphomolybdic acid. A yellow precipitate appeared immediately; the tubes were covered with aluminium foil and put in ice for 1 h. Thereafter the tubes were centrifuged for 2 min at 4000 r.p.m. The liquid in the centrifuge tubes was removed with a small capillary pipette, without disturbing the precipitate. The precipitate was washed twice, without disturbing it, with 1 ml of cold (10°) aqueous 10% sodium sulphate, centrifuging in between. Great care was taken to remove all traces of phosphomolybdic acid by rinsing the walls of the tubes. After the removal of the washing liquid, the tubes were filled to 4.1 ml with distilled water, and 0.5 ml of 10 N sulphuric acid was added. The tubes were provided with a small pointed glass rod and arranged in a boiling water bath. The choline phosphomolybdate was dissolved slowly in the hot 1 N sulphuric acid with continued stirring. The tubes were cooled to room temperature, and the phosphorus determination was started by the addition of 0.2 ml of aqueous 5% ammonium molybdate tetrahydrate and 0.2 ml of Fiske and Subbarow solution; the latter was prepared as described by Bartlett⁷.

The molybdenum blue colour was developed by placing the tubes in the boiling water bath for 7 min. The tubes were cooled in cold water, and the solution in each tube was transferred to a 50-ml volumetric flask, and diluted to volume with distilled water. The optical density at 830 m μ was determined against a reagent blank in a Unicam S.P. 500 spectrophotometer. The optical density of the reagent blank determined against distilled water was between 0.004 and 0.008.

2-Chlorocholine. The same procedure as that described for choline was followed. The dissolution of 2-chlorocholine phosphomolybdate, however, was accomplished in alkaline medium by the addition of 4.1 ml of 0.1 N sodium hydroxide. After the precipitate had completely dissolved (heating if necessary), the solution was acidified with 0.5 ml of 10 N sulphuric acid and phosphorus determined as described for choline.

Phosphorus. Amounts of 0.3 to 1 μ moles of phosphorus were used in preparing this curve, after a solution of potassium dihydrogen phosphate containing 0.323 μ mole of phosphorus/ml had been prepared.

Determination of choline in a phospholipid

In general, choline determinations can be divided into two parts. Firstly, the hydrolytic procedure, which liberates choline from the phospholipid; secondly, the determination of liberated choline. Hydrolysis in acid (HCl) as well as alkaline ($\text{Ba}(\text{OH})_2$) medium has been employed by different workers^{1,4,5,9,10}.

Hydrolysis. 50-600 mg of the phospholipid was hydrolysed with 2-25 ml of 2N hydrochloric acid in a sealed ampoule at 120° for 24 h. The ampoule was opened and the solution was filtered. The residual tar was thoroughly leached with hot water and poured into the same filter. If the residual tarry material passed through the filter, the water phase was extracted with some light petroleum. After evaporation of the filtrate on the steam-bath and drying in a vacuum desiccator, the material was taken up in 0.1-25.0 (exact) ml of water and an aliquot taken for analysis. Larger or smaller quantities of material can of course be hydrolysed. For instance, the total amount of choline liberated by hydrolysis of as little as 1 mg of phospholipid can be conveniently determined in this way.

Hydrolysis with 6 N hydrochloric acid or barium hydroxide was carried out as described by Levine and Chargaff⁴ and McKibbin and Taylor¹⁰, respectively.

Determination. An aliquot (e.g. 0.1 ml) containing between 0.1 to 3 μ moles of choline was treated as described for the preparation of the choline calibration curve. The amount of choline was obtained by reference to the choline calibration curve. If the presence of ammonia was suspected, 2 drops of a 38% formaldehyde solution in water were added 30 min before the introduction of phosphomolybdic acid.

Removal of amine acids. In some experiments it was necessary to remove amino acids before a choline determination. This was accomplished by adding to the solution in the centrifuge tube 0.2 ml of a 2% sodium nitrite solution and 1 drop of 4 N hydrochloric acid. The mixture was placed in a boiling water bath for approximately 15 min and subsequently analysed for choline.

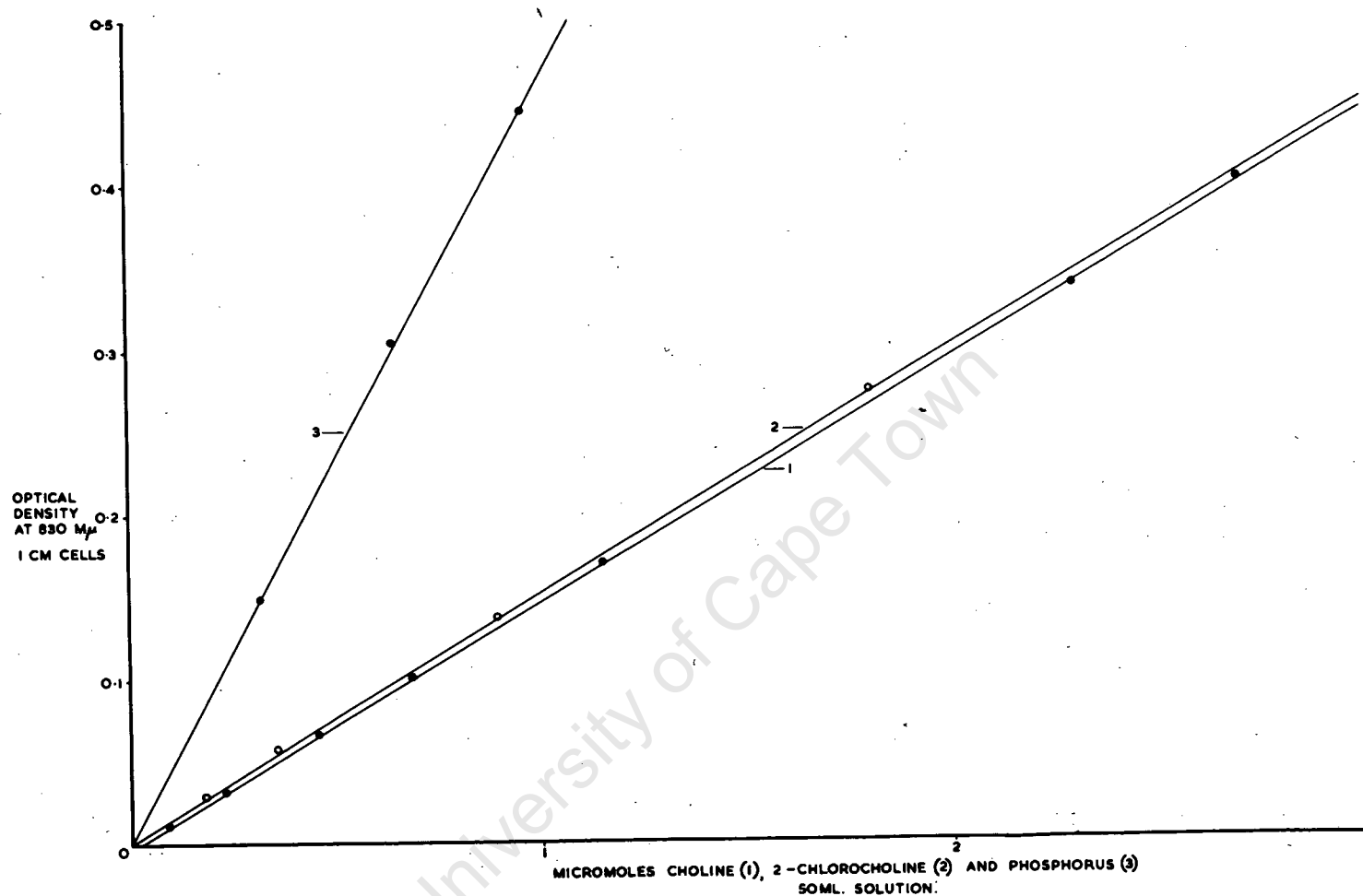
Paper Chromatography. Paper chromatograms were prepared as described in Chapter V. In one instance the staining procedure of Brante¹¹ with iodine vapour was employed.

RESULTS AND DISCUSSION

Calibration curves

Fig. 1 shows the optical density of the molybdenum blue solution at 830 m μ vs. μ moles of choline, 2-chloro-choline and phosphorus. The figure shows that under the conditions of the test choline phosphomolybdate was soluble to a very small extent. An amount of 0.05 μ moles (6 μ g) of choline dissolved during the procedure; this figure represents the lower limit of detection. The phosphomolybdate of 2-chlorocholeline was completely insoluble, dissolving only on prolonged heating in 1 N sulphuric acid, which was the method applied in the determination of choline in phospholipids. In the preparation of the calibration curve it was dissolved, in the first instance, in 0.1 N sodium hydroxide; subsequent addition of sulphuric acid did not result in re-precipitation of the phosphomolybdate. It can be seen from the curves that any conversion of choline to 2-chlorocholeline during hydrolysis with hydrochloric acid does not introduce any error in the determination.

Fig.1.



Optical density of the molybdenum blue colour
vs. micromoles of substance.

From the three calibration curves it can be calculated that one mole of phosphorus produces the same colour density as 3.1 moles of choline and 3.04 moles of 2-chlorocholine. The molar ratio of choline and 2-chlorocholine to phosphorus in their respective phosphomolybdates is therefore 3. At this stage it should be mentioned that reduction of choline phosphomolybdate to molybdenum blue, without prior addition of excess ammonium molybdate did not produce a straight line calibration curve. In our initial experiments the addition of ammonium molybdate was omitted, but the blue colours produced were very weak and did not follow Beer's law. Wheeldon and Collins¹², however, obtained a linear calibration curve at 630 m μ on reduction of choline phosphomolybdate without the addition of ammonium molybdate, by means of stannous chloride in acetone - alcohol (1:2) solution.

Recovery of choline

Different quantities of choline chloride were added to a commercial cooking oil; the material was hydrolysed with 2N hydrochloric acid as described in the experimental section. The recovery of choline was shown to be approximately 100% (Table I).

TABLE I

RECOVERY OF CHOLINE ADDED TO A COOKING OIL

Choline added (%)	Choline recovered (%)	% Recovery
2.73	2.74	100
6.31	6.25	99
8.75	8.90	102
12.57	12.63	103

Interference by compounds forming insoluble phosphomolybdates

Many nitrogenous compounds are capable of forming phosphomolybdates¹³⁻¹⁵. Extremely insoluble phosphomolybdates are found in the class of quaternary ammonium compounds¹³⁻¹⁵. These latter compounds, if present, will interfere in the determination of choline. It is shown below that, apart from ammonia, which is dealt with separately, no interfering compounds were present in hydrolysates of phospholipids.

Hydrolysates of pilchard phospholipids revealed on paper chromatograms one or two compounds forming an insoluble phosphomolybdate. The major compound was choline; the second compound, which sometimes occurred in small quantities on hydrolysis with aqueous hydrochloric acid, was 2-chlorocholine⁶. The remainder of the nitrogenous compounds in the hydrolysate consisted of amino acids and ethanolamine (Chapter VII).

Only the basic amino acids lysine, histidine, arginine, the sulfur-containing amino acid cystine and ethanolamine were capable of precipitating a reasonably insoluble phosphomolybdate (cf.¹⁶). These precipitates, however, were formed with quantities far in excess of those found in the hydrolysates. It was nevertheless decided to test for a possible interference of these substances. It was found that a choline calibration curve prepared from choline, to which lysine, histidine, arginine, cystine and ethanolamine were added, was identical to a curve prepared from choline only. The quantities of amino acid added to choline were more than ten times the actual amount found in a hydrolysate, while ethanolamine was added in approximately twice this amount. Moreover, the determination of choline, in a hydrolysate before and after treatment with nitrous acid gave identical results. This is clearly shown in Table II.

Betaine, which, as pointed out by Lovern³, is a likely interfering substance in the precipitation of choline as its reineckate was not detected. The staining procedure of Brante¹¹ failed to detect betaine on chromatograms of pilchard phospholipid hydrolysates treated with nitrous acid.

Interference by ammonia

Ammonium phosphomolybdate precipitates only slowly, hence it does not appear as a spot on paper chromatograms which are prepared by dipping the paper in a phosphomolybdic acid solution. The precipitate is much more soluble than choline phosphomolybdate. A calibration curve prepared with ammonium chloride appeared to be approximately a straight line parallel to the choline and 2-chlorocholine curves but cutting the abscissa at 0.3μ moles. Serious discrepancies were observed if, for instance, 2μ moles of ammonium chloride were added to choline in the preparation of the calibration curve. No interference, however, was noticed if formaldehyde was added before the precipitation with phosphomolybdic acid. Hexamethylenetetramine, the reaction product of ammonia and formaldehyde, did not precipitate as a phosphomolybdate, at least under the conditions of the test. The results in Table II demonstrate that hydrolysis in an alkaline medium¹⁰ probably gave rise to ammonia formation.

Effect of acidity

The effect of acidity in the precipitation of choline phosphomolybdate was studied by preparing calibration curves in 0.1 N and 1 N hydrochloric acid solutions. The precipitate in the latter solution settled more quickly, but identical curves were obtained.

Upper limit of determination

The molybdenum blue colour produced in a phosphorus determination is, according to Bartlett⁷, proportional to the concentration of phosphorus up to 1.5μ moles (corresponding to 4.5μ moles of choline) in the reaction mixture. We observed above 5μ moles of choline deviations from Beer's law and prefer to work in the range 0.1 - 3μ moles of choline.

Choline determination in a phospholipid

The results obtained in the determination are shown in Table II. Hydrolysis with 2 N hydrochloric acid, which shows approximately 100% recovery of choline added to a commercial cooking oil (Table I), is preferred. It should be noted that hydrolysis in an alkaline medium gave lower results than in an acid medium. This is undoubtedly due to decomposition of choline under alkaline conditions; it is well known that the free base is susceptible to heat, in contrast to choline chloride¹⁷.

TABLE II

CHOLINE DETERMINATION IN A PILCHARD PHOSPHOLIPID
(% P:3.3; % N:1.7)

Hydrolytic procedure	% Choline	Average
2N HCl at 120° for 24 h	7.84, 7.93	7.9
(cf. Lovern, Olley and Watson ³)	7.93, 8.04 (addition of HCHO)	8.0
	7.88, 7.79, 7.82 (HNO ₂ treatment)	7.8
6 N HCl at 100° for 48 h	8.03, 7.92, 8.03	8.0
(Levine and Chargaff ⁴)	7.89, 8.06 (addition of HCHO)	8.0
	8.06, 8.06, 8.06 (HNO ₂ treatment)	8.1
Saturated Ba(OH) ₂ at 100°	7.36, 7.51, 7.40, 7.48	7.4
for 10 h; followed by	6.97, 7.13, 6.82 (addition of HCHO)	7.0
± 1 N HCl for 1 $\frac{2}{3}$ h at 100°	7.32, 7.28, 7.32 (HNO ₂ treatment)	7.3
(McKibbin and Taylor ¹⁰) ^a		
		Standard Deviation = 0.06

^a Determination of choline by the method of Entenman et al.² in this sample,
i.e. reineckate precipitation, gave figures ranging from 8.8 to 12.7.

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CHAPTER VII

THE PRESENCE OF AMINO ACIDS IN PHOSPHOLIPIDS

An investigation in our laboratory of the nitrogenous constituents of the phospholipids extracted from the flesh of the South African pilchard (*Sardina ocellata*, Jengyns) indicated the presence of at least 16 amino acids. The amino acid serine has long been recognized as a constituent of the cephalin fraction of phospholipids¹. The original claim that ornithine and hydroxylysine are constituents of *Myxobolus* lipid phospholipids has been withdrawn by Lelander^{2,3}. Many workers, however, have described the presence of a large variety of amino acids in different phospholipids⁴⁻⁹. It has been suggested that these amino acids are bound in peptide structure to the phospholipids^{4,5,8,9} ("lipopeptides"). Doubt as to the existence of lipopeptides has been expressed by Bode and Ludwig¹⁰ on the ground that peptide and phospholipid can be separated by means of paper chromatography. These authors suggest that peptides are solubilized by phospholipids. Macfarlane¹¹ and also Houtzwaller and van Deenen¹² have recently demonstrated that amino acids are incorporated in several bacterial phospholipids as O-amino acid esters of phosphatidyl glycerol. In our case it was not certain whether the amino acids were linked in ester or peptide formation to the phospholipids, or were to be considered as a polypeptide impurity. The evidence described in this paper shows that polypeptide material was responsible for the presence of all amino acids, except serine.

EXPERIMENTAL

ISOLATION AND AMINO ACID ANALYSIS OF THE PHOSPHOLIPIDS

Lipids of the pilchard were extracted with chloroform-methanol (2:1, by vol.). The water-soluble impurities were removed by two washings according to the method of Folch et al.¹³. After taking the material to dryness in vacuo it was taken up in light petroleum (b.p. 40-60°). The phospholipids were separated from the triglycerides and cholesterol esters by chromatography over silica gel as described by Lovern, Olley and Watson¹⁴. The phospholipid mixture so obtained was a brown resinous material freely soluble in ether, light petroleum or chloroform. The N and P contents were respectively 1.7 and 3.3%. No free amino acids were detected before hydrolysis, but paper electrophoresis following Westley, Wren and Mitchell⁵ at pH values of 3.7, 4.15 and 7.8 indicated the presence of peptides. An hydrolysate of this material revealed, by chromatography according to Moore and Stein¹⁵, the presence of ethanolamine and at least sixteen amino acids. The amino acids corresponded to 0.3% N. The aminograms are shown in Fig. 1.

EVIDENCE FOR POLYPEPTIDE IMPURITY

In two different ways it was shown that the phospholipids still contained polypeptide material.

- A. The percentage amino N of the phospholipids was determined before and after hydrolysis according to Wheelan and Collins¹⁶ with 1-fluoro-2,4-dinitrobenzene. The percentages were respectively 0.34 and 0.76. Hydrolysis therefore liberated free amino groups. Treatment of the unhydrolysed phospholipids with 1-fluoro-2,4-dinitrobenzene followed by hydrolysis with HCl gave rise to only two DNP-amino compounds. These two compounds were identified by paper chromatography in 4 different solvent systems as DNP-ethanolamine and DNP-serine. The different R_f values are listed in Table I. An indication of the presence of ϵ -DNP-lysine was obtained in two solvent systems.

B. A portion of the phospholipids was washed in etheral solution with 0.1 N HCl, a treatment which removes ninhydrin-reacting substances from phospholipids¹⁶. After evaporation of the ether the washed material, which contained 1.3% N and 3.3% P, was hydrolysed. This hydrolysate contained only two ninhydrin-reacting substances: ethanolamine (1.6%) and serine (0.2%). This is illustrated in Fig. 1. The material which had passed into the 0.1 N HCl was shown to contain the remainder of the amino acids.

These results indicate that of the ninhydrin-reacting substances, originally detected in the phospholipid mixture, only serine, ethanolamine and lysine possess a free amino group. Serine and ethanolamine are bound in ester linkage to the phosphatidyl radical, leaving the amino group free for reaction with 1-fluoro-2,4-dinitrobenzene. The other amino acids are derived from polypeptides, containing the free Σ -amino group of lysine. These polypeptides are not linked in ester or peptide formation to the phospholipids, since washing with 0.1 N HCl removes this material. It is postulated that a saltlike linkage is present, similar to that in the inositol-containing phosphatido-peptides of Folch and Le Baron^{20,21}, and Hawthorne²². The presence of many amino acids detected by other workers in phospholipid hydrolysates can probably be explained in a similar way.

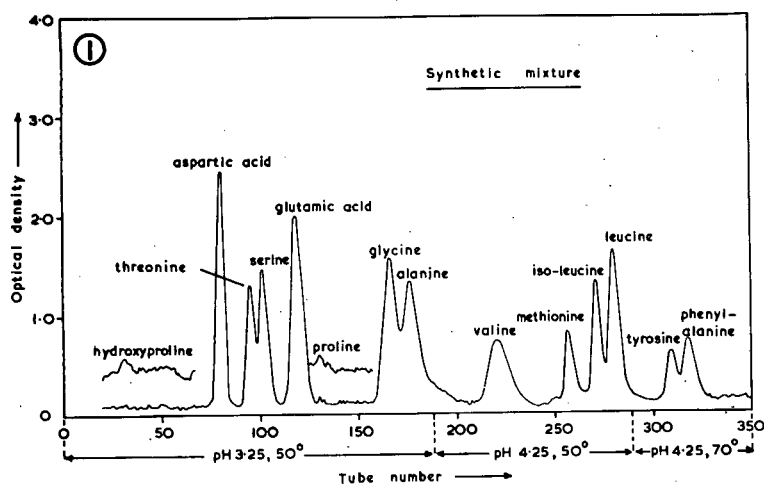
TABLE I

R_F VALUES OF DNP-ETHANOLAMINE, DNP-SERINE AND Σ -DNP-LYSINE IN
DIFFERENT SOLVENT SYSTEMS ON WHATMAN NO. 1 PAPER AT APPROX. 20°

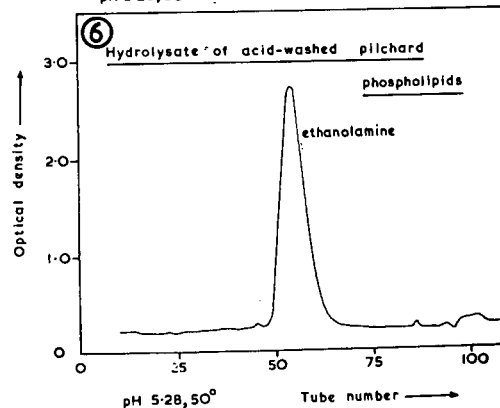
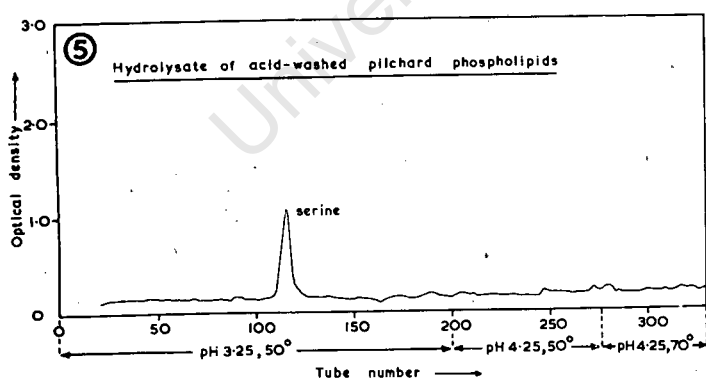
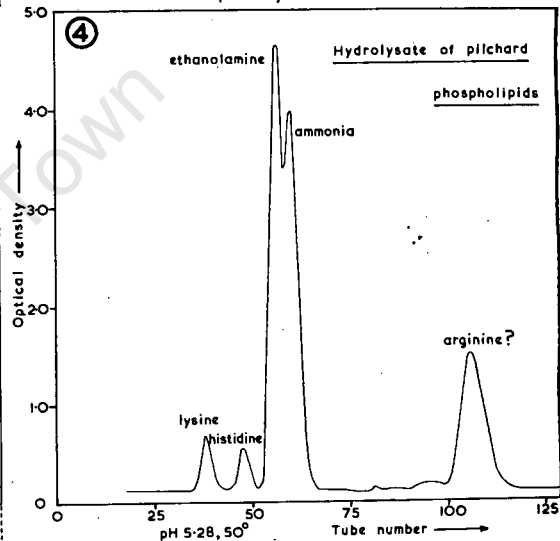
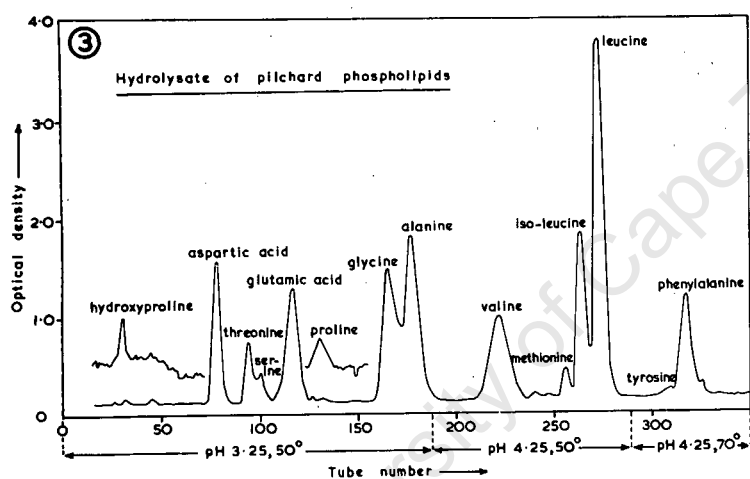
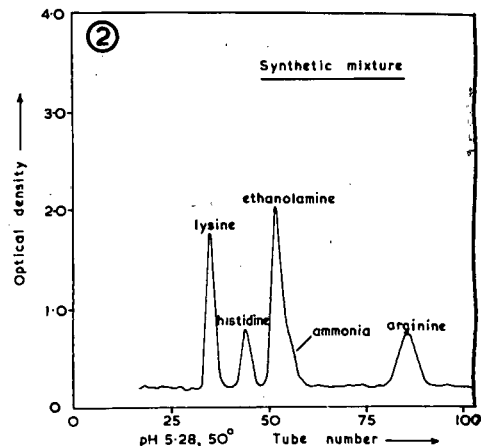
SOLVENT SYSTEM (by vol.)	DNP- ethanol- amine	DNP- serine	Σ -DNP- lysine	Hydrolysate DNP- pilohard phospholipids			REMARKS
1.5 N phosphate (Levy ¹⁷)	0.24	0.40	0.39	0.25	0.38		Ascending
tort-Pentanol-pyridine-water (5:1:5, upper layer) (Collins and Whealden ¹⁸)	0.97	0.61	-	0.97	0.60		Ascending
Toluene-pyridine-2-chloro-ethanol-0.8 N NH ₄ OH (5:1:3:3, upper layer) (Biserte and Osteux ¹⁹)*	0.94	0.04	0.10	0.94	0.04	0.10 (weak)	Descending, temp. 29°
	0.93	0.17	0.21	0.94	0.15		Descending, temp. 18°
Phenol-isoamylalcohol-water (1:1:1, upper layer) (Biserte and Osteux ¹⁹)	0.96	0.24	0.83	0.93	0.29	0.84 (weak)	Descending

* The R_F values of DNP-serine and DNP-lysine and consequently their separation in this solvent system are very temperature dependant.

FIG. 1. CHROMATOGRAMS OF THE ACIDIC AND NEUTRAL AMINO ACIDS.



CHROMATOGRAMS OF THE BASIC "AMINO ACIDS."



Hydrolysate of (3) and (4) was obtained by hydrolysis of pilchard phospholipids with 6 N HCl at 115° for 48 h. Amino acids are present and note the large quantity of ammonia. Hydrolysate of (5) and (6) was obtained by hydrolysis of acid-washed pilchard phospholipids with 2 N HCl at 120° for 24 h. Note the absence of amino acid impurities and ammonia. Details on pp. 66, 72 and 73.

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CHAPTER VIII

PHOSPHOLIPIDS OF THE HAKE

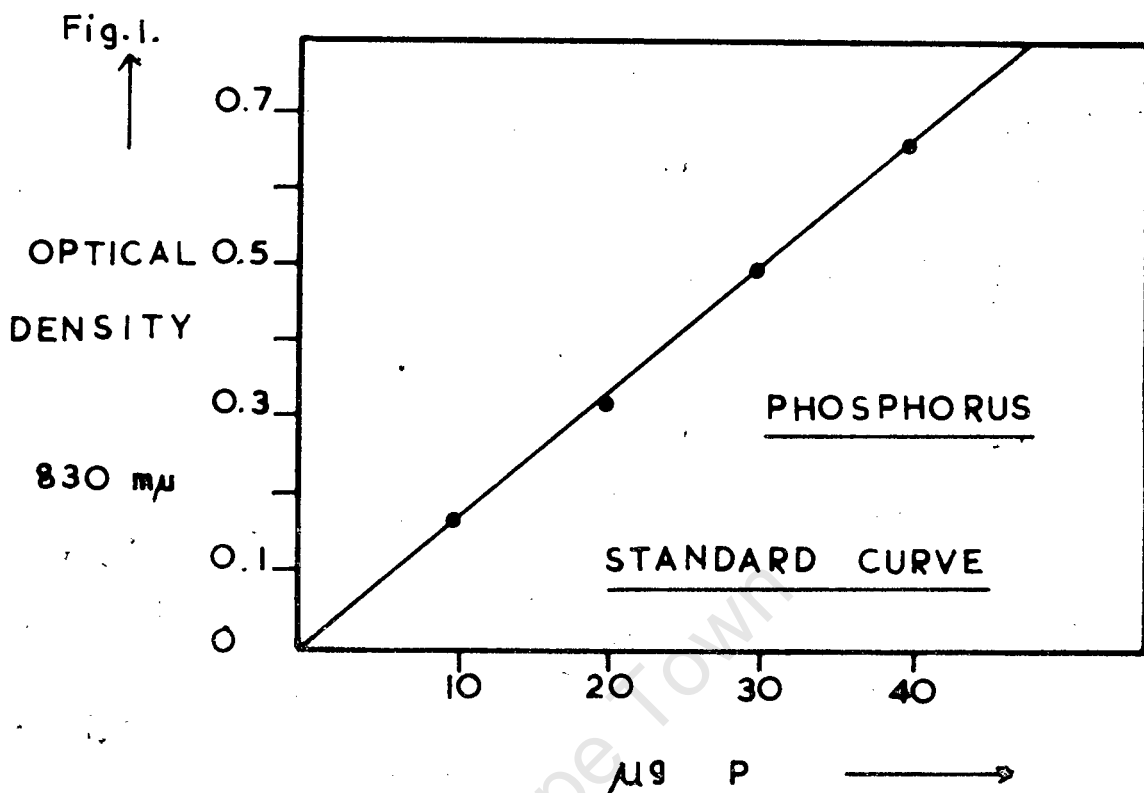
This chapter describes the analysis of the phospholipids extracted from the flesh and from the liver of the South African hake (*Merluccius capensis*, Castelnau). The composition of the phospholipids was determined by means of chromatography on silicic acid and by analysis of the hydrolytic cleavage products. The fatty acid distribution in the phosphatidyl choline, the cephalin and the sphingomyelin fractions was determined by gas chromatography of the fatty acid methyl esters. In addition the capacity of a phosphatidyl choline fraction to stabilize a supersaturated solution of cholesterol in a triglyceride oil is demonstrated. The fatty acid composition of hake liver oils has been investigated by van Rensburg, Rapson and Schwartz¹. Their work deals mainly with the triglycerides; the phospholipids were not isolated and studied. Phospholipids extracted from the flesh of the cod (*Gadus callarias*) and haddock (*Gadus aeglefinus*), species similar to the hake, have been investigated by Olley and Lovern² and by Garcia, Lovern and Olley³. These investigations together with reports concerning the phospholipid composition of other fish have been reviewed by Lovern⁴.

EXPERIMENTAL

Method

Operations were carried out under an atmosphere of carbon dioxide, produced by adding "dry ice" to all extracts. All lipids were stored in solution (chloroform or light petroleum) at -20° . Solvents were removed in vacuo in a rotary evaporator below 40° , the last traces of solvent were removed with a vacuum pump provided with a cold trap (-90°) at about 0.5 mm Hg.

Phosphorus was determined according to Bartlett⁵. The standard curve of the optical density of the molybdenum blue colour at 830 m μ versus μ g P is shown in Fig. 1.



Duman nitrogen was determined at the Chemistry Department of the University of Cape Town.

Extraction of the total lipids

Hake Flesh

The edible portion (5000 g, August) of fresh hake, caught during the previous 24 h, was extracted with 5 l chloroform:methanol (2:1, by vol.) in a Waring Blender. The mixture was filtered over a Buchner funnel and the extraction was repeated twice with a further 5 l chloroform:methanol. The combined extracts were washed twice at 0° according to the method of Folch et al⁶ in order to remove water soluble impurities. After dissolving the fluffy material which had separated at the interface with approx. 300 ml ethanol, the extracts were filtered. The chloroform was evaporated in a rotary evaporator at reduced pressure and below 40°. The weight of the brown oil obtained was 77.3 g with a P content of 1.2%.

Hake liver

Fresh hake livers (1000 g, March) were extracted four times in a Waring blender with 900 ml chloroform:methanol (2:1, by vol.). The extracts were treated as described for hake flesh. 460 g of a brown oil with a P content of 0.14% was obtained.

Separation of the phospholipids from the non-phosphorylated lipids

The phospholipids were separated from other lipids by chromatography in light petroleum on silica gel, a method introduced by Lovern, Olley and Watson⁷. These authors found that phospholipids dissolved in light petroleum are not absorbed by silica gel (B.D.H.) in contrast to other lipids. In our case several passages over silica gel columns were necessary in order to obtain phospholipids free from other lipids. The chromatography was stopped when no more material was absorbed by passage over silica gel. This is illustrated in the following experimental details.

Hake flesh

Silica gel (150 g, B.D.H. activated at 120° for 4 h) was slurried with 200 ml light petroleum (b.p. 40 - 60°) and packed into a column of 3.3 cm diameter. The mixture of total lipids (77.1 g) dissolved in 50 ml light petroleum (b.p. 40 - 60°) was carefully run into the top of the column. The "phospholipids" were eluted with 300 ml of the same solvent. The solvent was removed in vacuo below 40°, the weight of the material obtained was 56.5 g. This separation procedure was repeated three times, each time with a fresh amount (150 g) of silica gel; the weight of the "phospholipids" which passed unabsorbed through the column decreased from 56.5 to 34.4 g, from 34.4 to 23.1 g and from 23.1 to 22.9 g, whereupon the separation was considered complete. The final weight of the phospholipids, obtained as a brown resinous material, was 22.9 g with a P content of 3.7% and a N content of 1.8%. The non-phosphorylated lipids were recovered from the silica gel columns by washing with excess diethyl ether. The ether extracts were combined and after evaporation of the ether in vacuo 52.1 g of a yellow oil was obtained with P content of 0.03%.

Halibut liver

225 g of the total lipids were separated into 7.1 g phospholipids (P content 3.5%, N content 1.5%) and 139.1 g non-phosphorylated lipids (P content 0.01%), by chromatography on 10 columns of activated silica gel.

Determination of the phospholipid composition

The phospholipid composition was determined in three ways. Firstly, the phospholipid mixture was hydrolysed and the cleavage products identified and determined. Secondly, the mixture was separated into different components by chromatography on silicic acid impregnated paper. The components were characterized by appropriate reactions and quantitatively estimated by staining with Edicol Supra Ponceau 4 BS according to Hooghwinkel and van Niekirk⁸. Thirdly, the mixture was separated into fractions by column chromatography on silicic acid, following Hanahan, Dittmer and Harashina⁹. The first two methods of analysis are inter-dependent.

Hydrolytic procedure

For the identification and determination of the water soluble compounds choline, ethanolamine, serine and myo-inositol, the hydrolysis was accomplished with 2 N HCl at 120° for 24 h as described in Chapter VI.

Choline

This compound was detected and determined as described in Chapter VI. Choline was also characterized in the unsaponifiable material of pure phosphatidyl choline fractions (see under Fatty Acids). The solvent of the unsaponifiable fraction was removed in vacuo and the residue taken up in 5 ml water and filtered. Water was also evaporated and the residue treated with 1 ml of a saturated alcoholic solution of picric acid. After standing overnight at room temperature the crystals were filtered off and washed with a small quantity of alcohol; m.p. 247 - 249°. The m.p. of choline picrate was raised to 250 - 251° after one recrystallization from alcohol. Melting point, mixed melting point and infra-red spectra of authentic choline picrate were identical to the constants of the isolated substance.

The proportions of lecithin-choline, sphingomyelin-choline and lysolecithin-choline were determined by means of chromatography on silicic acid impregnated paper, as described in Chapter XI.

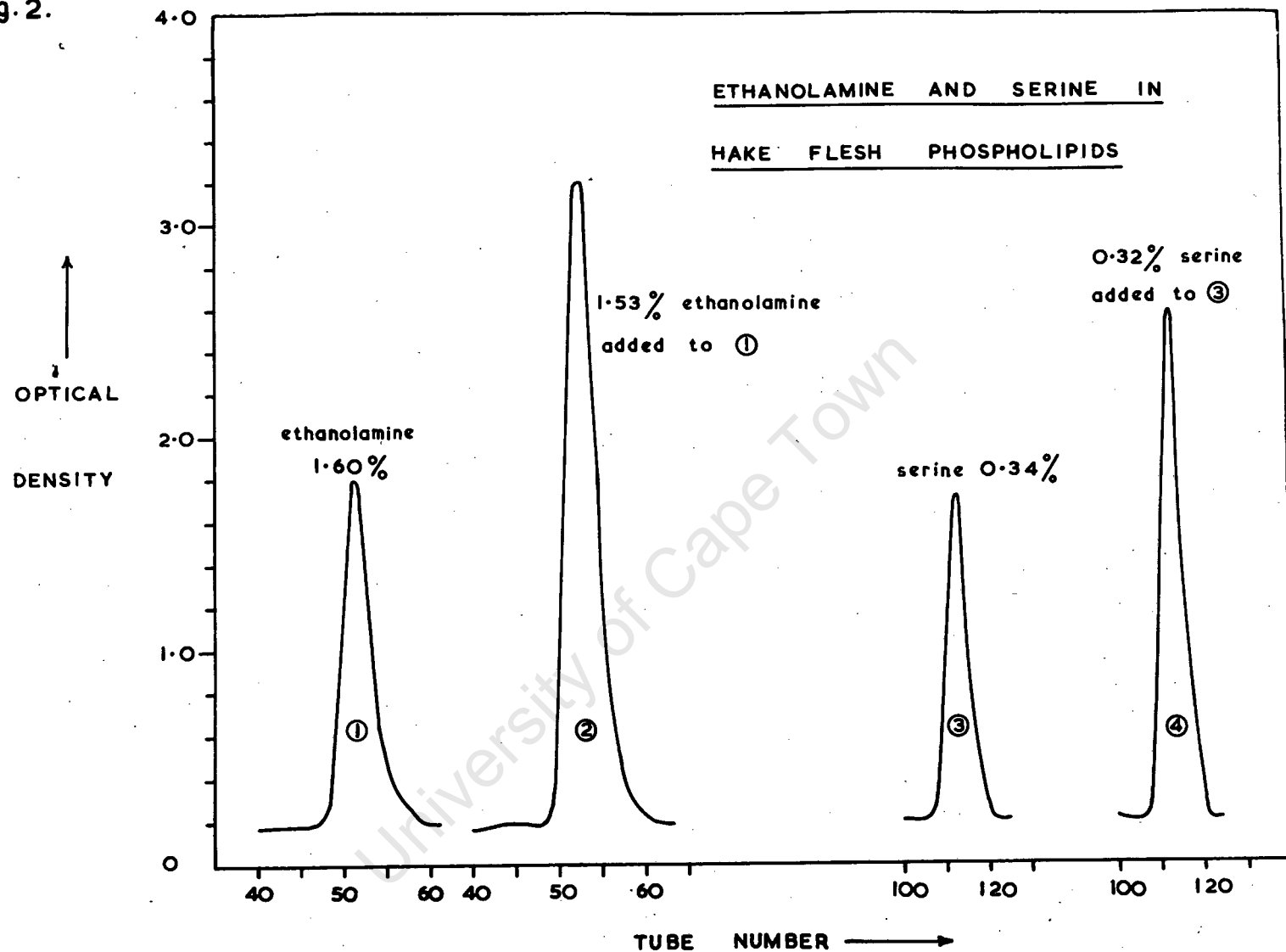
Ethanolamine and serine

These substances were identified as ninhydrin-reacting spots with respective R_f values of 0.22 and 0.17 on paper chromatograms developed with a descending solvent system of butanol:acetic acid:water (4:1:5, by vol., top layer). In contrast to our experience with pilchard phospholipids (Chapter VII) only traces of other ninhydrin-reacting substances were detected. These impurities were completely removed by washing the phospholipid mixture in ether solution with 0.1 N HCl.

Ethanolamine and serine were quantitatively determined by means of a column chromatographic separation on a cation exchange resin (Amberlite C.G. 120 Type 2) according to a modified Moore and Stein procedure¹⁰.

For the determination of ethanolamine the hydrolysate (containing 100 - 1000 μ g ethanolamine) was placed onto a short column (30 cm, ϕ 0.9 cm) and ethanolamine was eluted at 50° with a citrate buffer of pH = 5.28. Fractions of 2 ml were collected with an automatic fraction collector. Tubes 30-80 were treated with ninhydrin (4 ml of a 2% solution) and the colour developed at 100° for 20 min. The optical density of the colour was read after dilution with iso-propanol:water (3:7, by vol.) in an Evelyn colorimeter at 565 m μ . Under the conditions described ethanolamine was eluted in tubes 50-60; the extra tubes analysed served to obtain a blank value for the ninhydrin colour (see Fig. 2 and also Fig. 1, Chapter VII). Ethanolamine was quantitatively determined by plotting optical density vs. tube number and comparing the peak area with that of a known amount of redistilled ethanolamine (B.D.H. b.p. 64/4 mm n_D^{20} = 1.4538 lit.¹¹ 1.4539) or - more conveniently -²⁰ by summation of optical densities. It was necessary to treat ethanolamine in the same way as the unknown sample i.e. "hydrolyse" a weighed quantity with 2 N HCl at 120° for 24 h before chromatography. In addition a known quantity of ethanolamine was usually added to a sample and the increase in peak area determined; this served as a conclusive identification of ethanolamine.

Fig. 2.



Recovery of ethanolamine and serine added to hake flesh phospholipids. Ethanolamine was eluted from a 30 cm column of Amberlite C.G. 120 at 50° with a buffer of pH = 5.28. Serine was eluted from a 125 cm column of the same resin at 50° with a pH = 3.25 buffer.

Serine (100 - 500 μ g) was eluted on a long column (125 cm, ϕ 0.9 cm) with a citrate buffer of pH = 3.25 at 50°. Fractions of 1 ml were collected and tubes 90 - 140 were treated with 2 ml of the ninhydrin reagent and analysed as described for ethanolamine. DL-serine (California Foundation for Biochemical Research found: C, 34.35; H, 6.77; $\text{C}_3\text{H}_7\text{O}_2\text{N}$ requires C, 34.29; H, 6.72) served as standard compound. A serine recovery test was also included.

Typical recoveries are shown in Fig. 2 and Table I.

TABLE I

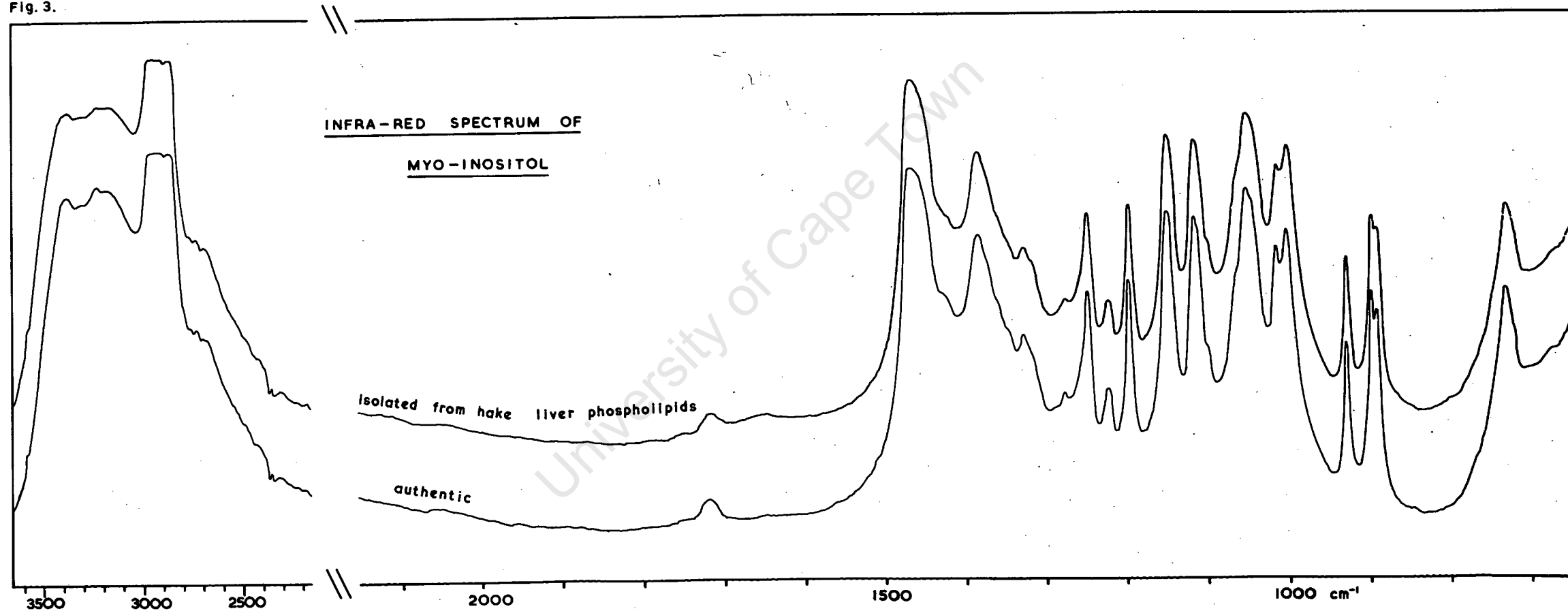
RECOVERY OF ETHANOLAMINE AND SERINE
ADDED TO PHOSPHOLIPIDS

SAMPLE	% present		% added		Total % determined		% recovery	
	Ethanol- amine	Serine	Ethanol- amine	Serine	Ethanol- amine	Serine	Ethanol- amine	Serine
hake flesh	1.60	0.34	1.53	0.32	3.07	0.69	98	105
hake liver	1.83	0.39	-	0.10	-	0.47	-	96
rock lobster hepato- pancreas	1.93	0.36	0.50	1.05	2.42	1.42	100	101
rock lobster roe	1.74	0.10	0.66	-	2.58	-	108	-

Myo-inositol

Myo-inositol was identified on paper chromatograms with a descending solvent system of acetone:water (4:1, by vol.). The air dried chromatogram was dipped in 50 ml aqueous acetone containing 2.5 ml saturated aqueous silver nitrate, and dried for 5 min at 60° following the method of Anet and Reynolds¹². The papers were then dipped in 1% sodium hydroxide in acetone:water (1:1, by vol.) and the spots allowed to develop for approximately 3 min in air. The chromatograms were fixed in 10% aqueous sodium thiosulphate and washed in running water before drying. The R_f value in this solvent system was 0.40.

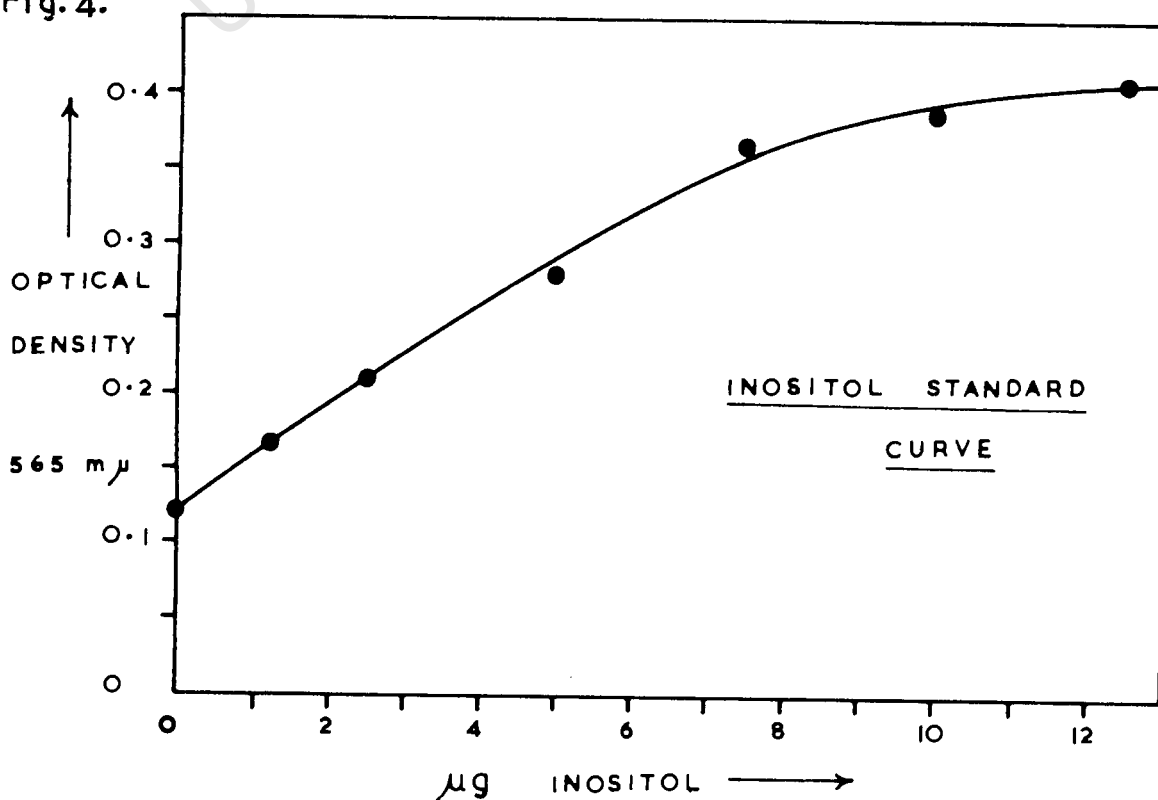
Fig. 3.



In the case of hake liver myo-inositol was isolated as a crystalline material of m.p $223-226^{\circ}$, undepressed by mixing with authentic myo-inositol (Lit.¹¹ : 225°). The infra-red spectra of the two compounds were also identical, this is clearly shown in Fig. 3. The isolation of myo-inositol was accomplished by hydrolysis of the lipids with 6 N HCl at 110° for 48 h, followed by removal of the fatty acids by filtration and HCl by evaporation in a rotary evaporator. The residue was taken up in water and passed over a mixed cation - anion exchange resin. The eluate containing myo-inositol was obtained crystalline by the addition of a small quantity of ethanol. The percentage yield of myo-inositol was about 40%.

Myo-inositol was assayed microbiologically with *Saccharomyces Carlsbergensis* (supplied by the South African Bureau of Standards). Full details of this method are to be found in the literature (e.g. Gyorgy¹³, see also Taylor and McKibbin¹⁴). Determinations were carried out in 2.5 x 17 cm test tubes with a B24 ground glass stopper. The standard inositol solution contained 2.5 μ g myo-inositol and 25 μ g choline per ml. Inositol solutions ranging from 0 to 12.5 μ g and unknown samples in triplicate were pipetted into the tubes. Sixteen tubes were conveniently done in one batch. Basal growth medium was added, the tubes were stoppered, sterilized by steaming for 10 min and inoculated with the yeast. After shaking the tubes overnight at 30° , the optical density of the turbid solution was read at 565 m μ in an Evelyn photoelectric colorimeter. A typical standard curve is shown in Fig. 4.

Fig. 4.



It has been shown by different workers¹⁵ that this method is specific for myo-inositol, but it was nevertheless decided to test (+) - inositol ($\frac{124}{356}$ - isomer) for its vitamin activity. (+)-Inositol was prepared from pinitol penta-acetate, which is the 3-methyl ether of (+)-inositol penta-acetate. Pinitol penta-acetate (m.p. 93-97°, Lit.¹⁶ 96-98°, 110 mg, supplied by Prof. A.M. Stephen), was deacetylated with 50 ml boiling 0.1 N sodium methoxide under reflux for 2 h. Methanol was evaporated in vacuo, the residue acidified with 5 ml 1 N HCl and passed over 5 g cation exchange resin. Water was removed in vacuo and the residue gave crystalline pinitol (40 mg; m.p. 177 - 181° Lit.¹⁶ 184 - 186°) after trituration with acetone. Pinitol (40 mg) was converted into (+)-inositol by treatment with 1 ml HI (s.g. 1.70) for 40 min over a small flame. Water and HI were removed in vacuo and the residue was recrystallized twice from 90% ethanol. (+)-Inositol (12.8 mg; m.p. 244 - 246°, Lit.¹⁶ 244°) was obtained as an off-white crystalline material, which did not sustain the growth of *S. Carlsbergensis* in quantities of 7 and 24 μ g.

Sphingosine

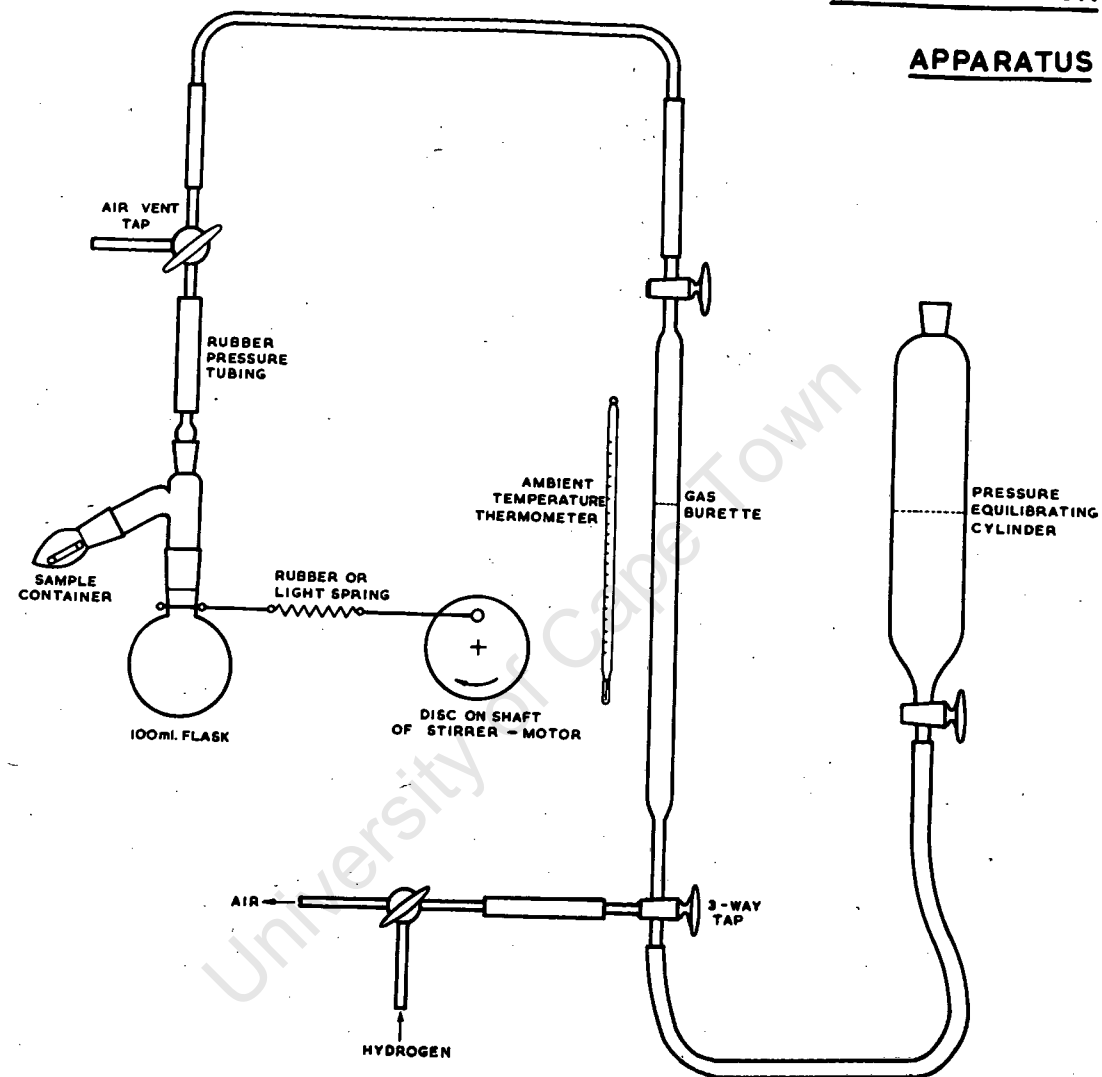
This substance was liberated by hydrolysis with 2 N methanolic-HCl according to Sweeley and Moscatelli¹⁷. The base was dissolved in chloroform and retained for identification by ascending paper chromatography with a solvent system representing the upper phase of butanol:acetic acid:water (4:1:5, by vol.) mixture. The chromatograms were dried in air for 0.5 h dipped in 0.25% sodium fluorescein according to Saito¹⁸ and washed 1 h in running water to reveal the sphingosine as an orange-pink spot with $R_F = 0.87$.

Sphingosine was initially determined according to McKibbin and Taylor¹⁹, but this method proved unreliable in our laboratory. It was subsequently estimated indirectly by determining the percentage sphingomyelin-choline. This will be described in Chapter XI.

Fig. 5.

HYDROGENATION

APPARATUS

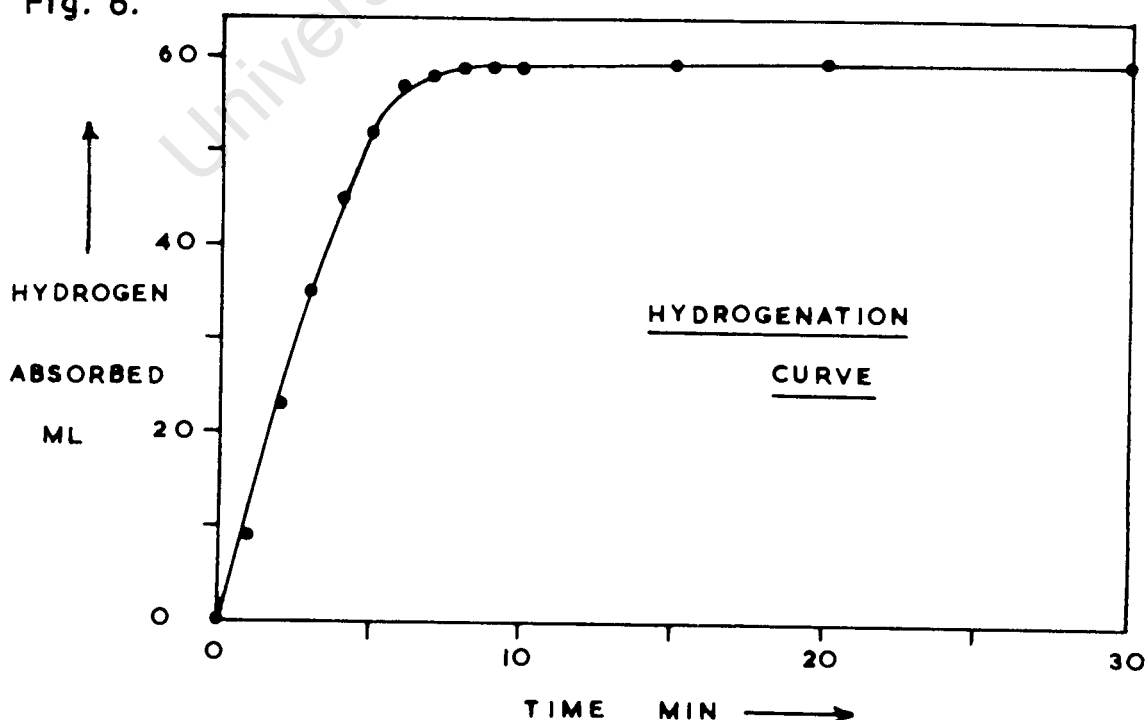


Fatty acids

Phospholipids were transmethylated with anhydrous methanol saturated with hydrogen chloride according to the method of Böttcher, Woodford, Boelma van Houte and van Gent²⁰. The unsaponifiable material was used for the identification of choline as choline picrate of m.p. 250 - 251° when pure lecithin fractions were hydrolysed. The methyl esters were saponified with 0.5 N ethanolic potassium hydroxide and the unsaturated fatty acids were retained for hydrogenation. Non-phosphorylated lipids were saponified with 0.5 N ethanolic potassium hydroxide without prior transmethylation. The unsaponifiable material in this case was used for the identification and determination of cholesterol of m.p. 145 - 147°. An anti-oxidant (3,5-di-tert. butyl-4-hydroxytoluene or hydroquinone; 2.5% of the weight of the lipids) was added to all refluxing solutions to prevent oxidation of unsaturated fatty acids.

The average unsaturation of the fatty acids was determined by quantitative hydrogenation in ethanol over a 2.5% Pd/BaSO_4 catalyst. The hydrogenation apparatus used was made in our laboratory and is shown in Fig. 5. The rate of hydrogen absorption depended on the rate of shaking, but it was usually complete within 60 min. A typical hydrogenation curve is shown in Fig. 6.

Fig. 6.



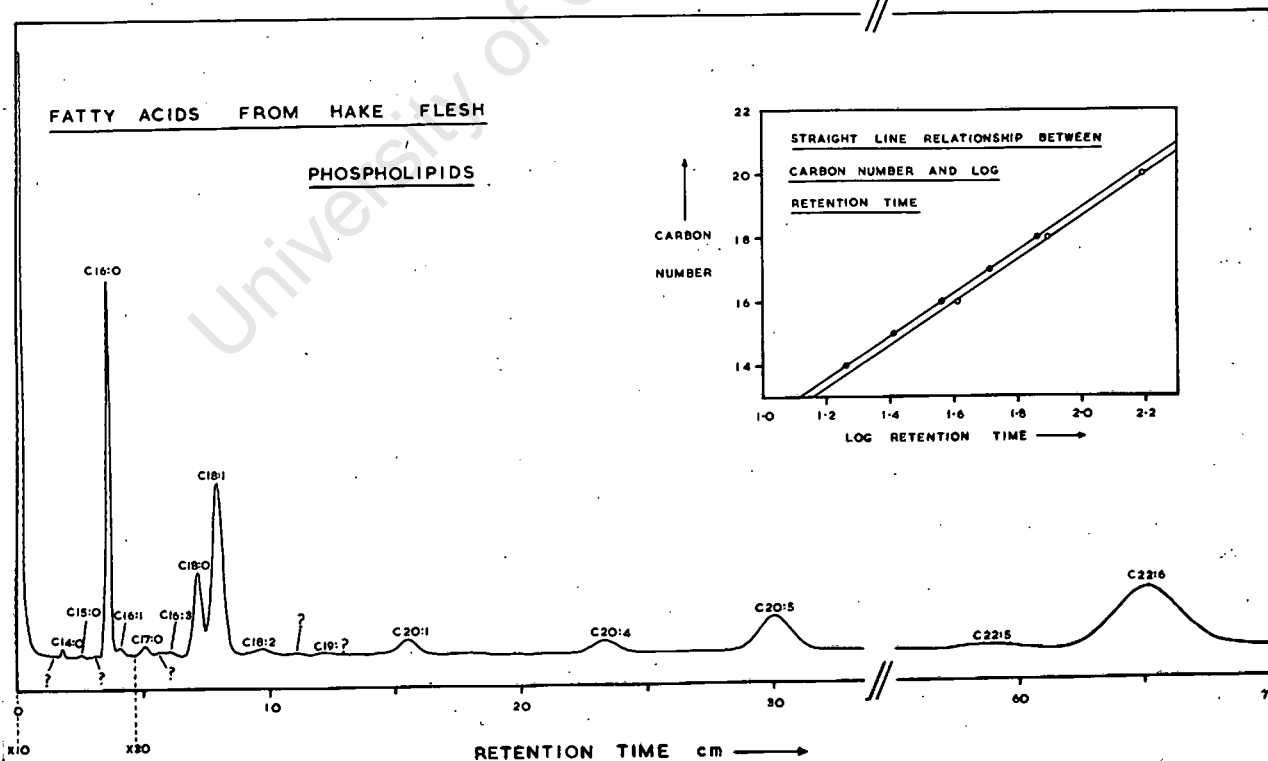
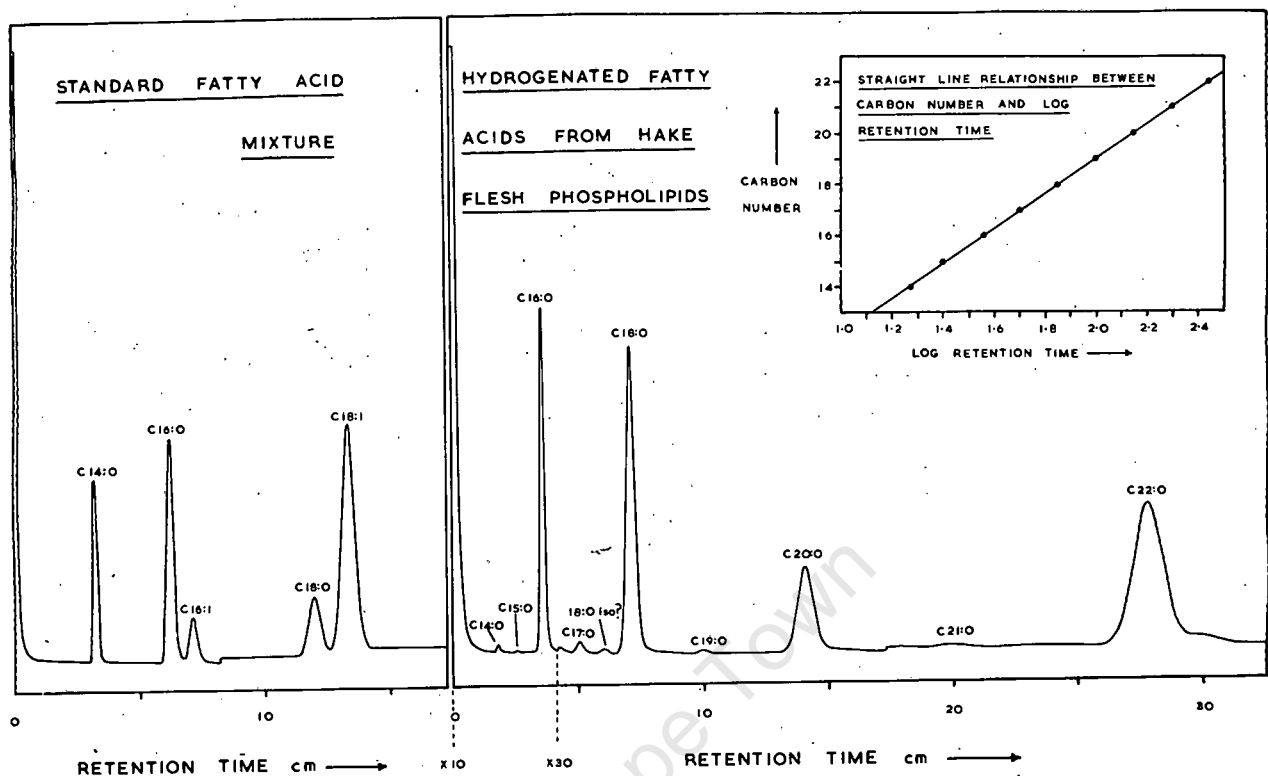
The hydrogenation reaction was carried out in duplicate and the results were in excellent agreement. For instance 261.5 mg and 302.9 mg hake flesh phospholipid fatty acids absorbed 54.9 and 64.3 ml hydrogen at NTP respectively; that is 0.210 ml/mg and 0.212 ml/mg, a deviation of only 1%. (The average deviation was 2%). The catalyst was filtered off on Whatman No. 42 paper and the alcohol removed in vacuo. The saturated fatty acids were dried in a vacuum desiccator over phosphorus pentoxide, and quantities of approximately 25 mg were titrated with 0.01 N sodium hydroxide in 20 ml hot methanol with phenolphthalein as indicator for the determination of the average equivalent weight. The accurate titre of the sodium hydroxide was determined by titrating known quantities of purified palmitic acid (this acid was purified by careful fractionation of methyl palmitate at approximately 5 mm Hg; its freezing or setting point - which can be determined more accurately than the melting point - was 62.3° Lit.²¹ 62.6°. Gas chromatography of the methyl ester showed only one peak).

The mean of five titrations was taken; the accuracy with which the equivalent weight could be obtained was usually 3%. The average unsaturation expressed in double bonds per molecule of fatty acid was calculated from the result of the hydrogenation and the equivalent weight. For example, the average equivalent weight of the hydrogenated fatty acids liberated from hake flesh phospholipids was 330 ± 9 ; the average unsaturation is therefore:

$$\frac{54.9}{22.4} \times \frac{330}{261.5 + 4.9} = 3.04 \text{ double bonds/molecule}$$

The complete composition of the fatty acid mixture was determined by gas chromatography of the methyl esters prepared from the unsaturated and saturated fatty acids. Methyl esters were prepared in more than 90% yield by refluxing for 30 min with approximately 0.06 N methanolic hydrogen chloride in the presence of an antioxidant (hydroquinone) according to Böttcher et al.²⁰. Gas chromatography was carried out at the Medical School of the University of Cape Town. A Barber Colman instrument was used with a polyethylene glycol glutarate stationary phase at 190° with Argon as carrier gas. An example of a graph obtained is shown in Fig. 7.

FIG. 7. Separation of fatty acid methyl esters by gas chromatography.



The fatty acids were identified by means of a standard mixture, literature data about relative retention times²², and the straight-line relationship between log retention time and carbon number. This last mentioned relationship was found to hold good for the saturated and for the monocyclic fatty acids; those with two or more double bonds showed discrepancies.

Cholesterol

This was identified and determined in the unsaponifiable fraction of the non-phosphorylated lipids (see under Fatty acids). After removal of light petroleum and one recrystallization from ethanol pure cholesterol of m.p. 145 - 147° (Lit.¹¹ 148.5°) was obtained. No depression of the m.p. was observed when mixing the material with authentic cholesterol, moreover the infra-red spectra of the two substances were identical. Cholesterol was quantitatively estimated in the unsaponifiable fraction by precipitation with digitonin²³.

Sugars

150 - 1000 mg of the phospholipid mixture was hydrolysed with 10% HCl for 30 min at 100° following the procedure of Klenk²⁴. The mixture was filtered and after removal of HCl in vacuo at 50° the residue was taken up in 5.0 - 10.0 ml H₂O and retained for analysis. Paper chromatographic examination of these fractions was done with a descending solvent system of n-propanol:H₂O (4:1, by vol.). Sugars were detected by spraying with aniline phthalate in water-saturated butanol (2% solution), followed by heating at 105° for 10 min. Standard glucose, galactose and mannose had R_F values of 0.28, 0.25 and 0.34 respectively.

Stabilization of a supersaturated cholesterol solution

These experiments were carried out by Dr. J.A. Wilkens at the Medical School of the University of Cape Town. Phosphatidyl choline fractions were tested for their capacity to stabilize a supersaturated solution of cholesterol in a triglyceride oil. The test consists briefly in comparing the crystallization rate of cholesterol in a cholesterol-triglyceride solution with that of an identical solution to which a small quantity of phosphatidyl choline was added. For experimental details we refer to the work of Wilkens and Krut²⁵.

Chromatography on silicic acid impregnated paper

Filter paper sheets (Whatman No. 2, 25 x 12 cm) were impregnated with silicic acid following the instructions of Hack²⁶. To each of four spots 2.5 cm from the lower edge of the paper were applied 0.02 - 0.04 ml of a 1% solution of the phospholipid mixture in chloroform. Chromatography was carried out according to Marinetti, Erbland and Kochen²⁷ with an ascending solvent system of di-isobutyl-ketone:acetic acid:water (24:15:2, by vol.) for 7 h. This solvent system contains less water than that of Marinetti et al (24:15:3, by vol.) but it gave better separations. The ninhydrin and phosphomolybdic acid reagents of Lea, Rhodes and Stoll²⁸ were used to distinguish between cephalins and compounds containing choline. The fuchsin-sulphurous acid reagent of Hack and Ferrans²⁶ was used for detection of plasmalogens. The quantitative composition of the phospholipid mixture was obtained by staining the developed papers with a solution of 0.01 N HCl containing 0.005% Edicol Supra Ponceau 4RS and 0.2% uranyl nitrate, following Hooghwinkel and van Niekark⁸. Red spots were obtained for cephalins, phosphatidyl choline, sphingomyelin and lyso phosphatidyl choline. Inositol phosphatides and cardiolipin (phosphatidic acids) do not show up by this procedure. The spots were cut out, the dye was eluted with 3 ml tert. butanol: 1.2 N HCl (1:1, by vol.) and the optical density measured at 510 m μ in a Unicam SP 500 spectrophotometer. The molecular percentage of each compound was calculated by expressing its optical density as a fraction of the sum of the optical densities. A correction for the presence of phosphatidyl inositol (from hydrolytic procedure) and cardiolipin (from column chromatographic results) was applied. Molecular percentages were converted to weight percentages.

Column chromatography on silicic acid

The separation of hake flesh and hake liver phospholipids into component fractions by column chromatography on activated silicic acid was carried out essentially according to Hanahan, Dittmer and Warashina⁹. The columns (3.3 cm diameter) were packed with 100 g silicic acid (Mallinckrodt A.R. activated for 16 h at 120°), which was slurried with 200 ml chloroform:methanol (7:1, by vol.).

The chloroform contained 2% ethanol as preservative. The columns were washed with 100 ml of chloroform:methanol (7:1, by vol.) before use. The phospholipid mixture (1.5 - 2.0 g) was placed on the column and the classes of phospholipids eluted with a series of chloroform:methanol mixtures, i.e. 7:1, 4:1, 3:2, 1:4 and 1:10 (by vol.). The columns were pressurized with nitrogen at 14 cm Hg and 15 ml fractions were collected with an automatic fraction collector. The progress of the fractionation was followed by analysis of P on 0.5 ml aliquots of each tube. Fractions belonging to a chromatographic peak were combined, the solvent evaporated in vacuo and weighed. A small portion of the material (5-40 mg) was used to identify each peak by paper chromatography after hydrolysis. Additional identification was obtained by chromatography of the intact phospholipid fraction on silicic acid impregnated paper.

The fatty acid composition of the phosphatidyl choline, cephalin and sphingomyelin fractions was obtained by gas chromatography.

RESULTS AND DISCUSSION

Hydrolytic procedure

In Table I are listed the analytical results of hake flesh and hake liver phospholipids.

TABLE I

MAIN CONSTITUENTS OF HAKE FLESH AND HAKE LIVER PHOSPHOLIPIDS

		<u>Hake Flesh (%)</u>	<u>Hake Liver (%)</u>
P	($\pm$ 4%)	3.7	3.5
N	($\pm$ 10%)	1.8	1.5
total choline	($\pm$ 1%)	9.56	9.15
lecithin-choline	($\pm$ 5%)	8.57	7.36
sphingomyelin-choline	($\pm$ 25%)	0.73	1.33
lyso lecithin-choline	($\pm$ 30%)	0.26	0.46
sphingosine	($\pm$ 25%)	1.81	3.29
ethanolamine	($\pm$ 8%)	1.60	1.83
serine	($\pm$ 6%)	0.34	0.39
inositol	($\pm$ 10%)	1.16	0.97
sugars		not detected	

TABLE II

AVERAGE EQUIVALENT WEIGHT AND UNSATURATION OF THE
FATTY ACIDS LIBERATED FROM HAKE PHOSPHOLIPIDS
AND CORRESPONDING NON-PHOSPHORYLATED LIPIDS

SOURCE	Equivalent weight ($\pm 3\%$)		Unsaturation (double bonds/molecule) ($\pm 5\%$)	
	Total phospholipids	Non-phosphorylated lipids	Total Phospholipids	Non-phosphorylated lipids
hake flesh	324	301	3.1	1.7
hake liver	322	298	2.9	2.1

Table II shows the average equivalent weight and the average unsaturation (double bonds per molecule) of the phospholipids as compared with the corresponding non-phosphorylated lipids. It can be clearly seen that the latter have a markedly lower equivalent weight and unsaturation. This phenomenon has been observed by many other workers (cf. Hilditch²⁹). The composition of the two phospholipid mixtures, calculated from the data of Tables I and II, is shown in Table III under hydrolytic procedure.

It is assumed in the calculation that the fatty acids are evenly distributed over the different classes of phospholipids. This is not correct, as will be shown in the present investigation (cf. Lovern³⁰), but the error introduced is small (see Table IV). Plasmalogens can be ignored since almost theoretical amounts of fatty acids were obtained after hydrolysis. Gray and MacFarlane³¹ detected only about 3% plasmalogens in trout flesh phospholipids and haddock flesh phospholipids contain about 2% plasmalogens (Olley and Lovern²). In our investigations an indication for the presence of small amounts of plasmalogens in the lecithin and cephalin fractions was obtained by a positive Schiff reaction (cf. Hack and Ferrans²⁶) on silicic acid impregnated paper chromatograms. The molecular weight of a "fatty aldehyde" is however not much different from that of a fatty acid and this is therefore hardly a source of error.

TABLE III

COMPOSITION OF HAKE FLESH AND HAKE LIVER PHOSPHOLIPIDS
(as per cent of total phospholipids)

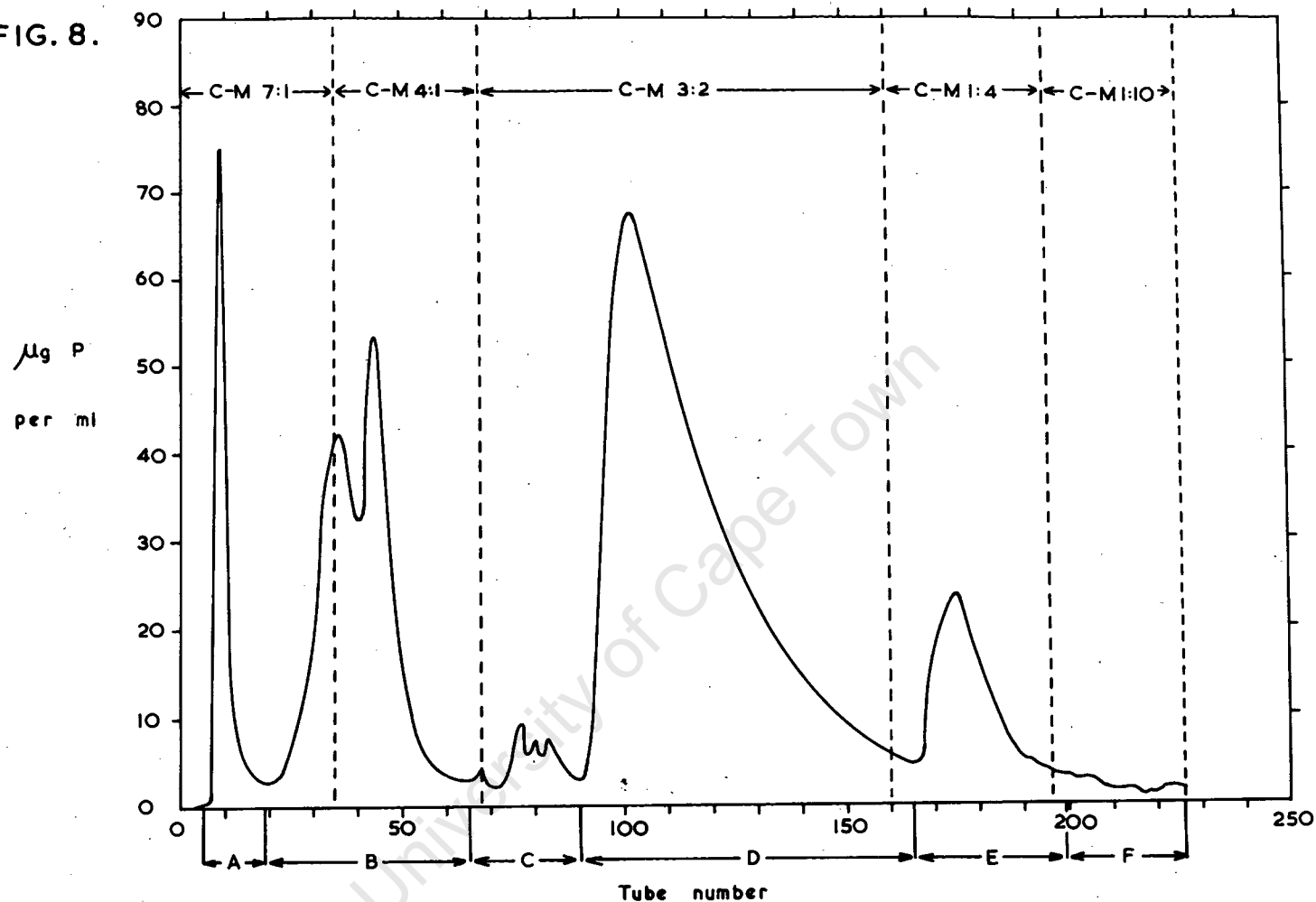
CLASS	HAKE FLESH				HAKE LIVER			
	1 Hydrolytic procedure	2 Chromatography on silicic acid impregnated paper	3 Column chroma- tography on silicic acid	Average of 1 and 3	1 Hydrolytic procedure	2 Chromatography on silicic acid impregnated paper	3 Column chroma- tography on silicic acid	Average of 1 and 3
phosphatidyl choline	62.8	64.8	62.4	63 ± 2	53.6	53.5	53.2	53 ± 2
sphingomyelin	4.8	5.2	3.9 ^{***}	4 ± 1	8.6	8.6	10.3 ^{***}	9 ± 2
lyso phosphatidyl ethanolamine	-		trace		-		trace	
phosphatidyl ethanolamine	21.7	18.6	28.0	21 ± 2	24.7	20.6	23.0	23 ± 2
phosphatidyl serine	2.8			2.8 ± 0.2	3.2			3.2 ± 0.2
phosphatidyl inositol	6.1	-	0.2	6.1 ± 0.5	5.1	-	1.0	5.1 ± 0.5
lyso phosphatidyl choline	1.2	1.3		1 ± 1	2.2	2.2		2 ± 1
cardiolipins	0.6 ^{**}	-	4.2 ^{***}	2 ± 3	2.6 ^{**}	-	10.1 ^{***}	6 ± 4

* Cardiolipins are obtained by difference

*** Contained a small quantity of phosphatidyl ethanolamine

*** Contained a small quantity of phosphatidyl choline

FIG. 8.



Separation of 1.55 g hake liver phospholipids on 100 g of silicic acid. Fractions were eluted with chloroform:methanol mixtures of increasing methanol concentration, and combined on the basis of P content. The composition of the fractions is given in the text.

It should however be realised that any plasmalogens present are included in the corresponding diester fractions. The hydrolytic procedure determines accurately the three components of the cephalin fraction, i.e. phosphatidyl ethanolamine, phosphatidyl serine and phosphatidyl inositol, which is a great advantage over the two chromatographic procedures. The disadvantage, however, is that cardiolipin (phosphatidic acids) may only be estimated indirectly by difference.

Chromatography on silicic acid impregnated paper

Chromatograms of the phospholipid mixtures on silicic acid impregnated paper showed four different spots when treated with the staining solution of Hooghwinkel and van Nisckerk⁸. Marker compounds, together with the ninhydrin and phosphomolybdic acid reagents of Lea et al²⁸ were used to identify these compounds as lysolecithin ($R_F = 0.29$), sphingomyelin ($R_F = 0.36$), phosphatidyl choline ($R_F = 0.46$) and cephalin ($R_F = 0.55$). These R_F values have only relative significance; marked differences of R_F values were observed due to temperature, water content of the solvent system, etc. (cf. Hack²⁶). The quantitative composition of the two mixtures obtained by eluting the four spots is shown in Table III under the appropriate heading. Phosphatidyl inositol and cardiolipins (phosphatidic acids) remained undetected; a correction was applied for the presence of these substances.

Column chromatography on silicic acid

Fig. 8 shows graphically the separation of hake liver phospholipids on a column of silicic acid; a similar graph was obtained for hake flesh phospholipids. It seems desirable to add the following short discussion to this graph.

Fraction A, which was eluted with chloroform:methanol 7:1 (by vol.), contained the pigments of the material and only traces of ethanolamine and serine. It did not stain with the staining solution of Hooghwinkel and van Nisckerk⁸ and was probably of the cardiolipin type (cf. Wren³², Gray and MacFarlane³³). Fraction B, eluted with chloroform:methanol 7:1 (by vol.) showed two peaks; the second peak is, however, of no significance but is due to the solvent change from the ratio 7:1 to 4:1.

This fraction showed the presence of ethanolamine, serine and inositol. Chromatography on silicic acid impregnated paper gave one spot in the cephalin position. This fraction was used to determine the average unsaturation and equivalent weight of the cephalin fatty acids (Table IV).

TABLE IV

AVERAGE EQUIVALENT WEIGHT AND UNSATURATION OF THE
FATTY ACIDS FROM PHOSPHATIDYL CHOLINE AND
CEPHALIN FRACTIONS OF HAKE PHOSPHOLIPIDS.

	Equivalent weight ($\pm$ 3%)		Unsaturation (double bonds/ molecule) ($\pm$ 5%)	
	Phosphatidyl Choline	Cephalin	Phosphatidyl Choline	Cephalin
Hake flesh	314	343	3.0	3.6
Hake liver	293	344	2.5	3.1

Fraction C, formed ethanolamine, serine and inositol on hydrolysis. It showed one spot in the cephalin position and one spot with $R_f = 0.37$ - probably due to lyso phosphatidyl ethanolamine (cf. Wron³², Hack²⁶) - when subjected to chromatography on silicic acid impregnated paper. The latter spot also stained with ninhydrin. A clear separation of the cephalin fraction into ethanolamine, serine and inositol-containing compounds was, therefore, not achieved. Fraction D, which was eluted with chloroform:methanol 3:2 (by vol.) consisted of phosphatidyl choline. Paper chromatograms of an hydrolysate only showed the presence of choline (% choline: 13.1 for hake liver, 13.6 for hake flesh.

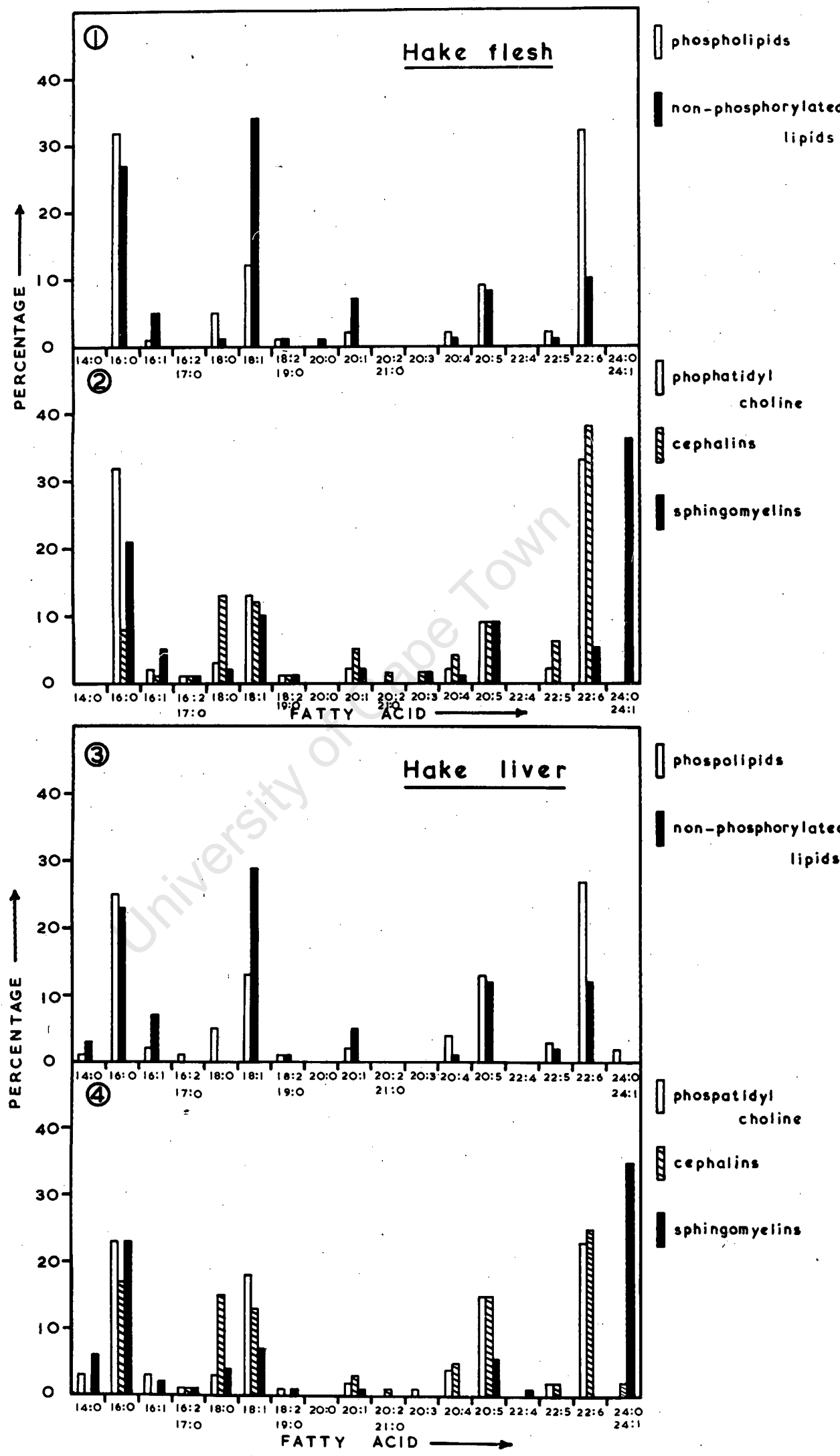
Chromatograms on silicic acid impregnated paper showed one spot in the phosphatidyl choline position. This fraction, which has always been obtained in an exceptionally pure condition, was used to determine the average equivalent weight and unsaturation of the phosphatidyl choline fatty acids. Fraction E, eluted with chloroform:methanol 1:4 (by vol.) was mainly sphingomyelin and the reaction for sphingosine was positive. A chromatogram on silicic acid impregnated paper indicated besides sphingomyelin the presence of small quantities of phosphatidyl choline. Fraction F, eluted with chloroform:methanol 1:10 (by vol.) consisted of approximately equal quantities of sphingomyelin and lyso phosphatidyl choline with traces of phosphatidyl choline; this was shown in the usual way by paper chromatography. The quantitative composition of hake flesh and hake liver phospholipids obtained by this separation is also shown in Table III. A good agreement exists between the results produced by the different methods. The least satisfactory analysis seems to be the chromatography on silicic acid impregnated paper. This method tends to give low values for the cephalin fractions, but it is very suitable to determine the choline distribution in the phosphatidyl choline, sphingomyelin and lyso phosphatidyl choline fractions (see Chapter XI^c). The final composition was obtained by taking the average of the hydrolytic and column chromatographic results. It should be noted that the quantities of lyso phosphatidyl choline and lyso phosphatidyl ethanolamine in the hake phospholipids is extremely small, which supports the theory of the structure of "complex" phospholipids recently postulated by us³⁴ (see Chapter XIII). The composition of the hake flesh and hake liver phospholipids is very similar; although the latter contain more sphingomyelin and cardiolipin type compounds at the expense of phosphatidyl choline. A similar composition was obtained by Gray and MacFarlane³¹ for trout flesh phospholipids and by Lovern et al⁷ for the phospholipids of cod flesh, after chromatography on silicic acid.

The last authors, however, failed to detect sphingomyelins which were probably combined with phosphatidyl choline by eluting the latter with chloroform:methanol 1:1 (by vol.). Results in our laboratory showed that sphingomyelins were eluted as a "flat" peak immediately after phosphatidyl choline if the latter were eluted with chloroform:methanol 1:1 (by vol.).

Fatty acid composition of the phospholipids

The average unsaturation and equivalent weight of the fatty acids from the phosphatidyl choline and cephalin fractions are recorded in Table IV. These results show that the cephalin fatty acids are much more unsaturated and have a higher equivalent weight than the phosphatidyl choline fatty acids. This is in agreement with the evidence obtained by other workers Gray and MacFarlane³³, Lea³⁵, Brockerhof, Ackman and Royle³⁶. Table V shows the fatty acid composition of the hake flesh and hake liver phospholipids together with the phosphatidyl choline, cephalin and sphingomyelin fractions. The fatty acid composition of the non-phosphorylated lipids is also included. A graphic representation of Table V is given in Fig. 9. It will be noticed that the sphingomyelin fractions contain a high proportion of lignoceric ($C_{24:0}$) and nervonic acid ($C_{24:1}$) and also higher chain length fatty acids (cf. Lovern³⁰). The cephalin fractions possess more polyunsaturated fatty acids than the phosphatidyl choline fraction, which is also demonstrated in Table IV. It should be mentioned here that the fatty acid composition of the non-phosphorylated lipids is similar to that obtained by van Rensburg et al.¹ for hake liver oil.

Fig.9. Fatty acid composition of hake lipids.



University of Cape Town

Stabilisation of a supersaturated solution of cholesterol

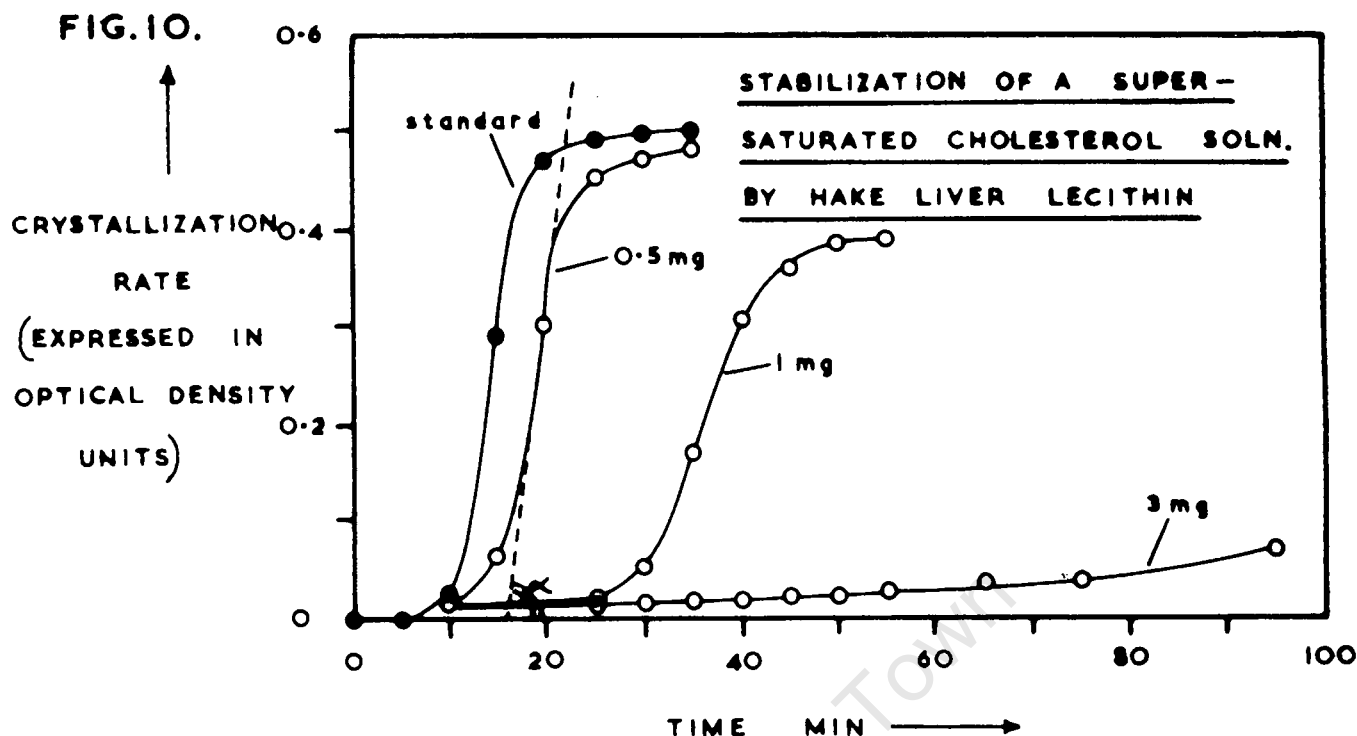


Fig. 10 shows the effect of 0.5 - 3.0 mg of hake liver lecithin on 2.5 ml of a supersaturated (approx. 7%) cholesterol solution in a triglyceride oil. The crystallisation of cholesterol was followed by means of optical density readings in a Klett colorimeter with a 540 m μ filter. It can be clearly seen that as little as 3 mg lecithin has a remarkable effect on the crystallisation rate of approx. 80 mg cholesterol. (A saturated solution contains about 3.5% cholesterol²⁵.) In order to express the results quantitatively the crystallisation rate index ("C.R. value"), which is the first derivative of the curve at the point of inflection (or $\tan \alpha$ see Fig. 10) was computed following Hilken and Krut²⁵. The results are shown in Table VI.

TABLE VI

"C.R. VALUES" OF DIFFERENT QUANTITIES OF HAKE LIVER LECITHIN

<u>Lecithin (mg)</u>	<u>C.R. Value</u>
0	5.3
0.5	4.7
1	2.5
2	0.27
3	0.17
5	0.17

The lecithin fraction of human serum phospholipids is of the same order of potency as our hake liver lecithin³⁸.

Cholesterol

Non-phosphorylated lipids were analysed for cholesterol. The percentages cholesterol in these lipids of hake flesh and hake liver were 4.1 and 1.8 respectively. It is not known how much of the cholesterol occurs in the free and the esterified condition. It should also be realised that the digitonin precipitation is not specific for cholesterol, other sterols also form an insoluble precipitate³⁷. Cholesterol was, however, undoubtedly the major component since it was the only sterol isolated in pure condition.

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CHAPTER IX

PHOSPHOLIPIDS OF THE ROCK LOBSTER

In the previous chapter we have described the analysis of the phospholipids extracted from the flesh and the liver of the hake (*Merluccius Capensis*, Castelnau), a marine teleostid. The present chapter describes the analysis of the phospholipids extracted from the roe (gonads) and the hepatopancreas of the rock lobster (*Jasus lalandii*), a marine crustacean.

Details of the lipid distribution in the rock lobster together with the fatty acid composition of the hepatopancreas oil have been published by Ligtholm, Novellie, Schwartz and von Beldt¹. These authors showed that the hepatopancreas and the gonads are the two main fat depots of the rock lobster; the phospholipids were not isolated and separately studied, although it was noticed that the gonad oil contained a much higher proportion of phospholipids than the hepatopancreas oil.

EXPERIMENTAL

Extraction and purification of the total lipids

400 g of ovaries were removed from rock lobsters caught during the previous 48 h (November) and extracted four times with 600 ml chloroform:methanol (2:1, by vol.) in a Waring blender. The extracts were filtered and purified as described. 47.3 g of a red viscous oil with a P content of 1.88% was obtained.

In the same way, 172 g of a brown oil with a P content of 0.54% was obtained by extracting 1380 g of rock lobster hepatopancreas (November) with a total of 6000 ml chloroform:methanol (2:1, by vol.).

Separation of the phospholipids from the non-phosphorylated lipids

The rock lobster roe oil (43.5 g) was separated into a phospholipid fraction (22.3 g, % P, 3.7; % N, 1.7) and a non-phosphorylated lipid fraction (20.1 g, % P, 0.05) by chromatography on silica gel in light petroleum. Similarly, 78 g hepatopaneas oil was separated into 10.4 g phospholipids (% P, 3.5; % N, 1.5) and 64 g non-phosphorylated lipids (% P, 0.01).

For the remaining experimental details we refer to the previous chapter.

RESULTS AND DISCUSSION

In Table I we have recorded the total lipid and phospholipid content of the various tissues investigated. These results show that although a large variation is observed in the total lipid content, the phospholipid content - apart from the rock lobster roe - remains fairly constant (cf. Lovorn²). The high phospholipid quantity (6%) in the rock lobster roe is noteworthy. Ligthelm et al¹ calculated from the percentage P of the gonad oil a phospholipid quantity of approximately 3.5%. Investigations by Zama³ have shown that fish roe in general is a very rich source of phospholipids.

The hepatopaneas had a very low phospholipid content, i.e. 1.7% (Ligthelm et al¹ : approx. 0.2%); this organ stores mainly neutral fat, similar to the hake liver.

TABLE I

TOTAL LIPIDS AND PHOSPHOLIPIDS IN VARIOUS TISSUES

(expressed as g per 100 g wet tissue)

	<u>Total lipids</u>	<u>Phospholipids</u>
rock lobster roe	12	6
rock lobster hepatopaneas	12.5	1.7
hake liver	46	1.5
hake flesh	1.5	0.5
total pilchard (see following chapter)	4.8	1.1

The phospholipid fractions showed after hydrolysis the presence of several ninhydrin-reacting substances, besides ethanolamine and serine (see Table II). These substances, which were due to polypeptide impurities, were readily removed by washing the phospholipids in ethereal solution with 0.1 N HCl.

TABLE II

MAIN CONSTITUENTS OF ROCK LOBSTER ROE AND HEPATOPANCREAS PHOSPHOLIPIDS

		<u>Roe</u>	<u>Hepatopancreas</u>
		(%)	(%)
P	($\pm$ 4%)	3.7	3.5
N	($\pm$ 10%)	1.7 [*]	1.5
total choline	($\pm$ 1%)	10.45	8.86
lecithin-choline	($\pm$ 5%)	9.70	8.41
sphingomyelin-choline	($\pm$ 25%)	0.75	0.22
lyso lecithin-choline	($\pm$ 30%)	0.00	0.52
sphingosine	($\pm$ 25%)	1.86	0.54
ethanolamine	($\pm$ 8%)	1.74	1.93
serine	($\pm$ 6%)	0.10	0.36
inositol	($\pm$ 10%)	0.25	0.89
sugars		not detected	

* This figure was reduced to 1.4 after removal of peptide impurities with 0.1 N HCl in ether.

The average unsaturation and equivalent weight of the fatty acids from the rock lobster roe and hepatopancreas phosphatides together with the non-phosphorylated lipids are shown in Table III. It appears to be the general tendency that the equivalent weight and unsaturation of the phospholipid fatty acids is higher than that of the corresponding non-phosphorylated lipid fatty acids (Hilditch⁴). Slight evidence for this tendency was shown by the hepatopancreas lipids, but the rock lobster roe lipids were decidedly an exception, i.e. the average unsaturation of the phospholipid and non-phosphorylated lipid fatty acids, was within experimental error, the same.

TABLE III

AVERAGE UNSATURATION AND EQUIVALENT WEIGHT OF THE
FATTY ACIDS FROM ROCK LOBSTER PHOSPHOLIPIDS AND
NON-PHOSPHORYLATED LIPIDS

Tissue	Equivalent weight ($\pm 3\%$)		Unsaturation (double bonds/molecule) ($\pm 5\%$)	
	Total Phospholipids	Non-phosphorylated lipids	Total Phospholipids	Non-phosphorylated lipids
Roe	319	315	2.3	2.4
Hepato-pancreas	323	316	2.4	2.2

Table IV shows the phospholipid composition determined according to the three procedures, outlined earlier. The phospholipid composition of the roe is outstanding, in that it contains a high percentage of phosphatidyl choline, very small quantities of phosphatidyl serine and phosphatidyl inositol and virtually no lyso phosphatidyl choline. It is well established that phosphatidyl choline forms the main part of fowl and fish eggs (cf. Deuel⁵), but a complete analysis of fish egg phospholipids has, to our knowledge, never been published. Zama³ has examined the eggs of shark, crab, pollack, salmon and carp; his work, however, can only be compared with ours on a qualitative basis. Thus crab egg phospholipids were found to consist of phosphatidyl choline, cephalin, sphingomyelin and phosphatidic acids and the cephalin fraction showed the presence of ethanolamine, serine and inositol. Rhodes and Lea⁶ examined hen egg phospholipids; the composition they reported for these phospholipids is very similar to that obtained by us for the rock lobster roe. The only significant difference is that hen egg phospholipids contain approximately 6% of lyso phosphatidyl choline, whereas the rock lobster roe contains only traces of this substance.

The phospholipid pattern of the hepatopancreas is similar to that obtained for hake liver, although it does not show the high sphingomyelin content of the latter.

TABLE IV

COMPOSITION OF ROCK LOBSTER ROE AND HEPATOPANCREAS PHOSPHOLIPIDS

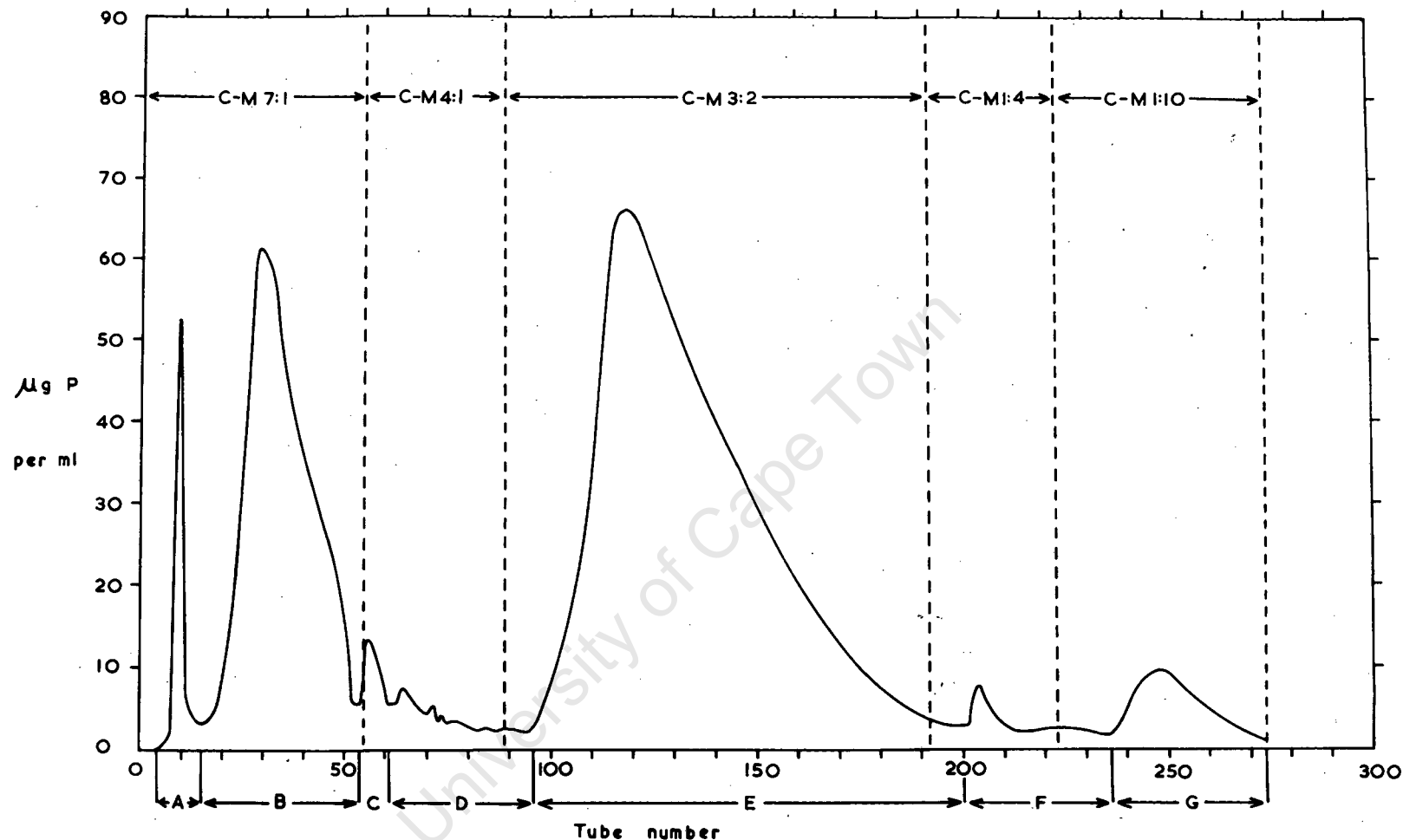
(expressed as per cent of total phospholipids)

CLASS	ROE				HEPATOPANCREAS			
	1 Hydrolytic procedure	2 Chromatography on silicic acid impregnated paper	3 Column chroma- tography on silicic acid	Average of 1 and 3	1 Hydrolytic procedure	2 Chromatography on silicic acid impregnated paper	3 Column chroma- tography on silicic acid	Average of 1 and 3
phosphatidyl choline	70.2	73.7	71.3	71 ± 3	61.2	68.0	59.7	60 ± 3
sphingomyelin	4.8	}	2.0	3 ± 2	1.4	}	1.0	1.2 ± 0.2
lyso phosphatidyl ethanolamine	-		}	trace	-		}	trace
phosphatidyl ethanolamine	23.3	27.0		23 ± 2	26.1	33.1		26 ± 2
phosphatidyl serine	0.8	18.1		0.8 ± 0.1	3.0	18.5		3.0 ± 0.2
phosphatidyl inositol	1.3	-		1.3 ± 0.1	4.7	-		4.7 ± 0.5
lyso phosphatidyl choline	0.0	0.0	trace	trace	2.5	2.5	3.2	2.8 ± 0.6
cardiolipin	0.0 ^{ms}	-	1.8 ^{ms}	1 ± 1	1.1 ^{ms}	-	5.0 ^{ms}	3 ± 2

* Cardiolipin is obtained by difference

** Contained a small quantity of phosphatidyl ethanolamine

FIG.1.



Separation of 1.97 g rock lobster hepatopancreas phospholipids on 100 g of silicic acid. Fractions were eluted with chloroform:methanol mixtures of increasing methanol concentration and combined on the basis of P content. The composition of the fractions is given in the text.

The Schiff reaction for plasmalogens was positive, but the same holds as described for lake phospholipids.

Fig. 1 shows graphically the separation of the hepatopancreas phospholipids by means of chromatography on silicic acid. A similar graph was obtained for the roo phospholipids. Fractions A - G, which were obtained by combining the appropriate tubes, were characterized by chromatography on silicic acid impregnated paper and by paper chromatography of the water soluble components after hydrolysis.

The results were analogous to those described for the lake (Chapter VIII) and will therefore only be briefly mentioned.

Fraction A consisted of cardiolipins.

Fraction B consisted of phosphatidyl ethanolamine, phosphatidyl serine and a small quantity of phosphatidyl inositol.

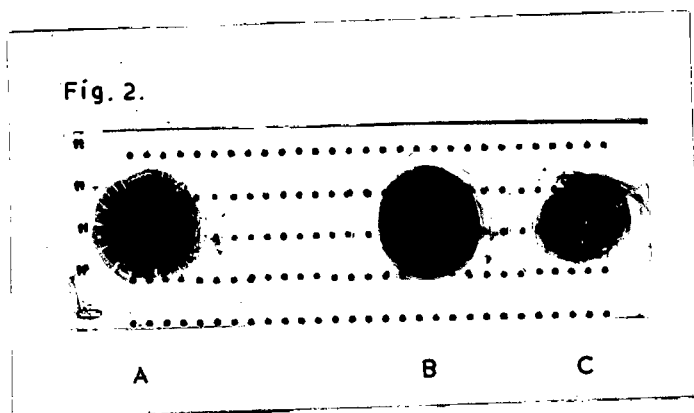
Fraction C consisted of phosphatidyl ethanolamine, phosphatidyl inositol and small quantities of phosphatidyl serine and probably lyso phosphatidyl ethanolamine.

Fraction D consisted of phosphatidyl inositol, small quantity of phosphatidyl ethanolamine, phosphatidyl serine and probably lyso phosphatidyl ethanolamine.

Fraction E consisted of phosphatidyl choline.

Fraction F consisted of sphingomyelin and phosphatidyl choline.

Fraction G consisted of lyso phosphatidyl choline. In this particular example a definite chromatographic peak was observed for lyso phosphatidyl choline. This substance was shown to possess a powerful hemolytic activity; small quantities hemolysed human blood within minutes, as is shown in Fig. 2.



Hemolytic effect of lyso phosphatidyl choline
on human blood

A : Control

B: lyso phosphatidyl choline added to blood -
note the transparency

C: phosphatidyl choline added to blood -
no effect.

The fatty acid distribution in the different rock lobster lipids is shown in Table V and in Fig. 3. Little difference is noticed in the fatty acid composition of the phospholipids and non-phosphorylated lipids, an indication for this was already obtained in Table III. A large difference, however, was observed in the fatty acid composition of the phosphatidyl choline and cephalin fractions, the latter containing more of the polyunsaturated fatty acids (cf. Chapter VIII and Table VI). It should also be mentioned that the roe and hepatopancreas lipids contained minor quantities (less than 1%) of numerous branched chain and odd numbered fatty acids. The chain length distribution in the rock lobster lipids is different from that of the hake, although the average equivalent weights are similar. The explanation for this is that in general the rock lobster lipids contain more C_{18} and C_{20} , but less C_{16} and C_{22} fatty acids than the hake lipids. The fatty acid composition of the prawn (*Leander serratus*) a marine crustacean examined by Klem⁷ is analogous to that of the rock lobster.

Fig. 3. Composition of fatty acids from rock lobster lipids.

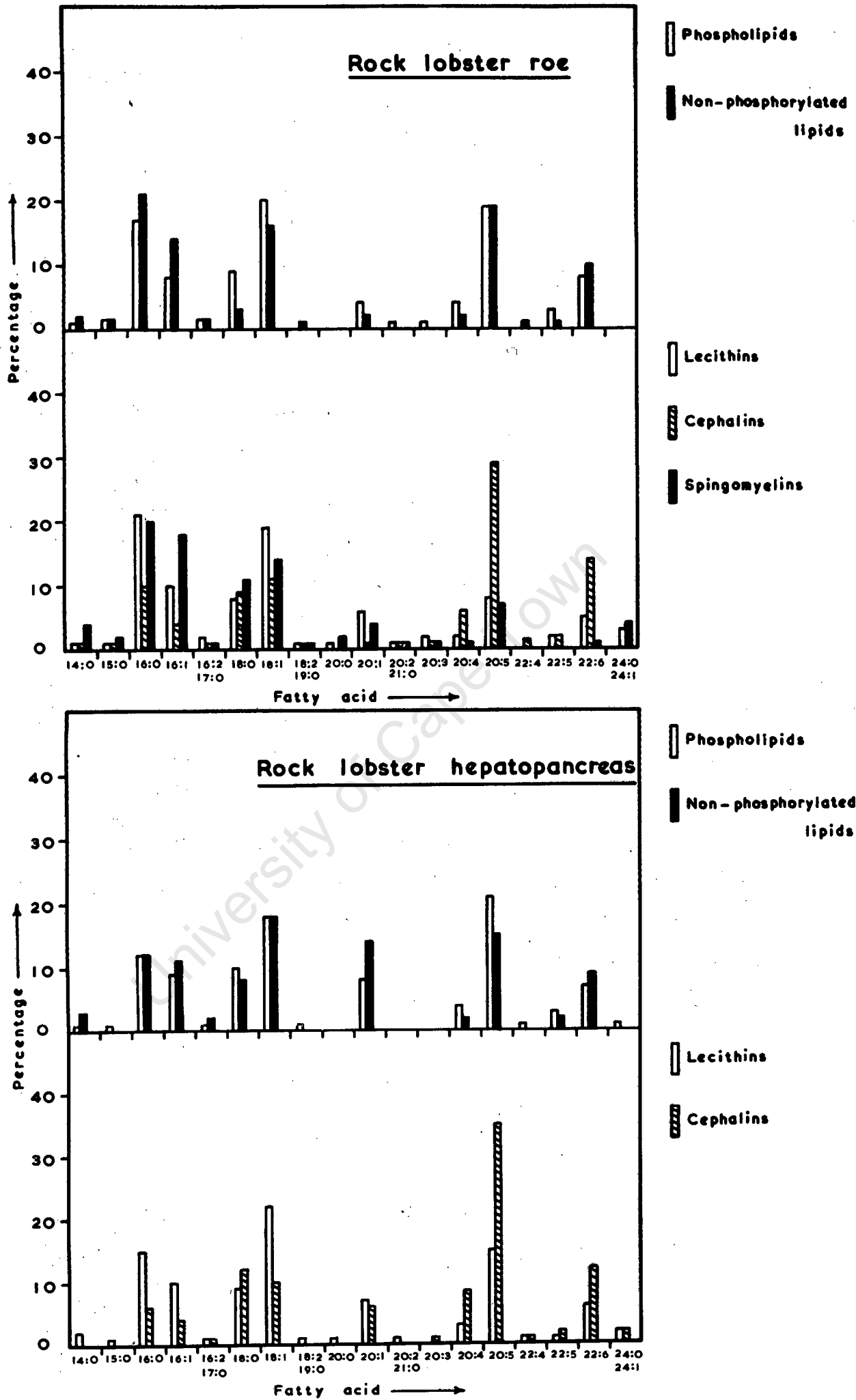


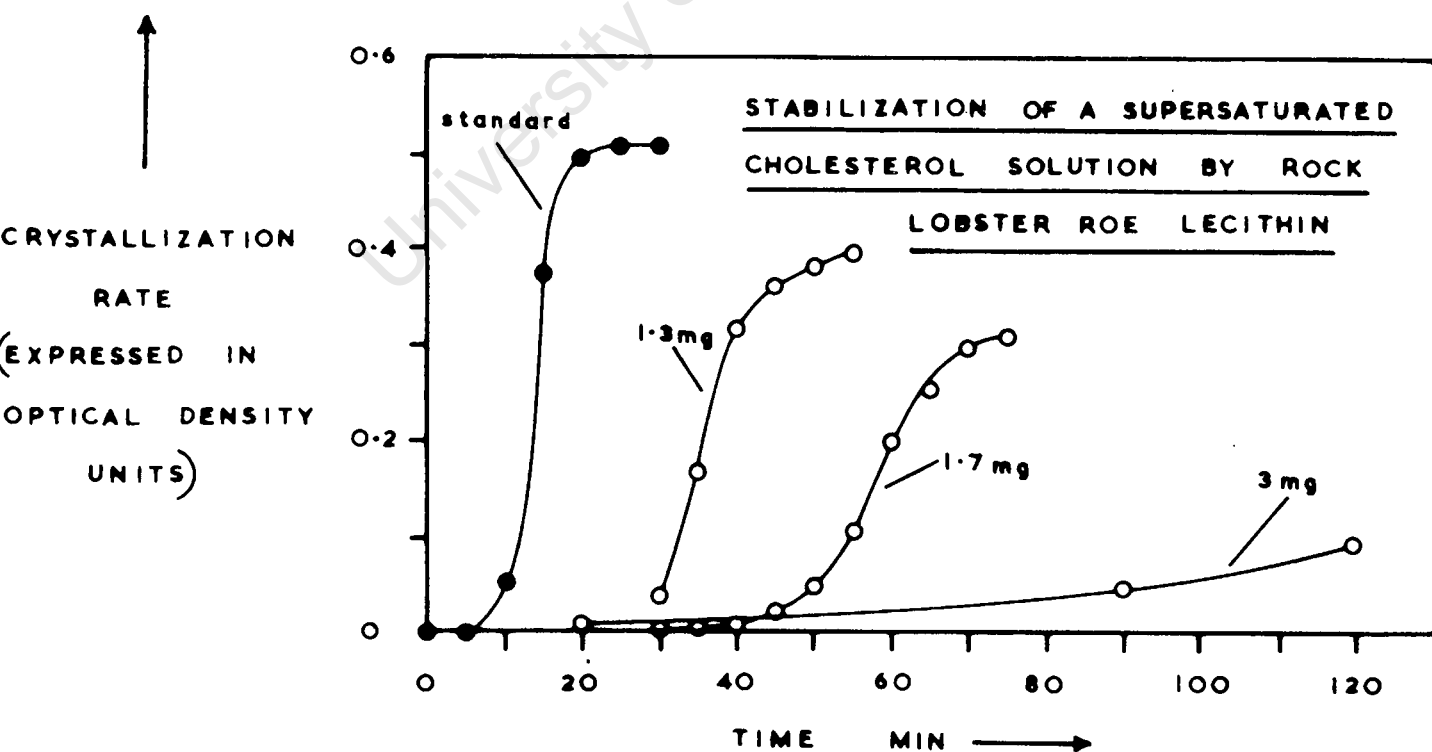
TABLE VI

AVERAGE UNSATURATION AND EQUIVALENT WEIGHT OF THE
PHOSPHATIDYL CHOLINE AND CEPHALIN FRACTION OF
ROCK LOBSTER ROE AND HEPATOPANCREAS PHOSPHOLIPIDS

	Equivalent weight ($\pm 3\%$)		Unsaturation (double bonds/ molecule) ($\pm 5\%$)	
	Phosphatidyl Choline	Cephalin	Phosphatidyl Choline	Cephalin
Roe	309	354	2.0	3.6
Hepato- pancreas	313	337	2.0	3.2

The effect of rock lobster roe lecithin on the crystallisation rate of cholesterol is similar to that of hake liver lecithin as is clearly shown in Fig. 4.

FIG. 4.



Finally, the amount of cholesterol in the non-phosphorylated lipids of the rock lobster roe was 5.3%. In this instance cholesterol was again obtained as a crystalline material with m.p. and mixed m.p. of 145 - 147°.

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CHAPTER X

PHOSPHOLIPIDS OF THE PILCHARD WITH PARTICULAR REFERENCE TO THEIR OXIDATION IN PILCHARD MEAL MANUFACTURE.

The pilchard (*Sardina ocellata*, Jenyns) is abundant in South African waters and is of considerable economic importance in the Republic. A large part of the pilchard catch is converted into fish meal by means of a cooking, pressing and drying process. A certain amount of oil (5-10% of the meal weight) remains in the fish meal and cannot be recovered by pressing. This residual oil contains virtually all the phospholipids and during the drying and subsequent storage of the meal autoxidation of the highly unsaturated oil takes place. Oxidation leads to destruction of vitamins A, D and E, reduction of protein quality and under certain conditions to spontaneous combustion¹. The addition of anti-oxidants such as 3,5-di-tert.butyl-4 hydroxytoluene (BHT) has only met with reasonable success¹.

In this chapter the progress of the oxidation of the phospholipids and the non-phosphorylated lipids in the manufacturing process of pilchard meal has been examined. In addition the complete analysis of pilchard phospholipids is given.

The composition of pilchard press oil, which is a non-phosphorylated lipid, has been investigated by Silk and Bahn² and Whitcutt³, but the phospholipids have not been examined previously. Lea⁴ has shown that phospholipids especially the phosphatidyl ethanolamine fraction oxidize rapidly in the presence of atmospheric oxygen.

EXPERIMENTAL

Manufacture of pilchard meal

Fresh pilchards (5300 g, July) were steamed for 30 min. at 100° . The mixture was pressed at 5000 lb/sq. inch in the canvas bag of a laboratory Carver press. The press oil (134 g, % P : 0.01) was separated from the press-water (stickwater) by decantation followed by extraction with light petroleum ($40 - 60^{\circ}$). The wet presscake (1990 g) was dried at 80° for $1\frac{1}{2}$ h in a laboratory drier with forced air circulation. Dried presscake (1120 g) was converted into fish meal by milling and was "cured" in a small hessian bag by storing at room temperature for 40 days. Samples of fresh pilchards, wet presscake, fresh fish meal and cured fish meal were taken for the extraction and analysis of the lipids. The figures quoted above are corrected for the material taken for analysis.

Extraction and purification of the lipids

I Fresh pilchards.

509 g fresh pilchards were extracted four times with 600 ml chloroform:methanol (2:1, by vol.) in a Waring blender. Crude lipids were purified by three washings according to Folch, Ascoli, Lees, Meath and Le Baron⁵. 25.5 g of a light oil (% P : 0.73) and 99.1 g protein was obtained.

II Wet presscake.

450 g wet presscake gave 27.4 g of a dark oil (% P : 1.57) and 185.7 g protein after four extractions with 800 ml chloroform:methanol.

III Fresh fish meal.

Similarly, 250 g fresh fish meal gave 27.1 g of a dark oil (% P : 1.50) and 196.0 g protein.

IV Cured fish meal

250 g cured meal gave 24.9 g of a dark viscous oil (% P : 1.31) and 204.0 g protein after an identical extraction and purification.

Separation of the phospholipids from the non-phosphorylated lipids

This separation was accomplished by chromatography on silica gel in light petroleum as described in Chapter VIII.

I Fresh pilchards

25.5 g mixed lipids were separated in 4.65 g phospholipids (% P = 3.7) and 20.2 g non-phosphorylated lipids (% P = 0.02).

II Wet presscake

27.4 g mixed lipids were separated in 12.0 g phospholipids (% P = 3.5) and 14.3 g non-phosphorylated lipids (% P = 0.03).

III Fresh fish meal

27.1 g mixed lipids were separated in 12.8 g phospholipids (% P = 3.3) and 12.5 g non-phosphorylated lipids (% P = 0.01).

IV Cured fish meal

Separation of the two lipid classes was impossible by chromatography on silica gel.

Amino nitrogen in unhydrolysed phospholipids

The free amino groups of ethanolamine and serine in the intact phospholipids were determined spectrophotometrically after treatment with 1-fluoro-2,4-dinitrobenzene following Wheeldon and Collins⁶ (see also Chapter VII).

Available lysine in protein

The free ϵ -amino group of lysine in the proteins was determined with 1-fluoro-2,4-dinitrobenzene according to Carpenter⁷.

For the determination of the average unsaturation of the liberated fatty acids and the phospholipid composition we refer to Chapter VIII.

RESULTS AND DISCUSSION

Oxidation studies.

In Table I we have recorded the average unsaturation of the phospholipid and the non-phosphorylated lipid fatty acids at the different stages of the manufacturing process.

TABLE I

UNSATURATION OF PHOSPHOLIPID AND NON-PHOSPHORYLATED LIPID FATTY ACIDS IN PILCHARD MEAL MANUFACTURE

Stage	Average unsaturation $\pm 5\%$ (double bonds/molecule)		% P in Phospholipids
	Phospholipids	Non-phosphorylated lipids	
fresh	3.2	2.3	3.7
wet presscake	3.1	2.4	3.5
fresh meal	2.8	2.2	3.3
cured meal		0.9 ^a	1.3 ^a
press oil		2.4 ^{aa}	

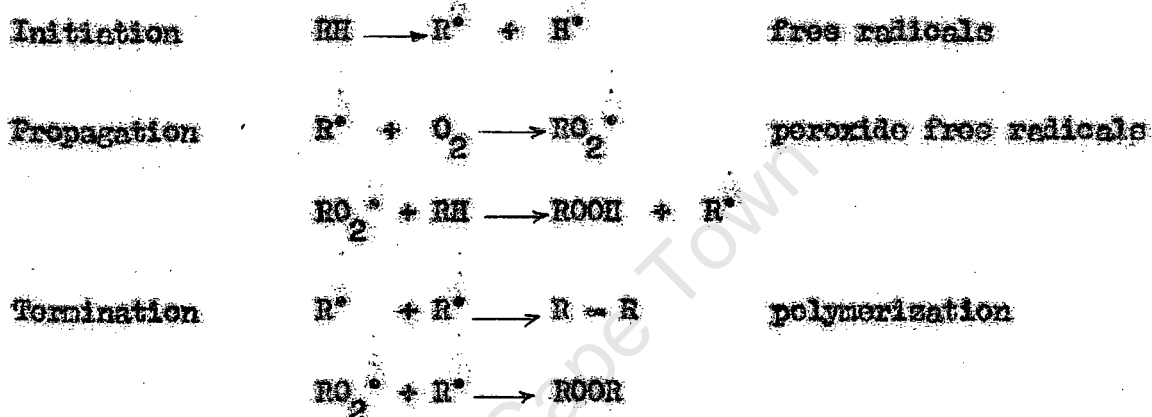
^a Total lipids

^{aa} Silk, Sephton and Hahn² : 2.4

The results show that fresh meal phospholipids had a significantly lower unsaturation than those extracted from the fresh fish, whereas the average unsaturation of the non-phosphorylated lipids remained - within experimental error - constant. The cured meal lipids were decomposed to a large extent, and could not be separated into a phospholipid and non-phosphorylated lipid fraction. The oxidation and polymerization between the two classes had proceeded to such an extent that separation proved impossible and the total lipids were therefore analysed.

Gas chromatographic analysis of the fatty acids liberated from these lipids indicated that almost all the polyunsaturated fatty acids were absent (Fig. 2). The fact that the phosphorus contents of the fresh meal and wet presscake phospholipids were lower than that of the fresh phospholipids, viz. 3.3, 3.5 and 3.7% respectively is an indication that to some extent polymerization reactions had already taken place in the initial stages of processing.

Autoxidation of unsaturated fatty acids proceeds according to a free radical mechanism, with the following reaction sequence^{8,9,10}:



For example in the case of linoleic acid $R^\bullet$ would be



i.e. the methylene group between the two unsaturated carbon atoms is the reactive centre. This free radical possesses two other resonance hybrid configurations, by virtue of which it is stabilized.



In the initial stages of autoxidation the double bonds remain intact, but secondary reactions due to peroxide degradation soon destroy the double bonds leading to chain scission and the formation of aldehydic decomposition products.

These results show that the available lysine of the cured meal protein is significantly lower than that of the fresh meal; no reduction in protein quality seems to take place in the steaming and drying process. A striking relation is apparent between the available amino nitrogen and the average unsaturation of the liberated fatty acids - shown in Table I - of a phospholipid.

After hydrolysis of the cured meal lipids with 2 N HCl at 120° for 24 h, ethanolamine, inositol and choline were recovered in amounts consistent with a P content of 1.3%, i.e. no serious chemical decomposition of these compounds had taken place (see Table III).

Phospholipid composition

In Table III are listed the main constituents of pilchard phospholipids.

TABLE III

MAIN CONSTITUENTS OF PILCHARD PHOSPHOLIPIDS

		Whole Pilchard	Total lipids of
			cured pilchard meal
		(%)	(%)
P	([±] 4%)	3.5	1.3
N	([±] 10%)	1.4 ^m	-
total choline	([±] 1%)	8.97	3.44
lecithin-choline	([±] 5%)	7.53	-
sphingomyelin-choline	([±] 25%)	0.91	-
lyso lecithin-choline	([±] 30%)	0.53	-
sphingosine	([±] 25%)	2.45	-
ethanolamine	([±] 8%)	1.94	0.74
serine	([±] 6%)	0.23	-
inositol	([±] 10%)	0.71	0.19
galactose		trace	

^m After washing with 0.1 N HCl in ether.

In our earlier experiments with pilchard phospholipids (Chapter V - VII) the separation from the non-phosphorylated lipids was accomplished by only one passage over silica gel and therefore this material undoubtedly still contained some non-phosphorylated lipids. A comparison of the analyses performed on the two samples clearly illustrates this, e.g. the percentages phosphorus, choline and ethanolamine were 3.3 and 3.5; 8.0 and 8.97; 1.6 and 1.94 respectively.

The average equivalent weights of the phospholipid, the corresponding non-phosphorylated lipid and the pilchard press oil fatty acids were 312 ± 8 , 292 ± 8 and 292 ± 8 respectively. Silk, Sephton and Hahn² found this last figure to be 282.

Table IV records the pilchard phospholipid composition determined as outlined in Chapter VIII.

TABLE IV
COMPOSITION OF PILCHARD PHOSPHOLIPIDS
(expressed as per cent of total phospholipids)

Class	(1) Hydrolytic procedure	(2) Chromatography on silicic acid impreg- nated paper	(3) Column chromatography on silicic acid	Average of 1 and 3
Phosphatidyl choline	53.2	60.6	52.6	53 ± 3
sphingomyelin	6.3	7.3	5.3	6 ± 1
lyso phosphatidyl ethanolamine	-			trace
phosphatidyl ethanolamine	25.5		29.7	25 ± 2
Phosphatidyl serine	1.9			1.9 ± 0.1
phosphatidyl Inositol	3.6	-		3.6 ± 0.4
lyso phosphatidyl choline	2.5	2.8	1.5	2 ± 0.5
cardiolipin	7.0 ^{ms}	-	10.9 ^{ms}	9 ± 2

^{ms} = cardiolipin is obtained by difference

^{ms} contained a small quantity of phosphatidyl ethanolamine.

It can again be clearly seen that chromatography on silicic acid impregnated paper tends to give low values for the cephalin fraction, this method has to be classified as semi-quantitative (cf. Chapters VIII and IX).

For reasons of comparison we have combined the compositions of all the phospholipids investigated in Table V.

The most striking feature of this table is the great similarity that exists in the different phospholipids; only those extracted from the rock lobster roe are to some extent different. Literature data of other fish phospholipids show the same pattern¹¹. Seasonal variations are therefore not likely to occur, even the amount of phospholipid present in fish seems to be fairly constant (cf. Table I of Chapter IX and Lovern¹¹). In this connection it may be mentioned that the same similarity has been noted in the phospholipids of mammalian tissues¹².

TABLE V

PHOSPHOLIPID COMPOSITION OF MARINE PRODUCTS
(expressed as per cent of total phospholipids)

SOURCE	PHOSPHOLIPID CLASS ^a						
	PC	PE	PS	PI	SP	PA	LP
whole pilchard	53	25	2	4	6	9	2
hake flesh	63	21	3	6	4	2	1
hake liver	53	23	3	5	9	7	2
rock lobster hepatopancreas	60	26	3	5	1	3	3
rock lobster roe	71	23	1	1	3	1	trace

- ^a PC phosphatidyl choline
 PE phosphatidyl ethanolamine
 PS phosphatidyl serine
 PI phosphatidyl inositol
 SP sphingomyelin
 PA phosphatidic acids (cardiolipins)
 LP lyso phosphatides

The separation of pilchard phospholipids by column chromatography on silicic acid is shown in Fig. 1. Fractions A - G were obtained by combining tubes on the basis of their phosphorus content. The fractions were characterized by paper chromatography of their hydrolysis products and by chromatography on silicic acid impregnated paper. Solvent changes were made as indicated in the figure. The results were similar to those described for the hake and the rock lobster.

Fraction A consisted of cardiolipins.

Fraction B consisted of phosphatidyl ethanolamine, phosphatidyl serine and a small quantity of phosphatidyl inositol.

Fraction C consisted of phosphatidyl ethanolamine, phosphatidyl serine, a small quantity of phosphatidyl inositol and probably lyso phosphatidyl ethanolamine.

Fraction D consisted of phosphatidyl inositol and small quantities of phosphatidyl ethanolamine, phosphatidyl serine and probably lyso phosphatidyl ethanolamine.

Fraction E consisted of phosphatidyl choline.

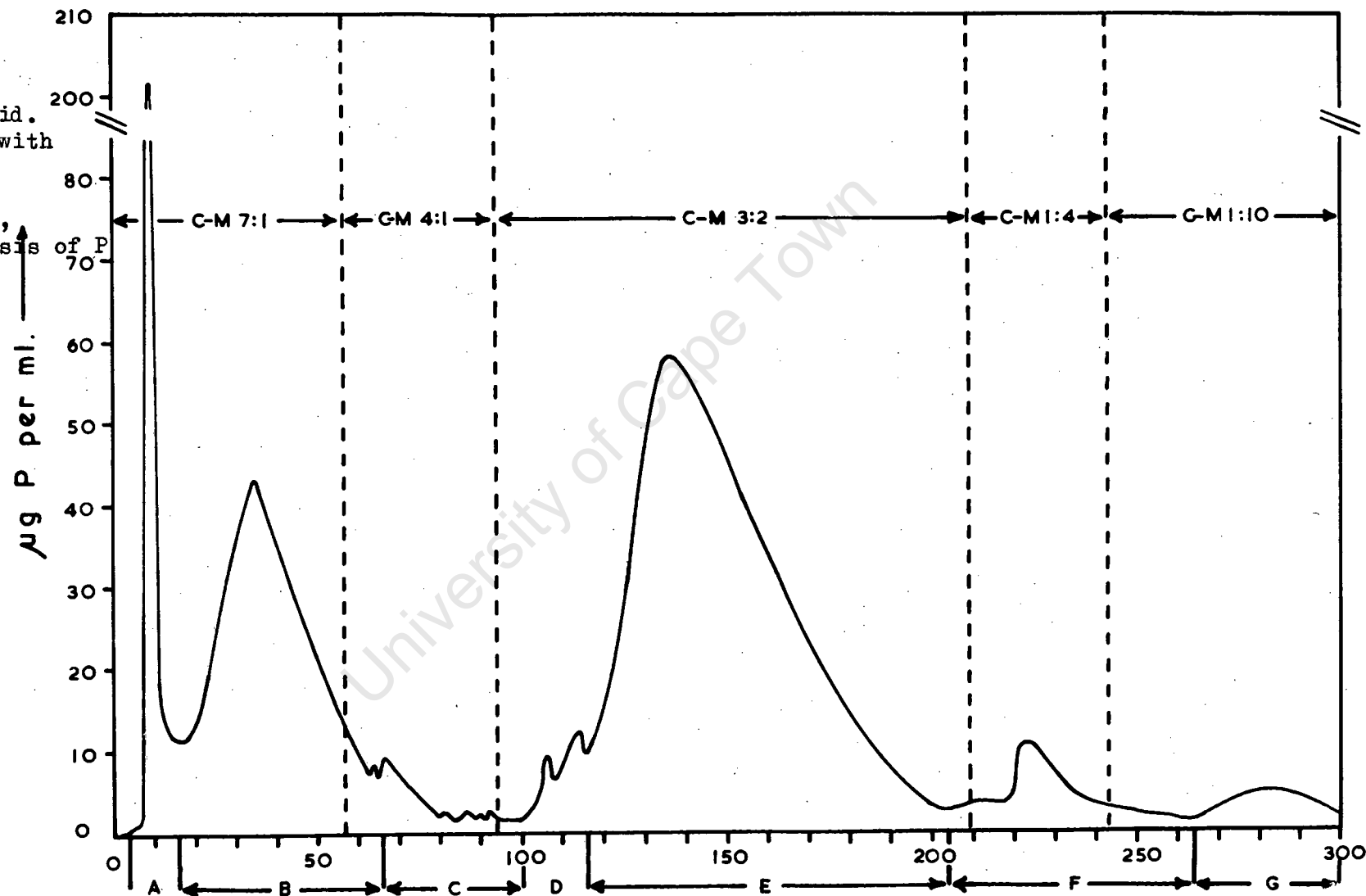
Fraction F consisted of sphingomyelin and a small amount of phosphatidyl choline.

Fraction G consisted of lyso phosphatidyl choline.

The fatty acid composition of the pilchard phospholipids and non-phosphorylated lipids together with the fatty acids of the lipids extracted from the cured meal are graphically shown in Fig. 2. It is evident from this figure that the phospholipids contain more C_{22} hexanoic and C_{16} saturated fatty acids than the non-phosphorylated lipids; the results of Table I demonstrate that the phospholipids are more unsaturated and have a higher equivalent weight than the non-phosphorylated lipids. A slight oxidation in the fatty acids cannot be entirely precluded since the lipids were extracted from the wet presscake. (See Table I). A marked reduction in the polyunsaturated fatty acids is noticed in the lipids of the cured meal.

Fig.1. Silicic acid chromatography of pilchard phospholipids (1.94 g)

Separation of 1.94 g pilchard phospholipids on 100 g of silicic acid. Fractions were eluted with chloroform:methanol mixtures of increasing methanol concentration, and combined on the basis of P content. The composition of the fractions is given in the text.



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CHAPTER XI

ESTIMATION OF SPHINGOMYELIN

In this chapter a new approach to the estimation of sphingomyelin in phospholipids is described. Sphingomyelin is frequently estimated by determining sphingosine according to the method of McKibbin and Taylor¹. This method consists of the separation of sphingosine from other nitrogenous bases by extraction from an aqueous acid solution with chloroform; total N is determined in the chloroform extract. This procedure has been criticised by several workers, e.g. Olley and Lovern², on the grounds that not all the chloroform soluble-N is in fact sphingosine-N. Modifications of the method have been suggested by Lovern³ and by Rhodes and Lea⁴, but substantially the original method is still commonly used in many laboratories^{5,6,7}.

The McKibbin and Taylor method proved unreliable in our laboratory, as is shown in Table I, and was abandoned.

A new routine procedure for the estimation of sphingomyelin was subsequently developed, by determining sphingomyelin-choline in the phospholipid mixture. This was found by determining the total choline in a phospholipid hydrolysate as described previously (Chapter VI) and the relative proportions of lecithin-choline, sphingomyelin-choline and lysolecithin-choline. The latter were readily obtained by chromatography of the phospholipid mixture on silicic acid impregnated paper, followed by staining with the tricomplex stain of Hooghuinkel and van Niekerk⁸. An example of the calculation is shown in Table II.

TABLE I

ESTIMATION OF SPHINGOMYELIN IN MARINE PHOSPHOLIPIDS
(as per cent of total phospholipids)

<u>Source of Phospholipid</u>	<u>McKibbin and Taylor's method^{1a}</u>	<u>Present Method</u>	<u>Column chromatographic procedure⁶</u>
Hake flesh	9.4	4.8	3.9
Hake liver	7.3	8.6	10.3
Rock lobster roe	2.2	4.8	2.0
Rock lobster hepatopancreas	9.4	1.4	1.0
Total pilchard	10.6	6.3	5.3

* Cerebrosides are not present in these lipids (no sugars detected) - the high values are therefore not due to cerebroside-sphingosine.

TABLE II

DETERMINATION OF THE PROPORTIONS LECITHIN-CHOLINE, SPHINGOMYELIN-CHOLINE AND LYSO LECITHIN-CHOLINE.

<u>Spot</u>	<u>Optical Density of the Excised Spot in 3 ml tert.butanol:1.2 N HCl (1:1) at 510 mμ</u>			
lecithin	0.215	0.135	0.252	0.153
sphingomyelin	0.028	0.018	0.031	0.022
lyso lecithin	0.013	0.013	0.016	0.010
Molar ratio:				
lecithin-choline:	215:28:13	135:18:13	252:31:16	153:22:10
sphingomyelin-choline:				
lyso lecithin-choline:				
	Total choline = 8.97%			
lecithin-choline	7.84%	7.30%	7.56%	7.42%
sphingomyelin-choline	0.66%	0.97%	0.93%	1.07%
lyso lecithin-choline	0.47%	0.70%	0.48%	0.49%
i.e. lecithin-choline	* 7.5 $\pm$ 0.3%			
sphingomyelin-choline	* 0.9 $\pm$ 0.2%			
lyso lecithin-choline	* 0.5 $\pm$ 0.2%			

After determining the average equivalent weight of the phospholipid fatty acids, the percentage sphingomyelin could be easily calculated from the sphingomyelin-choline figure.

The method has proved satisfactory in our laboratory; the results agree well with those obtained by an independent column chromatographic separation on silicic acid following Hanahan, Dittmer and Warashina⁶ (see Table I). In this connection a short discussion of the staining procedure of Hooghwinkel and van Niekirk⁸ seems appropriate. These authors determine the composition of a phospholipid mixture by staining a developed chromatogram with the tricomplex stain Edicol Supra Ponceau 4RS - Uranyl nitrate. The red spots obtained are cut out, the dye eluted and the optical density of the eluate measured spectrophotometrically. They have shown that the amount of dye absorbed by an "acid-base" type phospholipid is proportional to the molecular quantity. If a mixture consists therefore only of "acid-base" type phospholipids each component may be readily calculated. This method when applied to our phospholipid mixtures invariably gave - after a suitable correction for the presence of phosphatidyl inositol and cardiolipins - low values for the phosphatidyl ethanolamine plus phosphatidyl serine fraction (see Chapters VIII - X). It is suggested here that the proportionality between dye-uptake and molecular concentration does not strictly hold anymore when comparing phospholipids containing different nitrogenous bases, e.g. ethanolamine and choline.

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CHAPTER XII

HYDROLYSIS OF PHOSPHOLIPIDS WITH HYDROCHLORIC ACID.

The quantitative analysis of marine phospholipids as described in the previous chapters was largely based on the determination of the products they yield on hydrolysis, e.g. choline from the lecithins plus sphingomyelin and ethanolamine plus serine from the cephalins. Hydrolysis was effected with 2 N HCl in a sealed tube at 120° for 24 h. On the basis of literature data it was impossible to indicate the quantity of phosphorylcholine and phosphorylethanolamine formed during hydrolysis¹⁻⁴. It was, therefore, difficult to predict accurately the time necessary for complete liberation of choline and ethanolamine, which is a prerequisite in their determination. It was also not clear why the phosphorylcholine link in sphingomyelin is more resistant towards hydrolysis than in lecithins⁵.

It is the object of the present chapter to describe the progress of the decomposition of DL- α -lecithin (phosphatidyl choline), DL- α -cephalin (phosphatidyl ethanolamine) and sphingomyelin with 2 N HCl at 120°. An explanation of the reaction mechanism for the acid hydrolysis of these compounds is suggested. In addition the hydrolysis of phosphorylcholine and phosphorylethanolamine in 2 N HCl at 120° has been investigated.

MATERIALS

DL- α -lecithin, DL- α -cephalin, sphingomyelin, phosphorylcholine chloride (calcium salt) and phosphorylethanolamine were all obtained from L. Light & Co. Ltd., Colnbrook, England. The analyses of these compounds are shown in Table I.

TABLE I

ANALYSIS OF COMMERCIAL COMPOUNDS

	N (%)	P (%)	N/P	Remarks
DL- α -lecithin	1.8	3.9	1.02	
DL- α -cephalin	1.8	4.5	0.89	no serine present
sphingomyelin	3.4	3.7	2.03	
phosphorylcholine Chloride Ca-salt	4.3	8.6	1.11	chromatographically pure
phosphorylethanolamine				
	9.8	22.4	0.97	chromatographically pure

All other chemicals used were of A.R. quality.

EXPERIMENTAL

Phosphorus

This was determined according to Bartlett⁶.

Total glycerophosphate

Total glycerophosphate (α plus β) was determined by oxidation with periodic acid to glycolic aldehyde phosphate, which was then converted to inorganic P by hydrolysis in acid medium and determined (cf. Burmaster⁷, Olley and Blewett⁸). The oxidation with periodic acid was accomplished at 80° for 1 h in the case of the DL- α -lecithin hydrolysates. Burmaster⁷ found complete decomposition of α and β -glycerophosphate after oxidation with periodic acid at 100° for 1 h. Our experiments showed that the decomposition was equally effective at 80°, whereas the formation of inorganic P from phosphorylcholine under these conditions was negligible.

In the DL- α -cephalin hydrolysates, glycerophosphate was oxidised with periodic acid at room temperature for 16 h. Under these conditions the reaction was incomplete (95%) and a suitable correction was applied. It was, however, necessary to employ these milder reaction conditions, since phosphoryl-ethanolamine liberated inorganic P when treated with periodic acid at 80°. At room temperature the formation of inorganic P from this source appeared to be negligible.

Phosphorylcholine

Phosphorylcholine was quantitatively estimated (expressed as percentage P) by subtracting the inorganic P obtained after treatment with periodic acid plus the water insoluble P from the total P content.

In the hydrolysis of sphingomyelin no periodate labile P was detected therefore only inorganic P and water insoluble P were subtracted from the total P content.

Phosphorylcholine ($R_F = 0.14$) was characterized on paper chromatograms run in an ascending solvent system of butanol:diethylene glycol:water (4:1:1, by vol.). The compound was detected according to Burrows, Grylls and Harrison⁹ with colour development as described by Banduraki and Amelrod¹⁰.

Choline

This was detected and determined as described previously (Chapters V and VI).

Phosphorylethanolamine

This substance, formed during the hydrolysis of DL- α -cephalin, was estimated in the same way as phosphorylcholine. It was detected as a ninhydrin reacting spot ($R_F = 0.05$) on paper chromatograms run in a descending solvent system of butanol:acetic acid:water (4:1:5, by vol., upper layer) on Whatman No. 1 paper.

Ethanolamine

This was identified as a ninhydrin reacting spot ($R_f = 0.22$) on paper chromatograms as described for phosphorylethanolamine.

Nitrogen

Dumas nitrogen was determined at the Chemistry Department of the University of Cape Town.

HYDROLYTIC PROCEDURE

DL- α -lecithin and DL- α -cephalin

Weighed quantities (40.0 mg) were hydrolysed with 5.0 ml 2 N HCl in sealed tubes at 120° for 1, 7, 18, 24, 72 and 168 h. After hydrolysis the tubes were opened and the contents filtered over Whatman No. 42 filter paper. Aliquots were taken for the determination of inorganic P and total glycerophosphate. Choline was determined in an aliquot of the DL- α -lecithin hydrolysates. The residue was thoroughly washed with distilled water and the washings discarded. P was determined in the residue after digestion with sulphuric acid and hydrogen peroxide.⁶

Sphingomyelin

A different technique was adopted for the hydrolysis of this substance, since only small quantities were available. Weighed quantities of 10.0 mg were hydrolysed with 2.0 ml 2 N HCl in sealed tubes at 120° for 1, 7, 18, 24, 72 and 168 h. The contents of the tubes were filtered over a small (4.0 cm) Whatman No. 42 filter paper. The tubes and filter papers were carefully washed with small portions of distilled water and the filtrates made up to 5.0 ml. Aliquots were taken for the determination of inorganic P, total P and choline. The total P was determined after digestion of the material with sulphuric acid and hydrogen peroxide. The residue was retained for P determination as described for the DL- α -lecithin and DL- α -cephalin hydrolyses. Alkaline hydrolysis of sphingomyelin with saturated barium water at 110° was accomplished in the same way as acid hydrolysis. The contents of the tube were acidified with 0.3 ml conc. HCl before filtration.

Phosphorylcholine and phosphorylethanolamine

These substances were hydrolyzed in 10.0 mg quantities with 5.0 ml 2 N HCl at 120°. Inorganic P determinations were carried out at 1, 7, 18, 24, 72 and 168 h.

RESULTS AND DISCUSSION

DL- α -lecithin and DL- α -cephalin

The results relating to the hydrolysis of DL- α -lecithin and DL- α -cephalin are shown in Fig. 1. In order to indicate the accuracy with which these curves are drawn it seemed desirable to quote the results of the DL- α -lecithin hydrolysis in Table II.

TABLE II

HYDROLYSIS OF DL- α -LECITHIN WITH 2 N HCl AT 120°

Time (h)	Inorganic P (%)	Inorganic P after periodate treatment (%)	P as glycerophosphate (%) [±]	P as phosphoryllecithin (%)	P as phosphorylcholine (%)	Choline (%)
	± 0.1	± 0.1	± 0.2	± 0.1	± 0.2	
0	0.0	0.0	0.0	3.9	0.0	0.0
1	0.2	3.3	3.1	0.0	0.6	12.0 ± 0.6
7	1.0	3.5	2.5	0.0	0.4	12.1 ± 0.3
18	2.1	3.6	1.5	0.0	0.3	12.0 ± 0.1
24	2.5	3.6	1.1	0.0	0.3	11.8 ± 0.1
72	3.5	3.6	0.1	0.0	0.3	11.9 ± 0.1
168	3.3	3.7	- 0.1	0.0	0.2	11.9 ± 0.1

± A first order rate constant of about 0.04h⁻¹ was calculated for the hydrolysis of glycerophosphate.

$$K = \frac{2.303}{t} \log \frac{P_0}{P_t} \text{ where } P_0 = 3.1 \text{ o.g. } K_{24} = \frac{2.303}{23} \log \frac{3.1}{1.1} = 0.045h^{-1}$$

The DL- α -lecithin and DL- α -cephalin hydrolyses, as will be shown below, proceed similarly; a discussion of the DL- α -lecithin hydrolysis will therefore be sufficient. From the results in Table II it follows that after 1 h hydrolysis $80 \pm 5\%$ of the total P is present as glycerophosphate, $15 \pm 5\%$ as phosphorylcholine and $5 \pm 3\%$ as inorganic P. The amount of water insoluble P is negligible, this therefore excludes the presence of phosphatidic acid and unhydrolysed DL- α -lecithin. Glycerophosphorylcholine also need not be considered in the P balance since Dawson¹¹ has shown that it is completely hydrolysed in 15 min. to choline and glycerophosphate in 1 N HCl at 100° . Glycerophosphorylethanolamine is equally unstable (cf. Dawson¹¹) and will be excluded in the DL- α -cephalin calculations.

Inorganic P originates from the hydrolysis of glycerophosphate and phosphorylcholine which, as will be shown presently, have almost equal first order hydrolysis rate constants. This leads to the conclusion that in the hydrolysis of DL- α -lecithin $84 \pm 5\%$ of the P remains initially attached to the glycerol molecule and $16 \pm 5\%$ to the choline molecule, i.e. about 84% fission takes place at oxygen B and about 16% at oxygen A (see structure I). The corresponding figures for the hydrolysis of DL- α -cephalin are 80% and 20% (glycerophosphate and ~~glycerophosphate~~ phosphorylethanolamine have also almost equal first order hydrolysis rate constants). A similar result is obtained if the calculation is based on the figures of the 7, 18 and 24 h hydrolysates, which is a proof of its validity.

Bunton et al.¹², who investigated the kinetics of the acid hydrolysis of dimethyl phosphate, have shown that in acid medium (1-5 N) the reaction proceeds by protonation of the ester to give the conjugate acid. The conjugate acid is subsequently attacked by a H_2O molecule and converted to monomethyl phosphate and methanol. In our case two dissimilar oxygen atoms A and B are available for protonation. A priori A, which has the higher electron density, seems more liable to be protonated. The results, however, show that oxygen B, situated near to the positive quaternary ammonium group, is much more readily protonated than oxygen A.