A THESIS

SUBMITTED BY

MICHAEL IAN GURR

A CANDIDATE

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AT THE BEHEST OF THE UNIVERSITY, THE FOLLOWING PAGES OF THIS THESIS HAVE NOT BEEN SCANNED:

APPENDIX VII, Published Work

TO

ANNE

"I found myself hampered by so many doubts
and errors that my efforts to learn
seemed to have had no other effect than
to make me increasingly aware of my own
ignorance."

Descartes, Discourse on Method.

ASPECTS OF PHOSPHOLIPID METABOLISM IN LIVER AND INTESTINE.

SYNOPSIS

Few results have been published about the composition of individual phospholipids in liver cell nuclei.

Nuclei have been isolated in either 1% citric acid or 2.2M sucrose. Rigorous tests by chemical and microscopic methods showed them to be virtually free from contamination. The chemical composition of these nuclei has been compared.

Evidence from lipid analyses, surface area measurements of lipid films, and electron microscopy is consistent with the view that citric acid-isolated nuclei are bounded by a single unit-membrane, whereas in sucrose-isolated nuclei the typical double membrane is preserved.

The composition of glycerophosphatides in various tissues has been measured by paper chromatography of their water-soluble deacylation products. Both phospholipids and fatty acids are similarly distributed in nuclear, mitochondrial and microsomal fractions of rat liver. Only cardiolipin seems to have a unique site in the cell - namely in the mitochondrion. A survey of various tissues indicated that a particular phospholipid pattern is fairly widespread.

The ^{32}P uptake by phospholipids of rat liver cell fractions has been studied at different times *in vivo*. Whereas large differences have been observed in the uptake

of label by individual phospholipids, the pattern of labelling is similar in each cell fraction.

There was no significant change in the uptake of ^{32}P into phospholipids of regenerating liver over normal or sham-operated controls in any cell fraction under the conditions chosen for investigation. No specific phospholipid appeared to be concerned in cell division.

In the intestinal mucosa of the rat, phosphatidic acid was the most highly labelled phospholipid after a thirty minute isotopic exchange period. During the absorption of triglyceride, the labelling of this compound did not change significantly, but the uptake of isotope into phosphatidylcholine increased five-fold. This was probably due to the increased requirement of phosphatidylcholine for stabilisation of the chylomicrons during fat absorption.

ACKNOWLEDGEMENTS

This is an account of original work in the department of Medical Biochemistry and Pharmacology during the tenure of an M.R.C. research training scholarship, which I gratefully acknowledge.

What little merit may be found in this work is almost entirely due to my mentors and friends in the department. Dr. J.N.Hawthorne has blended his conscientious guidance with a degree of "laissez faire" as perfectly as I could have wished and his encouragement has been invaluable.

My many helpers have been thanked in footnotes to ease the burden on this paragraph. Dr. J.B.Finean has helped me with electron microscopy and problems related to ultrastructure; Dr. W.F.R.Pover, with radioisotope work and Dr. R.M.C.Dawson with advice on chromatographic techniques.

Thanks are due to Professor Frazer for his support and to Drs. G.Hübscher, Roland Ellis, and P.J.O'Brien and many other colleagues from whom I have learned much.

Mrs. Evans has taught me that washing-up is both an art and a science, and this work would not appear in such fine plumage without the skill of the typist. Finally, my

wife, Anne, has painstakingly drawn diagrams, checked references and generally encouraged. Thank you all.

CONTENTS

INTRODUCTION

The architecture of the living cell is an intricate mosaic of different chemical species, each playing its part in some individual way in the efficient running of the mechanism.

The "giant" molecules are built from a limited number of structural units, the arrangement of which determines the properties and function of the macromolecule. The structural framework of the cell is largely dependent on these macromolecules. Their dynamic role, however, is no less important than their structural one, if indeed such a distinction is valid.

Subtle changes in the arrangement of constituent amino acids yields proteins able to function in such widely different ways as structural materials, transporting substances (e.g., in the blood), enzymes, hormones, the contractile protein of muscle and as antibodies, combating invading organisms. Likewise, nucleic acids are composed of long chains of nucleotide sub-units; variation in the base sequence in these units in DNA is responsible for the

different genetic characters of each individual. A small chemical change produces quite a different molecule, namely, the RNA capable of performing the synthesis of a protein, specified by the DNA gene. Enormous carbohydrate molecules may form the basis of plant cell walls, or yield energy by the enzymatic splitting of their chains.

Phospholipids are universally distributed in all animal tissues so far investigated; they are present in all cells and in all parts of the cell. Some areas of the cell, such as the nucleus and the soluble supernatant, may be relatively poor in phospholipids, but nevertheless, they <u>are</u> there.

Unlike that of the nucleic acids, proteins and carbohydrates of the cell, our knowledge about their precise function is extremely slight, but it seems certain that, by virtue of their orientation and intercombination with other molecules, they may likewise exert quite a number of entirely different functions.

Unlike the nucleic acids, proteins and carbohydrates, too, they are not macromolecules. Nevertheless, they are invariably located in tissues in combination with proteins (lipoproteins) or to a lesser extent carbohydrates, (lipopolysaccharides). In this way they exist as

bimolecular layers on a protein base, as for example in the typical membrane structure and in this sense they achieve a macromolecular character.

Interest in phospholipids was stimulated by the work of Thudichum, (J.) a name revered in the realm of brain chemistry. He isolated and analysed lipids from many animal tissues, particularly brain. For several years, the list of "new" phospholipids grew to unwieldy proportions, until it was recognised that the solubility properties of these lipids depended on many factors, particularly the way in which they were bound up with other substances in the tissue. Consequently, the chemists of this time were analysing mixtures of large numbers of different lipids with non-lipid contaminants, whose properties varied according to the composition of the mixture.

When the full extent of the difficulties involved in the handling, extracting, and analysing of these lipid substances was appreciated, interest in the subject waned; they were either dismissed with only a brief mention, or at best, classified into three gross classes - lecithin (alcohol-soluble), cephalin (alcohol-insoluble) and sphingomyelin, (ether-insoluble). Another factor tending to discourage research into phospholipids was the very

prevalent but erroneous idea that they were metabolically inert. It was thought that once laid down during the initial growth of the tissue, their "turnover" was very slight and hence they were ascribed a purely structural significance.

The demonstration by Hevesy, using ^{32}P, that in fact phospholipids were metabolically active, exploded this myth. Interest revived and much later, improved methods of chromatography made possible the preparation of phospholipids in pure enough form for structural analysis. This revealed a whole spectrum of phospholipids having widely differing chemical structures and metabolic activities.

A study of the metabolic activities of a few of these molecular species, and the way in which they fit into some of the functional patterns which have been ascribed to them, forms the subject of this thesis.

Chromatographic separation of phospholipids

It is probably true to say that the greatest single factor limiting the scope of lipid investigations, until comparatively recently, has been the lack of suitable separation techniques. Lipids are extremely difficult

substances to handle, not only on account of their reluctance to dissolve in water, but because, once they have been dissolved by the variety of lipid solvents available, they are found to have drawn a collection of other, usually water-soluble substances into solution with them. The solubility properties, too, of a given lipid in a certain solvent are greatly altered by admixture with other components.

Great advances in lipid methodology during the 1950's have enhanced research and created new interest in lipid biochemistry.

Extraction of lipids

Before separation methods can successfully be applied, the lipids must be efficiently extracted from the tissue and freed as far as possible from non-lipid contaminants. There is no single standard method of lipid extraction; the choice depends rather on the type of tissue, and the nature of other compounds to be analysed. For example, there have been many analyses on the different classes of phosphorus compound in a tissue: DNA, RNA, inorganic phosphorus, acid-soluble organic phosphorus, phospholipid and phosphoprotein; as a consequence, methods were needed for the comprehensive extraction and separation of these

compounds. Methods due to Schneider (2.) and Schmidt and Thannhauser (3.) have been widely used; practical details are to be found in Part I, 'Methods'.

For most lipid work, a mixture of chloroform and methanol in the proportions 2:1 by volume has been found satisfactory. For a tissue such as liver, direct homogenisation of the tissue in the solvent is efficient, but in many cases, especially for ^{32}P incorporation experiments, the acid-soluble phosphorus fraction is also required; in this case, extraction is best done by initial precipitation of the lipid-containing material with 10% TCA, washing carefully with water to remove most of the acid, and then extracting the precipitate with chloroform-methanol. Getz and Bartley (4.) have compared different extraction methods and suggest that TCA precipitation causes slight loss of lipid material.

When dealing with brain, pretreatment with acetone (5.) has been found essential to ensure complete extraction of the higher inositides. Folch (6.) has found extraction of brain with chloroform-methanol, acidified with hydrochloric acid, to be satisfactory.

Owing to solubilisation of non-lipid compounds by the lipid, it may be necessary to wash the extract. Many

methods have been employed, but that due to Folch et al (7.) seems to be the most widely used and is very simple. The solvent extract is shaken with one-fifth of its volume of sodium, potassium, magnesium or calcium chloride. Care should be exercised in washing; emulsions are easily formed at the interface of aqueous and solvent layers, in which much lipid, especially inositides may be lost. Further purification of the washed extract is effected by reducing chloroform-methanol extracts to dryness under vacuum, and re-extracting the residue with pure, dry, chloroform. The method of Bligh and Dyer (8.) is also widely used.

Chromatography of intact phospholipids

Column chromatography

The use of column chromatography for the separation of phospholipids has one big advantage - it can be used preparatively, and it can yield enough material for extensive analyses. Early work used columns of magnesium oxide, alumina, and cellulose, but silicic acid has found the widest applications. The great disadvantage of silicic acid chromatography is that it separates phosphatides into groups rather than individual compounds, unless the starting material has a very simple composition; thus

the method is best used after initial separation by other means. It is advantageous to keep checks on the composition of peaks from silicic acid columns by the methods of paper or thin-layer chromatography. Hanahan et al (9) have described phosphatide separations on silicic acid-hyflo-super-cel columns, eluting compounds with different chloroform-methanol mixtures. Silicic acid chromatography has also found applications in the purification of phosphatidylethanolamine and phosphatidylserine, and is useful for removing triglycerides from tissue lipid extracts.

Silica-impregnated paper

This method has been used extensively by Marinetti (10.). Although many of the spots obtained seem to be pure compounds, others are undoubtedly mixtures and such separations (as indeed all phospholipid separations so far achieved) should be treated critically.

Formaldehyde-treated papers

These were described by Hörhammer et al (11.), and seemed to hold great promise for separating intact phosphatides, especially those of the brain inositide complex. They have proved disappointing in our hands; all methods

depending on impregnated paper seem to be prone to streaking. In the case of formaldehyde papers, spots were often heterogenous, phosphatidylcholine and phosphatidylethanolamine could not be separated, and a wealth of minor components seemed to simply get lost.

Thin layer chromatography

The use of thin layer chromatography for the separation of lipids has rocketed into prominence in the last year or so. It is versatile, quick, allows separations of μg or mg quantities of lipid, and can be used in conjunction with a large number of detection techniques, including charring. The procuring of adequate separations depends on subtle combination of a suitable solvent system with appropriate additives to the silica stationary phase. Methods have been covered adequately by Mangold (12.) and Skidmore (13.).

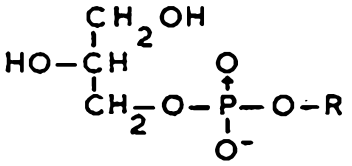
Separation of phospholipid hydrolysis products

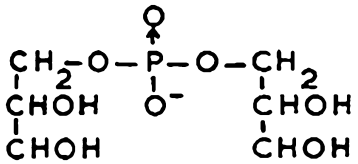
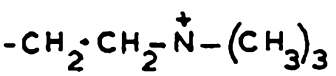
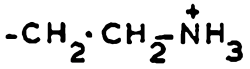
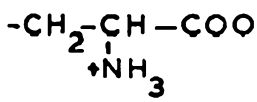
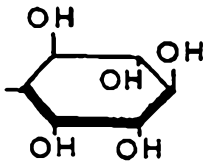
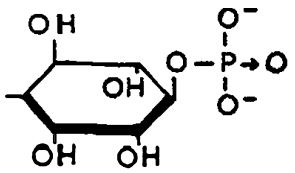
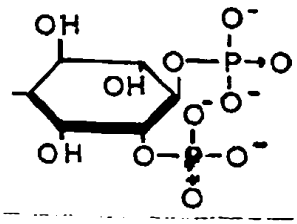
The most widely used method is that of Dawson, published originally in 1954 (14.), but enlarged and elaborated in 1960 and 1963 (15, 16.).

The initial procedure is a hydrolysis under specified conditions with mild ethanolic sodium hydroxide. This causes release of fatty acids from glycerophosphatides,

Fig 1 The mild alkaline hydrolysis products of phospholipids

General formula of glycerylphosphoryl derivatives



Group R	Symbol	Parent phospholipid
H-	GP	Phosphatidic acids (and Lyso-)
	GPGPG	Diphosphatidyl glycerol (cardiolipin, polyglycero-phosphatide) (and lyso-)
	GPC	Phosphatidylcholine (lecithin) (and lysolecithin)
	GPE	Phosphatidylethanolamine (and lyso-)
	GPS	Phosphatidylserine (and lyso-)
	GPI	Phosphatidylinositol (and lyso-)
	GPIP	Phosphatidylinositol phosphate (Diphosphoinositide)
	GPIPP	Phosphatidylinositol diphosphate (Triphosphoinositide)

The assistance of Mrs D. Clark and Miss P. Taylor is acknowledged.

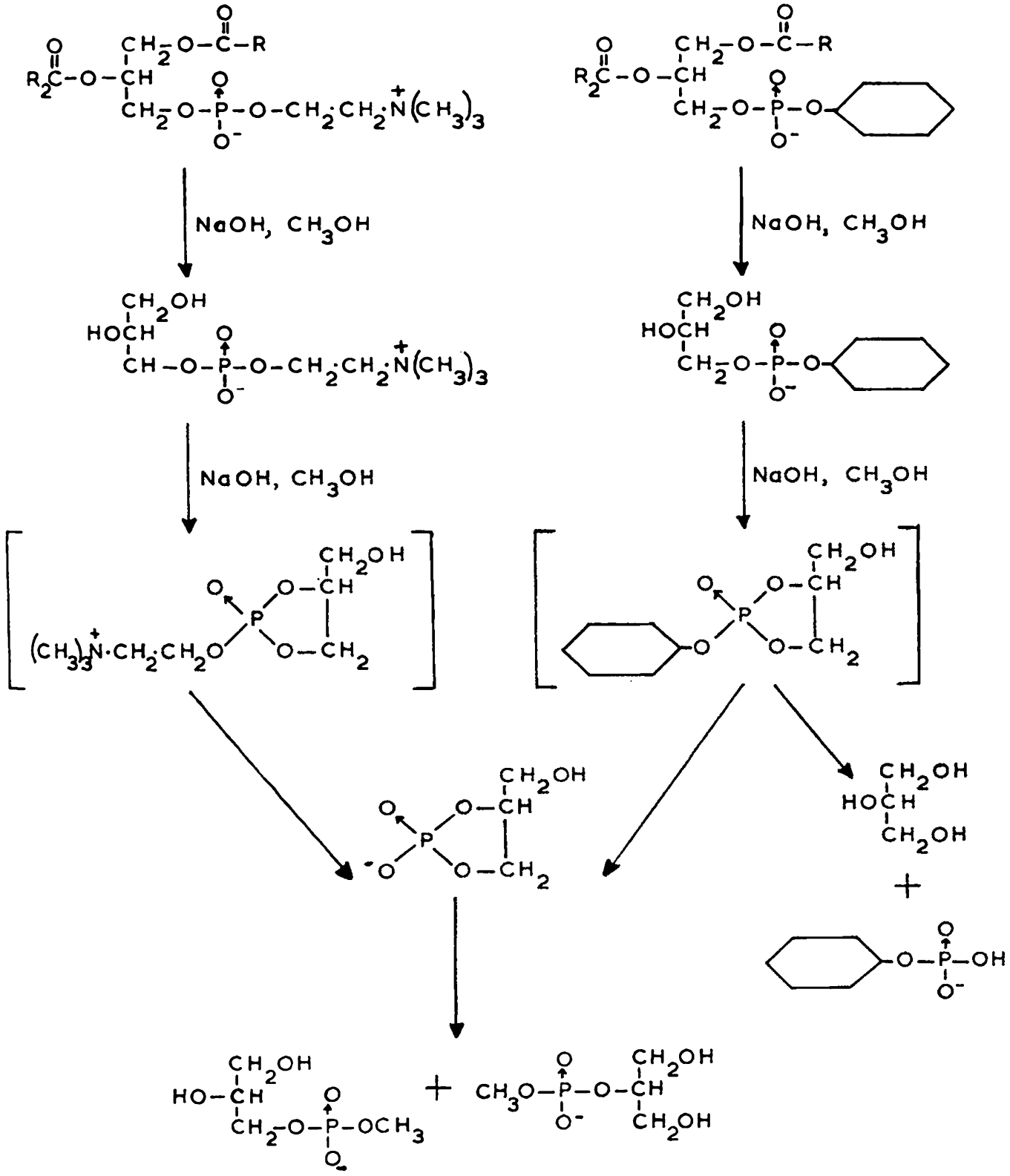
the remaining phospho-diesters being water-soluble and separable by chromatography in a variety of solvents, the commonest being water-saturated phenol-acetic acid-water, 100:10:12, or, preferably, by electrophoresis. The lipids which are resistant to mild alkali, are hydrolysed by mild acid, (plasmalogens) or finally, strong acid at high temperature in a sealed tube, (sphingomyelins). Hydrolysis products from the more common animal phospholipids are shown in figure 1.

Several criticisms can be levelled against the method; briefly these are:

1. Unless the conditions of the hydrolysis are controlled carefully, the mechanism in some cases is not a simple de-acylation; cyclic phosphate esters are formed (see figure 2), complicating the identification and analysis of these compounds. Prolonged alkaline hydrolysis results in cleavage of the nitrogen base to yield glycerophosphates. Phosphatidylcholine and phosphatidylinositol are especially susceptible to cyclic ester formation (figure 2) and in quantitative work correction must be made for breakdown of these compounds.

2. Since lysophosphatides yield the same diester as the analogous diacyl lipid, the method cannot distinguish between these phosphatides. In many tissues lysophosphatides

Fig 2 CYCLIC ESTER FORMATION DURING HYDROLYSIS OF LECITHIN AND MONOPHOSPHOINOSITIDE



may be safely ignored, but in others this cannot be the case and other separations must be made in conjunction with this one.

3. Plasmalogen is subject to cyclic acetal formation in mild acid conditions and the product is only further hydrolysed in strong acid. Consequently the recovery of plasmalogen is not quantitative. Dawson (16.) recognised this and subsequently included mercuric chloride in the mild acid hydrolysis to minimise cyclic acetal formation. Nevertheless, there is evidence that the degree of recovery of plasmalogen depends on the time of original incubation with mild alkali (17.).

On the credit side, the method is notable for the extremely good separations of all the hydrolysis products of common phospholipids; GPE* and GPS*, for instance, are far more easily separated than phosphatidylethanolamine and phosphatidylserine. The analysis of phosphorus in the spots is simple and in most cases yields excellent recoveries.

Ion-exchange chromatography

This method, originated by Hübscher et al (18.), extends the method of Dawson and enables larger quantities of lipids to be analysed. It is particularly useful for

* See abbreviations.

the characterisation of minor components and for the elucidation of the structures of new lipids.

Analysis by complete hydrolysis

Sometimes it is useful to completely break down the phospholipid molecule by hydrolysis in strong acid, and to analyse for such components as glycerol, phosphate, choline and other nitrogen bases, inositol etc. Detailed methods can be found in McIlwain's book (19.).

In summary, a considerable number of methods now exist for the separation of phospholipids, but no one method is good enough. While one method may separate only groups of phosphatides, others will not distinguish lyso-compounds, which are often marked out by their special properties. Others may cause breakdown or artifacts. The results from all methods need to be interpreted critically, and a method should be chosen which best suits the particular problem under investigation, and wherever possible, used in conjunction with other, different types of method.

Finally, we blithely talk about individual phospholipids when we really refer to a heterogeneous collection of molecules, each characterised by its own peculiar

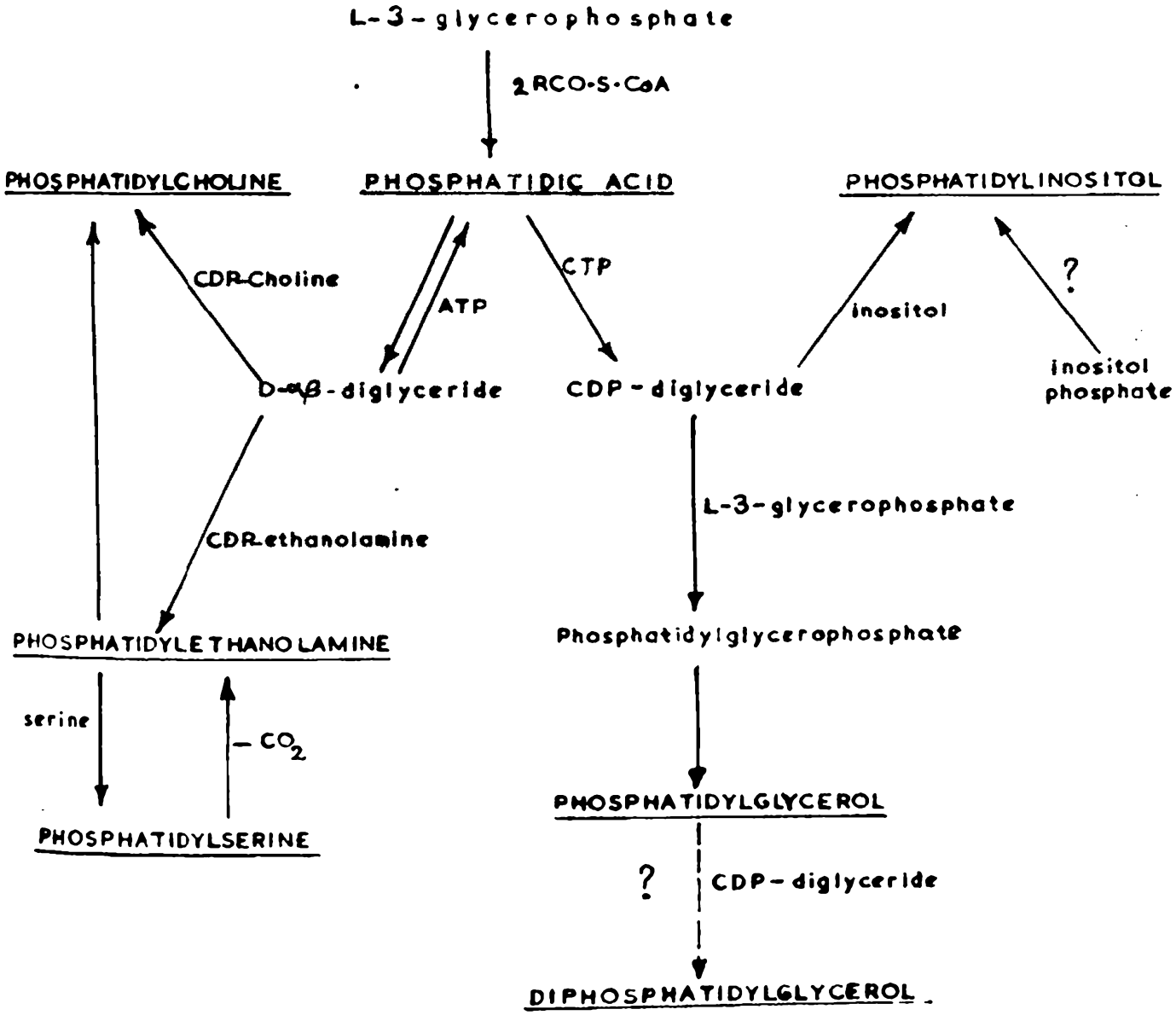
fatty acid pattern. Very few pure lipids have yet been obtained from living creatures though the skill of the synthetic chemists has produced many. It would need a latter-day H.G. Wells with biochemical leanings to describe the machine of the future for separating all the molecular species in a natural phospholipid mixture.

The biosynthesis of glycerophosphatides

The subject of phospholipid biosynthesis has been reviewed by Kennedy (20). The present state of our knowledge of the biosynthetic pathways for the most common glycerophosphatides of animal tissues is summarised in figure 3. As far as is known at present, the de novo generation of the phospho-diester linkages, characteristic of phospholipids, involves the cytidine coenzymes in every instance. Although two cases of phospholipid inter-conversion have been described, (the enzyme-catalysed methylation of phosphatidylethanolamine to phosphatidyl-choline, and the formation of phosphatidylserine from phosphatidylethanolamine and L-serine), the lipid in each case is ultimately derived from CDP-ethanolamine.

Introduction figure 3

General scheme for the biosynthesis of glycerophosphatides



Phosphatidic acid

Apart from the pathway in figure 3 in which L-3-glycerophosphate is the precursor, an alternative biosynthetic route has been described by Pieringer and Hokin (21.) involving the phosphorylation of ~~mono~~ di glyceride by ATP. The relative importance of these pathways is not known.

Phosphatidylinositol

As figure 3 shows, the pattern of reactions for the biosynthesis of phosphatidylinositol is different from those for phosphatidylcholine and phosphatidylethanolamine. Evidence that this pathway involved a cytidine diphosphate-diglyceride was first presented by Agranoff et al (22.) and at the same time, Paulus and Kennedy (23.) demonstrated the conversion of L-3-glycerophosphate (^{32}P) to phosphatidylinositol by a pathway specifically requiring CTP as a cofactor. Hence the phosphate moiety of L-3-glycerophosphate and phosphatidic acid is the direct precursor of the phosphatidylinositol phosphate and this is reflected in the high labelling of these two lipids when tissues are incubated with ^{32}Pi or (^{32}P)-glycerophosphate (14,24.). The results of Thompson et al (25.) suggest that there is a second pathway for the synthesis of phosphatidyl-

inositol in brain, not involving phosphatidic acid. During studies on the inositol compounds of rat liver, Galliard (26.) found that (^{14}C)-inositol phosphate had a higher specific activity than the lipid inositol. This points to the fact that the inositol phosphate is not solely a breakdown product of phosphatidylinositol, and again could suggest that an alternative to the Kennedy scheme is operating. Attempts to demonstrate an inositol kinase, however, have always failed.

Little precise evidence is available about the biosynthetic pathways for di- and triphosphoinositides. The experiments of Wagner et al (27.) and Ellis and Hawthorne (28.) in vivo, and those of Brockerhoff and Ballou (29.) in vitro, suggest that these compounds are synthesised by the stepwise phosphorylation of phosphatidylinositol:
Phosphatidylinositol $\xrightarrow{XP}$ diphosphoinositide $\xrightarrow{XP}$ triphosphoinositide
The phosphate donor is unknown, but is likely to be the same in each case (29.). Recently, evidence has been obtained for the biosynthesis of diphosphoinositide in the mitochondria of liver (30,31) and brain (32.).

Polyglycerophosphatides

The reaction sequence for the biosynthesis of polyglycerophosphatides (figure 3) was proposed by Kiyasu et al (33).

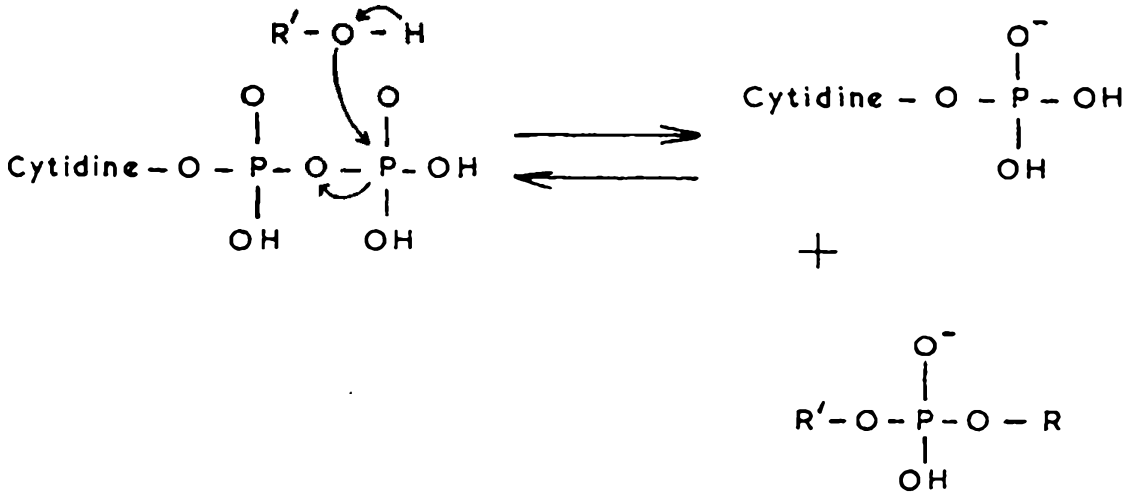
Phosphatidylglycerol does not accumulate in animal tissues and these authors suggested that it may act as a precursor of cardiolipin by reaction with a second mole of CDP-diglyceride, although there is no direct evidence of this step.

Methylation of phosphatidylethanolamine

Evidence for the methylation of phosphatidylethanolamine to phosphatidylcholine, with phosphatidyl (N-methyl) ethanolamine and phosphatidyl (N,N-dimethyl) ethanolamine as intermediates, has been presented by Bremer and Greenberg (34.). This provides an alternative means of biosynthesis of lecithin. The proportions of phosphatidylcholine synthesised by the two pathways are not known. Dawson (35.) showed that *in vivo* the phosphorylation of choline in liver proceeds at such a rate as to account for the overall synthesis of phosphatidylcholine from inorganic phosphate, but he pointed out that the kinetics of labelling of phosphorylcholine and phosphatidylcholine did not perfectly fit the criteria of Zilversmit *et al* (36.) for a precursor-product relationship, suggesting the possibility of alternative pathways. Dawson's work (37.) speaks against the conversion of phosphatidylethanolamine to phosphatidyl-choline as a predominant, or even significant, reaction for the formation of lecithin.

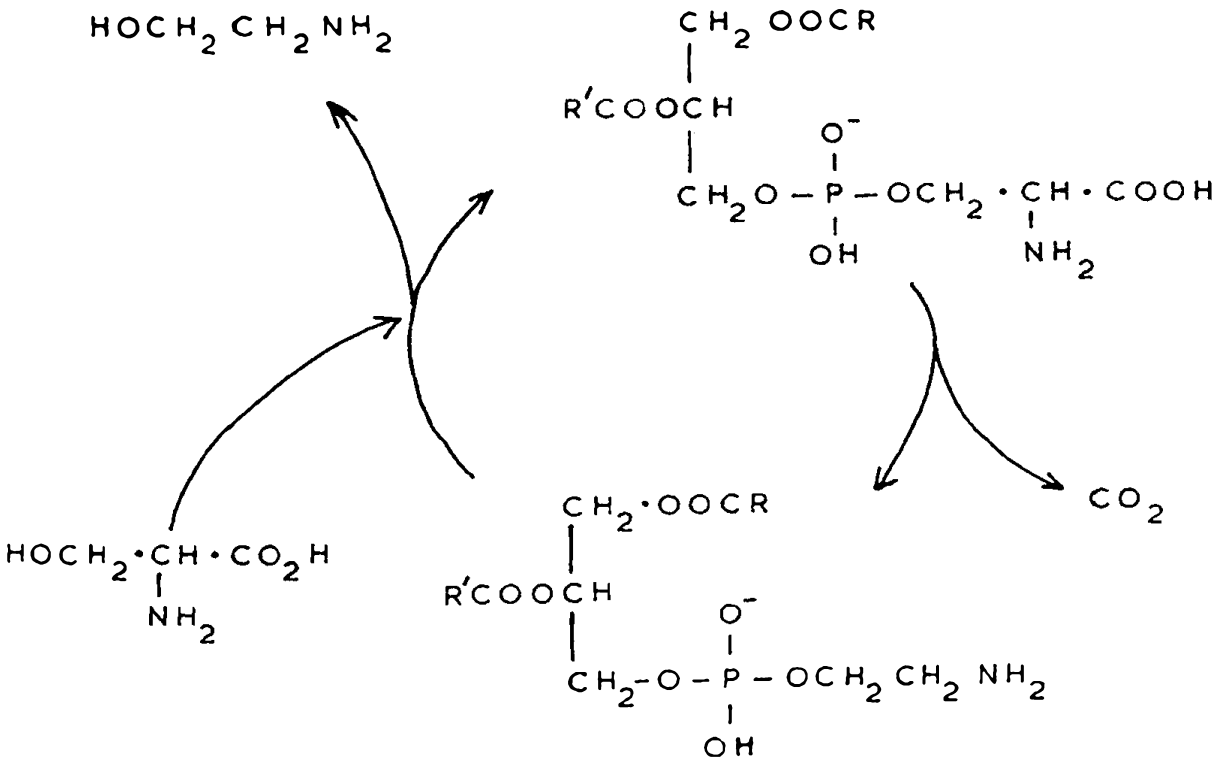
Introduction figure 4

Mechanism of formation of phosphodiester bonds



Introduction figure 5

Phospholipid cycle in decarboxylation of Serine



Acylation of lysophosphatidylcholine

The discovery by Lands (38) of an enzyme catalysing the acylation of lysolecithin is important in confirming the concept of the independent turnover of the fatty acid moieties of lecithin.

Phosphatidylserine

Hübscher et al (39,40) have studied the enzymatic synthesis of phosphatidylserine. Calcium ions stimulated the energy-independent incorporation of serine into phosphatidylserine, suggesting the reversible action of phospholipase D. Enzyme systems in liver (41.), can catalyse the formation of phosphatidylserine from L-serine and phosphatidylethanolamine and the decarboxylation of phosphatidylserine to phosphatidylethanolamine and carbon dioxide. Such reactions constitute a metabolic cycle (figure 5) for the interconversion of these lipids and cycles of this type may well be important for controlling phospholipid metabolism.

The composition and distribution of phospholipids in liver cells and their role in membranes

While figures can readily be quoted for the total lipid content of rat liver (42a), and a host of other

tissues, details of the individual lipid composition are much more scarce, especially with regard to the different phospholipids of the "cephalin" group, phosphatidylethanolamine, phosphatidylserine, and phosphatidylinositol.

Rat liver phospholipids have often been determined by total hydrolysis and detection of the nitrogenous bases (43-45). Marinetti et al (46) fractionated phospholipids of whole rat liver on silica-impregnated paper, and Getz et al (47) used both silicic acid column and paper chromatography. Another technique used by Strickland and Benson (48) has been the separation of deacylated lipids by paper chromatography and the detection of the phosphate esters by radioautography after neutron activation. The most recent studies have been by Biezenski et al (49.) using silicic acid papers for the separation of phosphatides and the determination of the nitrogenous bases for their estimation. In most of the work quoted here (43-45, 47-49.), the liver cells were fractionated into mitochondria, microsomes, and supernatant, but only in a few cases were nuclei analysed (see Part I: Introduction).

In addition to phosphatide composition, attention has recently been turned to the fatty acid composition of

individual rat liver phosphatides, and the distribution of fatty acids throughout the cell (47,50,51.).

From these results, one gets a picture of the phospholipid composition of rat liver as follows: Phospholipids comprise approximately 2.5 - 3% (W/W) of fresh liver. Of this, 50% is found in the microsomal fraction and 35% in the mitochondrial fraction. There is some considerable disagreement about the proportion of lipid in the cell nucleus, but the general opinion is that this organelle is relatively poor in lipid. The cytoplasmic particulates, which are rich in phospholipid, have little or no triglyceride, whereas the soluble supernatant fraction of the cell contains most of the cell triglyceride but has a low concentration of phospholipid.

About 50% of the lipid phosphorus is found in lecithin and lysolecithin, and 25% in phosphatidylethanolamine. The remaining fraction consists of phosphatidylserine, phosphatidylinositol, cardiolipin, phosphatidic acids and sphingomyelin, although the figures quoted in the literature for these minor components differ considerably. There are no exact data about the quantities of lyso-compounds present, and the rapid autolysis of tissues makes exact measurements of these compounds difficult; diacyl lipids

tend to be broken down very slowly during storage to yield lyso-compounds, the rate of breakdown depending on the polarity of the solvent. The plasmalogen content of liver is negligible (52.).

It has been stated earlier that phospholipids are present in most tissues as definite lipid-protein complexes. The evidence for this view comes from many sources. In general, phospholipids are not extractable from tissues by chloroform alone, even though this solvent is a perfectly adequate one for dissolving almost all lipids. When methanol is added to the extraction solvent most of the lipid can be removed from the tissue; this is interpreted as meaning that the more polar solvent destroys the lipid-protein bonds, thus enabling the complete removal of the lipid into the organic solvent. As an illustration of the tightness of binding of the lipid to protein, an experiment of Chargaff and Cohen (53.) may be cited. When lipoproteins from egg yolk were treated with lecithinase A, more lipid was extractable than by solvent action alone.

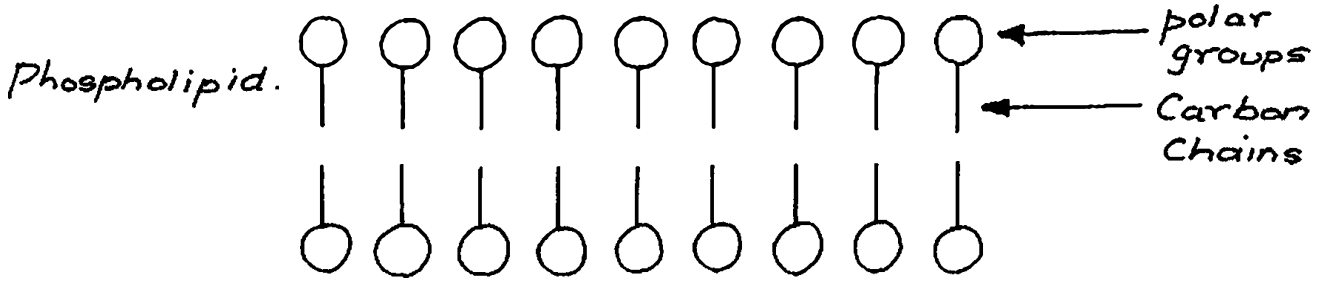
The type of bonding between lipid and protein is not known with certainty. Dervichian (54.) presented evidence that the basic unit of a lipoprotein is an ionic compound,

while the units are bound to each other by Van der Walls forces. Chargaff (55) pointed out that since lipoproteins contain a variety of lipid types, it was likely that different types of bonding occur. Thus phospholipids, possessing functional groups and ionic centres, could bond by primary covalent or ionic linkages, whereas sterols could only make use of secondary valence forces, either with the protein or, more likely, with the other lipid molecules. This may be the reason why in some cases sterols may be removed without disrupting the lipoprotein, while phospholipids can never be liberated leaving the sterol still bound.

A great deal of evidence has accumulated to prove beyohd all question that lipoprotein complexes of the type described above, are of prime importance in cell membrane structures. Gorter and Grendel (56) extracted the lipids from red cells and related the surface area which would be occupied by this lipid in a monomolecular film, to the total surface area of the red cells. The lipid was found to be sufficient to provide a bimolecular leaflet around the red cell. Although Gorter and Grendel's extraction has been criticised (57), the experiment has been repeated by Van Deenen (58.) using refined methods

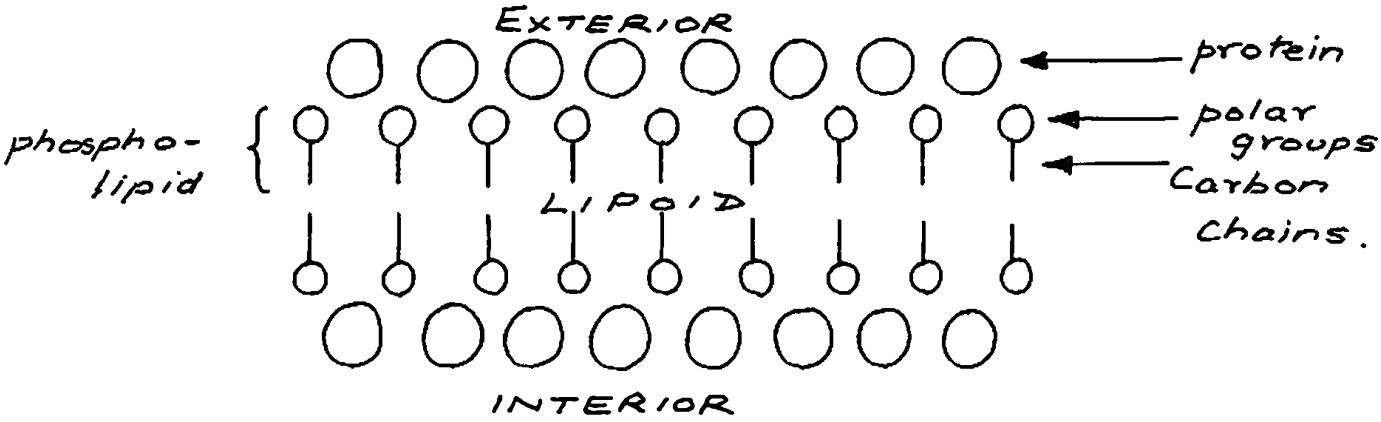
and the result proved substantially correct. Arguing from chemical and optical data, Ponder (57.) concluded that the surface membrane of erythrocytes did not contain a continuous layer; he regarded the surface layers as much more complex and argued against the rigid distinction between surface layers and the interior.

The first, and simplest idea of membrane structure was put forward by Gorter and Grendel in view of their results. The membrane was thought to consist simply of a bimolecular leaflet of phospholipid, with the hydrophilic groups orientated towards the plasma and interior of the cell respectively.



Introduction fig.6.
Simple membrane model of
Gorter and Grendel.

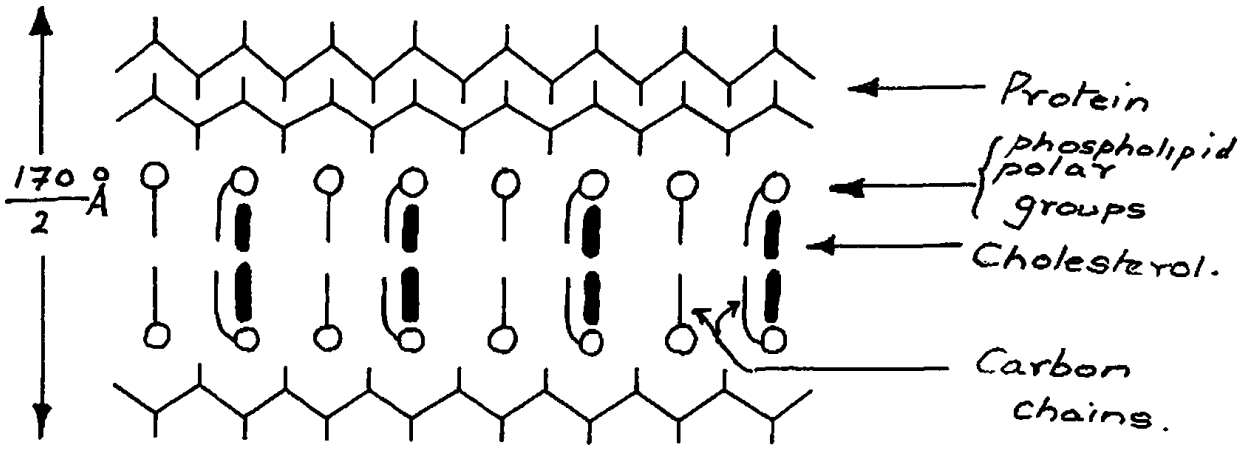
Danielli et al (59,60), realising that protein must be associated with the lipid in the membrane, owing to the low surface tension values they obtained at the membrane-water interface, formulated the pauci-molecular theory of membrane structure.



Introduction fig.7.

The pauci-molecular theory of membrane structure (Danielli and Davson)

The evidence on which our present knowledge of membrane structure is based, has been largely accumulated by the study of nerve fibres with the electron microscope. Finean (61.) believes that the myelin sheath structure consists of an arrangement of lipid bimolecular leaflets and protein monolayers. Cholesterol molecules are inserted between every other phospholipid molecule.



Introduction fig.8.
One-half of the radially repeating unit of the myelin sheath structure (Finean).

After the introduction of $KMnO_4$ as a fixative for the electron microscopy of tissues, Robertson (62.) noted the occurrence of the 75 Å thick layer featuring two narrow dense lines separated by a light region of similar thickness; this has been called a unit membrane. Such a structure is represented in OsO_4 -fixed tissue as a single line. The nuclear membrane was first found by Hartman (63.) to be a double membrane, i.e. to be composed of two unit membranes or four lipid monolayers. One other point of interest in relation to the lipid components of nuclear structures is that the nuclear membrane is at many points continuous with the endoplasmic reticulum (64.), which also has the 75 Å unit structure.

The studies of Palade (65), Sjostrand (66) and others

have indicated that the mitochondrial membranes are in every way similar to those seen in cell surfaces, endoplasmic reticulum and nuclear membranes. The mitochondrion contains some 28% of its weight as lipid, and Fleischer et al (67.) have shown that the phospholipid composition is essentially the same in all submitochondrial particles. Green (68.) considers that the mitochondrial lipid serves as the medium in which the oxido-reductions of the structured segments of the electron transport chain take place. In addition to this function, the lipid fraction contains co-enzyme Q and a cytochrome-c-lipid complex. These substances appear to be capable of shuttling electrons between pairs of fixed oxidation-reduction components.

From this summary of the lipid components of membranes, it can be seen that although there is a great deal of evidence that phospholipids are important structural components of the cell, they can by no means be regarded as inert building blocks. Their very presence in the membrane confers on them an additional "active" or "metabolic" significance and the more our knowledge grows, the harder it becomes to make any real distinction between "structural" and "metabolic".

Phospholipids and the transport and binding of ions

The series of papers by Hokin and Hokin which have stimulated so much interest in the role of phospholipids as ion carriers across membranes, began with their original observation that acetyl choline increases the turnover of phosphatidic acid and phosphoinositides in certain tissues (69). These compounds have a noticeably high turnover even before stimulation with the hormone and all tissues in which this high turnover can be demonstrated, appear to be secretory in nature, (Pancreas, thyroid, adrenals, salt gland, parotid, (70), and also brain (69), leucocytes (71) and intestine - see Part III). Broadly speaking, there seem to be two types of tissue, those showing the phosphatidic acid effect, and those in which phosphoinositides and phosphatidic acid turn over at rates similar to the other phospholipids. This tends to suggest a special function for these lipids in this type of tissue and the Hokins have suggested that they are concerned with the transport of ions in and out of cells, since lipids are located largely in membrane systems.

Transport of sodium

Most living cells have a higher potassium content and lower sodium content than the fluid surrounding them;

therefore the transport of sodium out of the cell requires metabolic energy. The "membrane hypothesis" holds that a membrane constituent combines specifically with the ion to be transported; the complex diffuses across the membrane, releases the ion at the opposite face, and in so doing, sets up a concentration gradient for the complex within the membrane in the direction of transport.

Active transport is known to depend on respiration, glycolysis or a supply of ATP. A sodium-dependent ATP-ase system exists in various membranes, which is in many respects similar to the sodium transporting system, and Jarnefelt (72) has suggested that there is a phosphorylated intermediate in ATP breakdown which is concerned in sodium transport. The ATP-ase in brain microsomes is adequate for the observed ion transport in brain tissues (73).

Hokin and Hokin (74), after experiments showing acetyl choline stimulation of phosphatidic acid turnover coupled with sodium secretion in the albatross salt gland, suggested phosphatidic acid as the carrier molecule. The cycle would be:

ATP + diglyceride ⟶ phosphatidic acid + ADP

phosphatidic acid + Na^+ ⟶ sodium phosphatidate

sodium phosphatidate ⟶ diglyceride + Na^+ + PO_4^{3-}

Sodium phosphatidate diffuses through the membrane and is hydrolysed at the outer surface; this is in effect, the ATP-ase system. The question of how the phosphate is transported back across the membrane is not clear.

The criticisms which have been levelled against this scheme are, firstly: the observed turnover of phosphatidic acid is not enough to carry the amounts of sodium known to be excreted, (kidney can transport up to 28 sodium ions for every molecule of oxygen consumed, whereas the above system could only manage 12 of these). Secondly, phosphatidic acid is unlikely to be specific for sodium rather than potassium. Lately, the theory has been modified to include a carrier protein (75.), whose configuration is altered by the alternate phosphorylation and reformation of phosphatidic acid, thus supplying the energy to pass sodium ions across the membrane; criticisms concerning the kinetics of the process have been answered by the speculation that the phosphatidic acid in a particular "compartment" of the cell or cell fraction turns over faster than that in another "compartment".

The role of phosphoinositides in nervous tissue

The "acetyl choline effect" has been investigated by

the Hokins in nervous tissue, as well as a host of others. Hokin has suggested that the phosphatidic acid pump is involved in the restoration of Na^+ and K^+ ion levels after a nervous impulse (76).

By electrically stimulating cat cervical ganglia, Gardiner (77) was able to show that although there was no change in phosphatidic acid turnover, two more lipids became radioactive after incubation with ^{32}P. Larrabee (78) obtained an increase of more than 50% in the ^{32}P uptake of phosphatidylinositol in stimulated rat cervical ganglia; labelling of phosphatidic acid was not affected. Hawthorne (79) thinks that this might be due to hydrolysis of the higher inositides, the build up of monophosphoinositide accounting for the increase in radioactivity.

Brossard and Quastel showed that addition of potassium ions and the removal of calcium ions stimulated the ^{32}P incorporation into the phosphatidic acid and phosphoinositides of brain slices (80). These authors think that the stimulation by acetyl choline is due to the production of a local concentration of potassium ions, possibly stimulating ATP turnover. It seems likely that a role in the transport of calcium or magnesium in nervous tissue could be assigned to triphosphoinositide. Calcium

ions play an important part in determining the permeability of nerve membranes. The monoesterified phosphate groups of triphosphoinositide would have greater affinity for divalent than monovalent cations (79.); this also applies to phosphatidic acid, (81.). The two monoesterified phosphates of triphosphoinositide have a higher turnover than the diester phosphate (28.) and these would no doubt provide greater capacity for cation transport than phosphatidic acid.

The recent work of Galliard and Hawthorne (31.) (following an observation of Garbus et al (30)) shows a rapid incorporation of ^{32}P into only the diphosphoinositide (and to a lesser extent, the phosphatidic acid) of rat liver mitochondria after only five minutes incubation with Tris/HCl buffer, potassium glutamate, magnesium chloride, sucrose and radiophosphorus. Hawthorne has suggested that the behaviour of these lipids may be connected with the restoration of ionic levels in the mitochondria when they are first incubated.

Interest in the lipid field now seems to be swinging very much in this direction, and the next few years should see some exciting developments in this aspect of phospholipid function.

Phospholipids and fat absorption

Lipids are by definition, fatty substances, soluble in fat solvents. The phospholipids derive their distinctive physical properties from two contrasting regions of the molecule: their fatty nature derives from the long-chain fatty acid residue while the polar region confers on the molecule a certain hydrophilic character. Hence they are soluble in fats and fat solvents, but readily form emulsions or micelles in which the polar regions of the molecule are orientated towards an aqueous medium. In consequence, fatty acids would be more readily solubilised in a predominantly aqueous system, when esterified to a phospholipid, than to a triglyceride.

In view of these properties, it is perhaps inevitable that many biochemists should have postulated phospholipids as intermediates in processes of absorption and resynthesis of fat in intestinal cells. Experiments designed to show such a relationship began with those of Bloor in 1915 (82.) and have been performed by many in the intervening years. At the present time there is no support for the view that phospholipids in general must be involved as intermediates, but it is realised that in certain instances a particular

phospholipid molecule may be involved in processes concerned with the absorption of fat, or in the resynthesis of triglyceride from absorbed components.

Before reviewing the work in this field, it is pertinent to mention the general theories about the way in which fat is absorbed in the gut.

A number of experiments performed in the latter half of the nineteenth century left the question "Is it necessary for fat to be completely hydrolysed to its components glycerol and fatty acid, before it can be absorbed by the intestine?" open to no small degree of doubt. In 1900 Pflüger (83) stated quite dogmatically that "All fat in the intestine must be hydrolysed before absorption". This statement was based on certain observations of his own and of others, but there was no proof for such a bold statement. As part of his support for this theory, Pflüger quoted the results of Frank (1898) (84.) who found that ethyl esters of fatty acids were totally hydrolysed before absorption. He had also demonstrated that synthesis of triglyceride was taking place during the period when fat was being absorbed. These ideas were challenged by Munk (85.) and others, but Pflüger ably defended his theory and soon received support from the experiments of Henriques and Hansen (86.). They showed that by feeding emulsions of

equal parts of lard and paraffin to animals, only the lard was absorbed from the gut while the paraffin was quantitatively recovered from the faeces; hence they conlcuded that droplets of emulsion could not be absorbed and that only the fat could ~~only~~ be taken up by the gut after hydrolysis. This "Lipolytic" theory held pride of place for many years and was given great support by Verzar (87.) and others as late as the 1930's and 1940's.

Evidence from a number of sources that hydrolysis of fat, before absorption, was incomplete led Frazer (88) to postulate what has been called the "Partition" theory (1938). Briefly this theory holds that fat may be absorbed in the intestine without prior hydrolysis, in the form of a finely dispersed emulsion, or in the hydrolysed or partially hydrolysed form as fatty acid, mono-, di- and triglyceride, and glycerol. After absorption, the shorter chain fatty acids pass through the portal vein to the liver, while the triglyceride, in the form of chylomicrons, stabilised by phospholipid and protein, finds its way via the lymphatics to the thoracic duct and thence to the fat depots. Frazer has comprehensively reviewed the two theories (89.).

Some experiments of Frazer's may be briefly quoted

as lending support to this theory. Finely dispersed paraffin oil fed to rats resulted in 60% absorption, as much as the absorption of an equivalent amount of olive oil in the same period (90). A marked increase in the number of chylomicrons in human subjects given a test meal of olive oil, was curtailed by addition of lipase and by feeding oleic acid and glycerol (91.). Lastly, the absorption of neutral fat resulted in a milky appearance of the lacteals, whereas absorption of fatty acid did not have this effect. There have been some minor alterations to the theory and much discussion about the ideal particle size for absorption, but in general, the theory has now gained wide acceptance.

To summarise, fat is now considered to be absorbed as a mixture of glycerol, free fatty acids, mono-, di-, and triglycerides. Some resynthesis of triglyceride is necessary, but the precursors are mono- and diglyceride as well as glycerol and fatty acid.

Among the earliest experiments on the theme of phospholipids and fat absorption were those of Bloor (1915) (82). He observed an increase in blood phosphatidylcholine and concluded a) that blood corpuscles take up fat from plasma and transfer it to phosphatidylcholine, and b) that lecithin

is therefore an intermediate. This makes interesting reading in the light of this present and other work, but Bloor's general theory had to be discarded when Sinclair later showed high labelling of plasma fatty acids with elaidic acid but none in red corpuscles (92).

The evidence about whether phospholipids form an obligatory step in fat absorption seems to have been based, since 1929, on four types of experiment:

1. labelling with specific fatty acids.
2. inhibition and acceleration of lipid phosphorylation.
3. observation of lymph phospholipids.
4. labelling with radioactive phosphorus.

Sinclair's (92 - 94.) are among the classical experiments in this field, and his reasons for believing that phospholipids might be important in fat absorption are worth relating. An accumulation of data from experiments of Munk (1880) (95) and others had shown that there is a resynthesis of fat from absorbed constituents in intestinal mucosa, and attempts to show reversal of lipase action had been unsuccessful. Krehl (1890) (96) had shown that mitochondria of epithelial cells were active agents in fat resynthesis and Mayer et al (1914) (97) had shown mitochondria to be composed largely of phospholipids.

Sinclair reasoned that the phospholipids of intestinal mucosa might be concerned in fat absorption processes and that if this were so, then their fatty acids should assume the characteristics of the fat being absorbed.

In Sinclair's experiments (93), cats were fed a constant fat diet for four days and then cod liver oil or cocoanut oil in 50% emulsion by stomach tube. The absorbed fatty acids (measured by their "iodine numbers") were found to be incorporated in mucosal phospholipids. Two parts of the mucosa were studied and although there were differences in the % amounts of fatty acids in phospholipids in the two sections of mucosa, the iodine numbers were the same. To summarise Sinclair's results, he found that during the absorption of fat there was (1) a pronounced change in composition, but not in amount of phospholipid fatty acid of intestinal mucosa and liver, and (2) no significant change in amount or composition of phospholipid within intestinal muscle. His hypothesis was that within the epithelial cells, there is a "specific" phospholipid, (note that he did not say phospholipids in general), which is intermediate in the sequence:

Fatty acid ⟷ Phospholipid ⟷ Neutral fat

The amount of this compound, it can be seen, should

remain constant; such a compound was regarded by Sinclair as being an essential step in the resynthesis of fat. Bloor, writing in 1943 (98), enlarges on this idea: "As soon as fatty acids are absorbed into epithelial cells, molecules of phospholipid react with free fatty acid or soaps to form neutral fat; immediately, however, the residual phosphoric acid-base complex unites with newly absorbed fatty acid and glycerol to form new phospholipid." This theory was not entirely new. Meigs et al (1919) (99) had presented evidence to the effect that phospholipid is removed from circulating blood and transformed into milk fat by secreting mammary glands and Frank (1898) (84.) had suggested that fats were formed by the reaction:

lecithin + soaps = fat

The views of Sinclair held sway for many years and were strongly upheld by Verzar. Verzar and Laszt (1934) (100) showed that a more rapid absorption of oleic acid in isolated intestinal loops of anaesthetised rats, occurred when glycerophosphate, or glycerol + phosphate were added; they performed experiments designed to abolish phosphorylation (adrenalectomies (101, 102) and 'poisoning' (103, 104) with phlorizin) and because these resulted in decreased absorption of fat, he concluded that phosphorylation was essential for

such a process.

Sinclair's methods of tracing fatty acids by their degree of unsaturation (iodine numbers) and later by labelling with elaidic acid, had their limitations, and later the study of phospholipid transformations by labelling with ^{32}P became fashionable. By this means, Fries et al (1938) (105) found that among the diverse organs of the animal body, the small intestine plays a major role in phospholipid turnover in the absence, as well as the presence of ingested fat. A larger part of the radiophosphorus was found in the phospholipids of the small intestine when olive oil was given simultaneously.

Schmidt-Nielsen (106) has reviewed the position up to 1946 excellently and describes a series of his own experiments, designed to throw more light on the subject. One can only be impressed by the careful way in which he critically sets forth his data.

He performed experiments of several types. In one group he fed oleic acid half an hour before injecting ^{32}P into rats, and studied the variations in phospholipid specific activity between these animals and controls in different loops of intestine of the same animal. He used glucose-fed controls to eliminate the possibility that

generally increased absorptive activity of the cells might account for any increases in phospholipid turnover. In another series, peanut oil was fed by stomach tube, ^{32}P injected five hours after feeding and the animals killed at different times after injection. Schmidt-Nielsen recorded that: (1) there was no increase in amount of phospholipid during fat absorption in any part of the intestine. (2) The fatty acid content of the mucosa increased considerably, due to higher concentration of neutral fat. (3) The rate of formation of phospholipids as measured by ^{32}P was considerably higher when oleic acid or peanut oil was absorbed.

He concluded that phospholipids are synthesised in intestinal cells as an intermediate step in the uptake of fatty acid and resynthesis of triglyceride. The fatty acid-glycerol part of the molecule, he thought, might be split off, leaving the phosphorus-containing part more or less undisturbed. It is noteworthy that he considered it likely that such an increase of phospholipid synthesis might be confined to epithelial cells alone and that further information would be gained by analysing mucosa. He also speculates on the possibility that one, several or all of the phospholipids may be involved in the higher

turnover rate.

Quite a different standpoint was taken by Zilversmit et al (107) as a result of their series of experiments in 1948.

Using ^{32}P they studied the effects on phospholipid turnover of feeding different fatty diets to dogs and rats. The conclusions were that in the dog, neither the amount nor turnover of phospholipid of the mucosa was affected by absorption of cream, corn oil or fatty acids. In the rat, fairly consistent, but small, increases in the incorporation of ^{32}P into mucosal phospholipids at different times, were far too small to account for all the absorbed fat having passed through the phospholipid stage.

Nor was Zilversmit the only one to oppose the "obligatory intermediate theory". Barnes et al (108) found that neutral fat in mucosa contained large amounts of administered labelled fatty acid, but only small quantities were found in the phospholipids thereof. They concluded that phosphorylation was not essential. Even more striking was the evidence of Reiser et al (1954) (109). After feeding glycerol-(^{14}C)-tripalmitin and 1-(^{14}C)-fatty acids, it appeared that resynthesised triglycerides were

precursors of mucosal and lymph phospholipids rather than the other way round.

Another series of experiments which is of interest here is the type performed by Artom and others. Artom and Cornatzer (110) found no increased phospholipid turnover when cottonseed oil was fed to rats on a low protein, low fat (choline-deficient) diet. The turnover could be increased above the normal again by feeding choline with the oil. Similarly, lecithin was shown to stimulate glyceride absorption by Augur et al (111.) and by Sagrott (112).

The experiments described in the foregoing paragraphs have dealt with the study of changes in total extracts of phospholipids, mainly because refined methods were not available for the separation of individual phospholipids.

Recently, Johnston and Bearden (113.) have looked at the problem again and employed chromatographic methods to identify individual lipid classes. Their experiments showed that by using (^{14}C)-palmitic acid and (^{32}P)-inorganic phosphate tracers in an 'everted sac' of hamster intestine, phosphatidic acids could be identified as the most highly labelled of the mucosal phospholipids. When fatty acids were included in the incubation system, the incorporation

of ^{32}P in phosphatidic acids increased 2 to 3-fold.

It is against such a background of investigation that the work described in section III was undertaken.

Phospholipids and cancer

Much of the data concerning the composition and metabolism of phospholipids in tumours has been reviewed by Haven and Bloor (114).

Out of a mass of involved and sometimes obscure data, several things emerge as significant or at least interesting. While, in general, there are few striking differences in composition between normal tissues and the corresponding tumours, cancerous tissue seems to have a higher phospholipid content, and of this, the proportion of "cephalins" seems to increase the most. The fatty acids of hepatoma and ascites cells have been studied by Veerkamp et al (51.) and Gray (115) respectively. Some differences in composition were observed, but the most noticeable feature was the rather less selective distribution of fatty acids among the phospholipids and neutral fats in the tumour as compared with the normal tissue, suggesting a less specific enzyme system.

Secondly, there is evidence of some metabdic abnormality

in the biochemistry of ethanolamine in tumours. A patient with primary liver carcinoma excreted much ethanolamine, and phosphoryl ethanolamine is known to accumulate in the livers of rats injected with hepatocarcinogens.

The enzyme system methylating phosphatidylethanolamine to phosphatidylcholine in hepatoma, has only one tenth of the activity of that in normal liver, yet the proportions of these lipids did not alter greatly, (Figard and Greenberg, (116)). Veerkamp et al (51.) compared the uptake of ^{32}P by phospholipids in liver and hepatoma; phosphatidylcholine became more highly labelled in the tumour than in the liver, while the reverse was true of phosphatidylethanolamine. These turnover studies are consistent with the findings of Figard and Greenberg (116). Depressed methylation of phosphatidylethanolamine would reduce the need for its biosynthesis from phosphoryl ethanolamine, while synthesis of phosphatidylcholine would be stimulated. "Carcinolipin" has been claimed as a new lipid which enhances amino-acid incorporation into protein and promotes the growth of tumours when injected into rats. Malignolipin, a "new lipid" characteristic of tumours, has been described. Few people other than the authors can

substantiate these claims.

Considerable weight of evidence implicates phospholipids in the process of cancer growth but no firm theory can be postulated yet.

The implication of phospholipids in blood clotting and ischaemic heart disease

It was known many years ago, that alcoholic extracts of animal tissues accelerated blood clotting. MacFarlane (117) extracted oxalated plasma with petroleum ether and found that its ability to clot on addition of calcium ions was lost, but could be restored by adding back the extracted lipid to the plasma. 'Thromboplastic factor' was found to be in platelets and to be inactivated by phospholipase A (118); phosphatidylethanolamine could replace platelets in the thromboplastin test.

In the clotting process, prothrombin is converted to thrombin, which in turn converts fibrinogen into the fibrin clot; the agent is a thromboplastic particle composed of antihaemophilic globulin, "Christmas factor", platelets, and calcium ions. In natural thromboplastin, the lipid is probably an unsaturated phosphatidylethanolamine linked through calcium to a protein. The claim that phosphatidyl-

serine is also active may be due to the difficulty in separating these two lipids.

Phospholipids are contained in the "fatty streaks" which are the first signs of atheroma, and increased sphingomyelin content seems to be characteristic of atheromatous lesions. Zilversmit (119) found that turnover of aortic phospholipids increased after feeding of cholesterol. He thought that this phospholipid was formed <u>in situ</u> as part of the defence mechanism against influx of cholesterol from plasma. Cholesterol is transported in the blood as part of the plasma lipoproteins which contain phospholipids. In atheroma, cholesterol might be deposited in arteries if there was insufficient phospholipid, or phospholipid of the wrong type to hold cholesterol in solution. Phospholipids might also be connected with heart disease in view of their role in blood clotting.

Such speculations are based on the most fragile of evidence; it is quite possible that the primary cause of ischaemic heart disease does not directly involve lipid metabolism at all. Phospholipids are so universal in their occurrence, and their metabolism is so intimately bound up with that of other substances, that it would

indeed be surprising if some secondary effect on phospholipid metabolism, ~~caused~~ was not observable in the course of a bodily disorder of this type.

Phospholipids as energy reserves

It is unusual to think of the phospholipid molecule as a storehouse of energy in the same way as the triglycerides of the fat depots.

In 1941, Lardy and Phillips (120) noticed that phospholipids were effective in maintaining the motility of bull spermatozoa in a sugar-free medium under aerobic conditions; in the presence of lipids both rate and duration of oxygen consumption was increased.

Hartree and Mann (121) found that ram spermatozoa were rich in plasmalogen, with 1.2 grammes of the lipid per 100 grammes of spermatozoa. Either aerobic or anaerobic incubation caused a reduction in acyl-ester bonds but not in fatty aldehyde or lipid phosphorus content. This indicates a release of fatty acids (leaving lysoplasmalogen) which act as the oxidisable substrate for the endogenous respiration of sperm cells.

It seems therefore that plasmalogen can function as the intracellular energy reserve of the spermatozoon.

Phospholipids and protein synthesis

A number of instances of the isolation of "phosphatido-peptides" (122) and "lipo-amino-acid complexes" (123.) have tempted speculations about a dynamic role for phospholipids in protein synthesis. Originally, claims for the existence of such compounds were received with scepticism by phospholipid chemists, who knew that phospholipids could easily take up amino acids into solution in such solvents as chloroform. Many of these compounds are quite stable, however, and the amino acids are not removed by washing in the Folch manner.

Bacterial systems, can, in certain conditions, incorporate radioactive amino acids into such complexes, and the process can in many cases be inhibited by chloramphenicol, an inhibitor of protein synthesis. MacFarlane (124) has described bacterial compounds in which amino acids are covalently linked to phosphatidylglycerol. The subject has been reviewed by Hawthorne (125).

In the preceeding pages, I have ~~talked~~ written about some of the functions which have been ascribed to phospholipids. Some have been well substantiated, others have not; but in no case is our knowledge of their precise function very extensive.

In the following pages, Part I looks at the phospholipids in compartments of the liver cell; the relationship between the amount of lipid in the nucleus and the amount required to form the typical bimolecular leaflet in the membrane has been investigated. In Part II, the metabolic activities of different phospholipids in these cell compartments are compared and Part III tries to probe a little further into the behaviour of different phospholipids in the intestine during fat absorption.

PART I

THE PHOSPHOLIPID COMPOSITION OF RAT LIVER CELL FRACTIONS

INTRODUCTION

The first part of this thesis will deal with the isolation of different cell fractions of rat liver, the extraction of lipids from these fractions and the analysis of the phospholipids by means of chromatography.

Much greater emphasis has been placed on the lipids of the cell nucleus because of the comparative lack of accurate or detailed information on this subject in the literature; in any case, the available information is conflicting. Since membranes of cellular structures are largely lipid-protein in nature, and since there is considerable weight of evidence that the nuclear membrane is continuous with the endoplasmic reticulum (64.), it was thought that comparative studies of the phospholipid composition (Part I) and turnover (Part II) of the cell fractions from which these membranes are derived would be of interest.

Methods for preparing mitochondrial and microsomal "fractions" are now fairly standardised and most workers

use modifications of the basic Schneider and Hogeboom technique (126). For reasons to be discussed later the problems involved in isolating nuclei are considerably more complex, so I will devote some space to a discussion of these problems.

In the last two decades, so many methods for the isolation of cell nuclei have been described that it is difficult to take stock of all of them. Many of these methods, however, are merely variations on one of a group of basic methods, evolved to suit the investigator's own particular needs, so that the biochemist who wishes to choose a method for a particular topic has in fact only half a dozen or so of these basic methods to consider. No one method is ideal for all considerations, each tending to be a compromise, and one can only consider the advantages and disadvantages of each method and finally select the one suited to one's own particular needs. An excellent series of reviews helps enormously in this choice (127-132).

Evaluation of the isolation medium

All isolation media tend to produce serious changes in either the structure, composition or properties of the nucleus(133), and the ideal method would produce nuclei which are:

(a) unaltered in appearance

(b) unchanged in chemical composition

(c) obtained in good yield

(d) free of contamination

and which,

(e) retain full biological activity (132).

The morphological appearance of the nuclei is probably not such a useful criterion for evaluating the isolating medium, since it is probable that the granularity of the nucleoplasm and degree of shrinking or swelling are derived from reversible changes in the physico-chemical state of the DNA-histone complex and not irreversible damage to structure (132).

In the work to be described, the retention of nuclear lipid, uncontaminated by lipid from other parts of the cell or indeed, from any other source, was of primary importance. In comparison with mitochondria and other cell particles, little work has been reported on the lipid composition of isolated cell nuclei (4,43,45,49,127,134-142) The available methods are not easy to compare and also include a large range of values for the lipid content of the cell nucleus. One of the most poignant reasons for this has been the difficulty of isolating nuclei completely free from other cell particles rich in

phospholipids. It will be shown that the isolated cell nucleus contains only a very small fraction of the cell phospholipid and hence even slight contamination with other particulate fractions will give a false picture of nuclear phospholipids.

Methods employing organic solvent mixtures (144.) to separate particles according to their densities, whilst having the advantages that little adsorption of material occurs on the nuclear surface, are obviously of little use in quantitative lipid studies owing to extraction of lipid by the solvent; hence one is obliged to use one or other of the aqueous media. These have the disadvantages that (a) extraction of protein and other high molecular weight material, and (b) absorption of cytoplasmic constituents on the nuclear surface, can be considerable. In the present studies objection (a) is only of importance if lipoprotein is extracted from the nuclei (see discussion).

Many more new methods have been described since I made my choice of medium for this work, and their usefulness is difficult to assess until they have been more extensively tried. At the time, the three most popular methods, which reduced objection (b) to a minimum were:

1. 1% citric acid (Mirsky and Pollister) (145.)
   A wide range of citric acid concentrations has been used and Dounce has studied the effect of pH on the composition of nuclei isolated in citric acid. (137)
2. Sucrose-citrate at pH 6.0 - 6.2 (Dounce) (146)
3. 2.2M Sucrose (Chauveau) (134.)
   At the present time this method is becoming very widely used and many other workers have introduced their own modifications. (147,148)

Choice of the isolation medium

For the routine work of extracting lipid from nuclear fractions followed by the chromatographic separation of the lipids, the 1% citric acid medium was chosen, since:

1. It gives nuclei of a high degree of purity; later microscopic and chemical studies confirmed this.
2. The low pH creates "tough" nuclei and protects them from damage and breakage during the homogenisation process. Mitochondria and erythrocytes are destroyed. Working at low pH, however, has several disadvantages which will be discussed later.
3. The method gives a fairly high yield and is simple to use on a large scale. This is necessary, to obtain enough lipid for analytical purposes.

4. Several other workers have found it suitable for lipid work ([43,140] / [141,143]), and therefore some comparisons may be made with other results. Useful comparisons, however, can be made in one or two cases only, since the way in which results are expressed varies so widely - a strong indication that some sort of standardisation of tabulation of results is necessary.

Most enzymes are destroyed at the pH of 1% citric acid; hence enzymatic methods for investigation of cytoplasmic contamination are out of the question. Citric acid is also known to extract up to as much as 50% of the protein from the nuclei (131). In short, citric acid fulfils only two of the criteria listed above for the ideal isolation medium, ((c) and (d)), but it is thought that the advantages listed above make it suitable for the problem in hand, namely the chemical determination of the lipid components of the cell nucleus.

Chauveau's method, while fulfilling most of the criteria for the ideal medium, has the disadvantages that the conditions for isolating uncontaminated nuclei are more rigorous (fine density and temperature control) and it is not so easily applied to large scale preparations.

Improvements on the original method have been made by Dr. P.J. O'Brien.(148) Filtering through absorbent

lint immediately after homogenisation reduces contamination, especially by erythrocytes, and addition of bivalent cations (149) and sodium β-glycerophosphate (149) help to prevent swelling, gelation, and agglutination. There is some evidence that Mg^{++} ions play some part in nuclear ribosome structure, chromosome integrity, and the binding of nucleotides for nuclear phosphorylation (150).

The method has been used in this work for purposes of comparison with results obtained from citric acid nuclei.

Purity of the fraction

Several methods are in common use for the examination of nuclear fractions for contamination, and for the estimation of the amount of contamination present. Of these, examination under the light microscope is both simple and reliable and has been used in this work fairly extensively. By this means, unbroken cells, connective tissue, erythrocytes (improbable contaminants in the case of citric acid isolations), mitochondria, cytoplasmic membranes and broken nuclei should be readily seen. Identification of unwanted materials is very easy if crystal violet is used as a nuclear stain. By placing a drop of the stain by the side of the nuclear sediment on the slide, and then applying the cover-slip, the stain

diffuses gradually through the material and is taken up by nuclei much more rapidly than by unbroken cells.

For more detailed examination of the fractions, the electron microscope is very useful. Davison and Mercer claim to be able to detect cytoplasmic contamination in nuclear fractions which appear extremely clean under the light microscope (151).

Enzymes provide a convenient means of identifying the nature and extent of contamination in cases where an enzyme is known to be typical of a particular cellular region, providing of course, that the enzyme is not destroyed by the isolation medium, as is often the case with citric acid. Thus succinoxidase (132) (1.3.99.1 succinate:O_2 oxidoreductase) and cytochrome oxidase (152) (1.9.3.1 cytochrome C:O_2 oxidoreductase) may be used as mitochondrial markers in nuclei isolated in sucrose, and glucose-6-phosphatase (153) (3.1.3.9 D-glucose-6 phosphate phosphohydrolase) as an indication of microsomal contamination. Care must be taken in interpreting these results, however, since not much information is available about the possibility of some enzymes being small but true components of the nucleus. While Hogeboom et al. (152) have shown conclusively that cytochrome oxidase is confined

to the mitochondria, many workers are not at all certain whether the small amounts of glucose-6-phosphatase observed in nuclear fractions are nuclear in origin or due to microsomal contamination (154). O'Brien (148) is convinced that slight glucose-6-phosphatase activity in his nuclear fractions comes from genuine nuclear material.

Chemical analysis, too, can be of value in assessing contamination. Since Boivin, Vendrely, and Vendrely's observation in 1948 (155) that for a given species, the amount of DNA is constant in somatic cells of different tissues, it has become an accepted procedure to express results of chemical analyses of tissues or isolated nuclei on a DNA basis. This is preferable to the practice of referring such results to dry weight or wet weight of tissue or to tissue protein, as in these cases variations are likely to be very large, especially where large and variable quantities of protein have been extracted. For the use of DNA as a standard, one must assume that no DNA has been extracted in the isolation procedure. The method raises problems when used for liver nuclei due to the heterogeneous nature of liver, resulting in variation of cell type, chromosome complement and composition (131).

As an example of the use of the DNA standard, a high ratio of protein/DNA might indicate a heavily contaminated fraction. On the other hand a low ratio might reflect loss of protein. The ratio RNA/DNA, quoted by many workers, is variable, but a lower value indicates low contamination. There is less information on the ratio: lipid/DNA, but a high ratio would indicate a grossly contaminated fraction. Values from 0.1 - 0.3 can be found in the literature. This ratio has been used in the present work as an indication of contamination. Since the lipid content of nuclei is generally agreed to be the lowest of all commonly studied cell fractions, I think it provides a fairly sensitive indication of contamination. Until, however, the vexed question of extraction of proteins from nuclei during isolation is settled, the use of such ratios must be cautious, but in combination with other methods may be a very useful guide.

DNA measurement has also been used here to calculate the yield of nuclei, rather than the method of counting; but since fragments of broken nuclei are likely to contain DNA, it is not necessarily a measure of intact nuclei.

Finally, the efforts devoted to scrutinising nuclei for contaminants can be successfully reduced by paying

greater attention to homogenising techniques. The most popular type of homogeniser is that described by Potter and Elvejhem (156) and such a machine is used in this work. Very few quantitative data are available on the optimum conditions for cell breakage with minimum damage to nuclei, such as gap between plunger and vessel, shearing forces, tissue concentration, and time of homogenisation. However, many different figures are available in the literature and Dounce and Allfrey discuss quite extensively the problems involved in homogenisation (127,131). The "Waring Blendor" type of homogeniser was thought to have a much too vicious action on the tissue, resulting in the breakage of large numbers of nuclei.

Removal of contamination by red cells (rat liver has 20 ml blood per 100g tissue) (157) may be effected by preliminary perfusion of the liver with saline. This is unnecessary where nuclei are prepared in citric acid, but even with sucrose, perfusion may be objected to on the grounds that it is time-consuming and may adversely affect cells and intracellular structure (158). The condition for isolation of nuclei in 2.2M sucrose can be arranged to eliminate most, if not all, red cells.

Extraction of nuclear lipid

Having determined to one's satisfaction the purity of the nuclei, the problem is then to (a) extract the lipid, (b) to fractionate the lipid by a suitable chromatographic technique. The various methods of extraction of lipids have been mentioned in the main introduction; three different methods are compared in Part I and the results found to be essentially the same. In the last section of Part I, I will discuss more fully the various extractions quoted in the literature.

General aspects of lipid separation have also been discussed. The method of Dawson (15) was chosen as (a) being suitable for dealing with moderately small quantities of lipid (200-500 μg phosphorus), and (b) giving a good separation of the main glycerophosphatides, which are the principal components of liver phospholipids. It is relatively simple to cut out spots from paper chromatograms and estimate phosphorus content quantitatively. The disadvantages of the method are the extra procedure involved in de-acylating the lipids, the fact that "lyso" compounds cannot be identified and the problems involved in breakdown of various lipids by other pathways than simple de-acylation. These and other points are discussed in the section on "lipid separations".

MATERIALS AND METHODS

Materials

All water used was demineralised with an Elgastat Deioniser, E.101, (Elga Products Ltd., Bucks.).

All reagents and solvents were ANALAR grade where available. These were purchased from:

British Drug Houses, Ltd., Poole

Hopkin and Williams, Ltd., London

May and Baker, Ltd., London

Philip Harris, Ltd., Birmingham (Laboratory grade reagents)

Ethanol was supplied by J. Burroughs, Ltd., London.

Diphenylamine was recrystallised from petroleum ether.

Diazomethane was prepared from p-nitrosomethyltoluenesulphonamide (May and Baker, Ltd., London) (159).

Glass wool was treated with nitric acid and then washed with demineralised water to pH 6-7 before use.

Amberlite IRC-50 ion-exchange resin was obtained from BDH, Ltd, Poole, and was washed first with 2N hydrochloric acid and

then with water to pH 6-7 before use.

Biodeminrolit mixed-bed ion-exchange resin was supplied by The Permutit Co. Ltd., London.

Filter and chromatographic papers were Whatman grade and obtained from H. Reeve Angel and Co. Ltd., London.

Spectrophotometric determinations were made with the Unicam SP-500 or SP-600 spectrophotometer, Unicam. Instruments, Ltd., Cambridge, England.

Isolation of subcellular particles

Isolation of nuclei in citric acid

The method of Mirsky and Pollister (145) was used and details are given by Allfrey (131). It has been found necessary to modify the method in one or two small details.

The nuclei were isolated from the livers of rats fed on the normal laboratory diet, up to 30 animals being used for each experiment. The animals were killed by a blow on the neck, and the livers immediately removed, without perfusion, and put on ice. All subsequent operations were performed at 0°-4°, usually in a refrigerated room. The livers were dried off on tissue paper, sliced with scissors and homogenised in

100 ml of 1% citric acid for every 10g of liver. The homogeniser was the Potter-Elvejhem co-axial type, made of Perspex, not a Waring Blendor as described by Allfrey. The difference in diameter between the plunger and the vessel was 0.038 cm, the diameter of the plunger was 3.7 cm and the speed of rotation, 470 revs/min; 6-10 strokes of the plunger were required for complete homogenisation.

The homogenate was centrifuged at 2,000 x g (4,000 revs/min) in the MSE "13,000" refrigerated centrifuge (Measuring and Scientific equipment, London) for 10 minutes. The supernatant was discarded, and the sediment resuspended in a volume of 0.2% citric acid 1.5 times the original volume of homogenate by stirring in an "Atomix" high speed homogeniser (MSE Ltd. London) at the lowest speed. 2-3 drops of octyl alcohol were added to prevent foaming. The suspension was filtered through a single thickness of absorbent lint and the filtrate was centrifuged at 2,000 x g (4,000 revs/min) for 5 minutes. After resuspending the sediment as before and filtering through two layers of absorbent lint, the filtrate was centrifuged at approximately

300 x g (1,200 revs/min.) for 5 minutes.

The white nuclear sediment was then washed by re-suspension and low speed centrifugation until little or no contamination could be seen by light microscopy.

Usually six washings were sufficient to obtain nuclei of high purity, but the washing process was controlled by light microscopy in each case.

Isolation of nuclei in 2.2M sucrose*

For routine work, and in all cases where lipids extracted from nuclei were separated by chromatography, the method of isolation of nuclei described above was used, owing to the ease with which the method could be used to yield clean nuclei and because this method was easily adapted to large scale preparations.

Some samples were isolated in a 2.2M sucrose medium, essentially as a comparison with nuclei isolated in citric acid. The method is basically that of Chauveau et al. (134), but has several modifications by Dr. P.J.O'Brien (148). The medium consisted of 2.2M sucrose, 0.002M $MgCl_2$, and 0.05M sodium glycerophosphate. The specific gravity, which is critical, was 1.273, and was checked with a hydrometer. The animals were fasted

*footnote: the assistance of Dr.P.J.O'Brien is acknowledged.

for 20 hours previous to death in order to deplete the cells of glycogen; this has the effect of lowering the specific gravity of the cytoplasm, and ensures better separation of the nuclei from cytoplasm. The fresh livers from not more than three rats were weighed, freed from all pieces of hair, etc., and then dried thoroughly on pieces of tissue paper. The sliced tissue was then homogenised in 20 volumes of the above medium in a perspex Potter-Elvejhem homogeniser, (details above); 3 strokes of the plunger were required for complete homogenisation. After straining through one thickness of absorbent lint, the homogenate was centrifuged at 40,000 x g (18,250 revs/min.) in the MSE "40,000" ultra-centrifuge, ("30" head). The nuclei were obtained as a transparent pellet at the bottom of the tube, and were not subjected to any washing procedure. All operations were carried out at 0^{o}.

The cytoplasmic debris adhered to the top of the tube and was removed quickly and carefully with Kleenex tissues after pouring off the bulk of the supernatant. The nuclear pellet was removed on a spatula, preferably without letting it touch the sides of the tube at the upper end.

Isolation of mitochondrial and microsomal fractions

Mitochondrial fraction

The method is basically that of Schneider and Hogeboom (126).

The medium consists of 0.3M sucrose, containing EDTA (0.002M), and potassium bicarbonate added until the pH of the solution is 7.2-7.4. The liver from one rat was sliced with scissors and homogenised in 9 volumes of the above medium. The homogeniser was of the Potter-Elvejhem type with a glass tube, (pestle diameter, 0.736"), and a Teflon-tipped plunger with stainless steel spindle, (difference between diameter of vessel and plunger was 0.005"-0.007"). The speed of rotation of the plunger was 470 revs/min. and 12 strokes were used to obtain complete homogenisation. All steps in the procedure were carried out at 0-4°. The homogenate was centrifuged in the MSE "13,000" refrigerated centrifuge at 1,200 x g (3,000 revs/min.) for 10 minutes. The supernatant was carefully poured off, making sure that none of the debris was taken off with it, (the last few drops of supernatant were generally rejected), and recentrifuged at 12,000 x g (10,000 revs/min.) for 10 minutes. The sediment was yellowish

in appearance and was washed by resuspending in about 25 ml 0.3M sucrose and gently homogenising by hand. Finally the pellet obtained by spinning this suspension at 17,000 x g (12,000 revs/min.), was taken as the mitochondrial fraction.

Microsomal fraction

The supernatant after centrifuging at 12,000 x g was a cloudy brown liquid with a layer of fat at the surface. The fat layer was removed with a pipette or by absorbing into a tissue paper and the suspension centrifuged at 104,000 x g (40,000 revs/min.) in the MSE "40,000" ultracentrifuge,("40" head). The pellet after decanting the clear supernatant was tightly packed and red in colour and was taken as the microsomal fraction.

Methods for determining the purity of cell fractions

Nuclear samples were examined by three methods to investigate possible contamination by other cell particulates, especially those rich in lipid. These were:

(i) Light microscopy

(ii) Electron microscopy

(iii) Enzymatic methods.

Examination of nuclei by light microscopy

Nuclei were examined by using a small light microscope the total magnification of which was x 400. A small spot of a fairly dilute suspension of the nuclei in 0.2% citric acid was placed on the slide and stained by applying an equal volume of crystal violet (0.1% in 0.2% citric acid) from a Pasteur pipette. A cover slip was carefully placed over the spot and the nuclei observed under direct illumination.

Nuclei isolated in citric acid

Most of the nuclei appeared as discrete particles, almost circular in shape, with nearly all the material taking up the stain. Occasionally "clumps" or large aggregates of nuclei could be seen but these were rare if nuclei had been washed at least six times as described above. Special care was taken to look for whole cells, erythrocytes and pieces of membranous material, and any contamination which might have come from other parts of the cell. By placing the sample and a drop of stain side by side on the slide, and then covering carefully with a cover slip, the stain could be observed to progress through the nuclei. In this way, contamination

such as whole cells could be easily picked out, since it would take up the stain much less readily. No whole cells or erythrocytes were seen in washed samples and only very occasionally were strands of membrane seen. More common were broken nuclei, either completely disintegrated without a membrane, or those in which part of the outer edge of the nucleus seemed ruptured and the contents were spilling out. Such broken nuclei, however, were a minute proportion of the many thousands examined. In many experiments, the course of the purification was tracked by examining samples at each stage of the procedure. The marked fall in contamination could then be observed at the washing stage, but this was only attained by greatly sacrificing the final yield.

Nuclei isolated in 2.2M sucrose

The sediments in the tube after centrifugation were vastly different in appearance. The 'citric acid' nuclei were obtained as a white, opaque sediment, almost "powdery", (but sometimes discoloured by a brownish material, especially in the case of rabbit liver nuclei), whereas 'sucrose' nuclei formed a translucent jelly which was very difficult to see. Under the light microscope, however, these latter took up the crystal violet stain and had a very similar shape and appearance to 'citric

acid' nuclei, except that they had a greater tendency to clump in large aggregates. In each case this tendency increased with time and usually after half an hour the slide was useless; the 'citric acid' nuclei, however, were preserved for longer periods.

Examination of nuclei by electron microscopy*

Nuclear samples were fixed in osmium tetroxide, embedded in araldite, and examined under the Siemens-Elminskop Mk.II electron microscope.

Nuclei isolated in citric acid. (Plates I, IA, II)

The electron micrographs revealed very little contamination of the nuclear material. There were no whole cells, mitochondria, or erythrocytes; small fragments of granular material occasionally observed between intact nuclei appeared to have come from a few disrupted nuclei. The individual nucleus had a well-rounded shape and a sharply defined surface, but there was no indication of a discrete double membrane such as is normally observed in sections of intact tissue. Within the membrane, the distribution of stained material was diffuse. A sample of the crude homogenate before

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* The assistance of Mr. A.Smallwood is acknowledged.

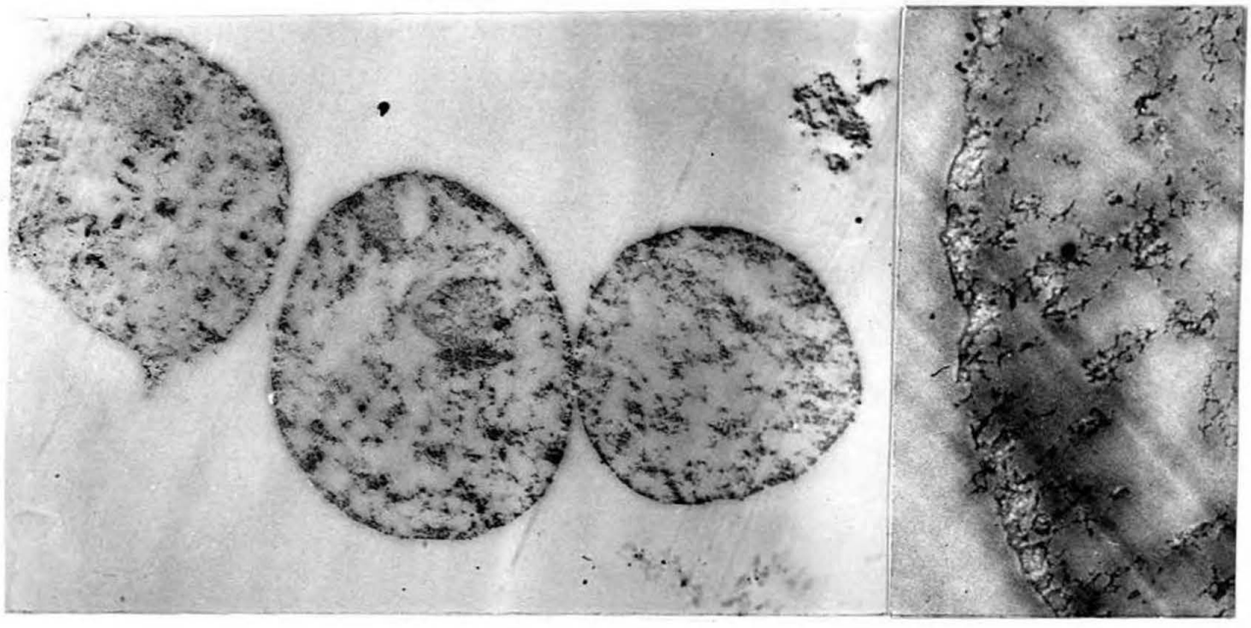
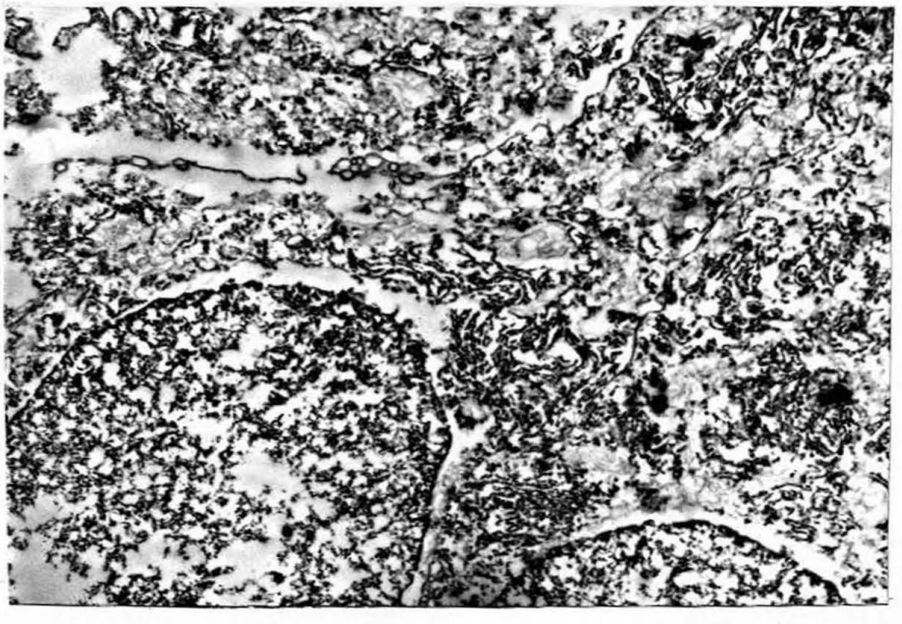


PLATE I. IA.

NUCLEI ISOLATED IN CITRIC ACID

Plate I: Rat liver nuclei, isolated in citric acid; OsO_4 -fixed, araldite-embedded, magnification: 7,500 times.
IA: Portion of the membrane of the nucleus. Magnification: 20,000 times.
II: Liver homogenate in citric acid. Magnification: 7,500 times.

PLATE II.



NUCLEI ISOLATED IN

2.2M SUCROSE

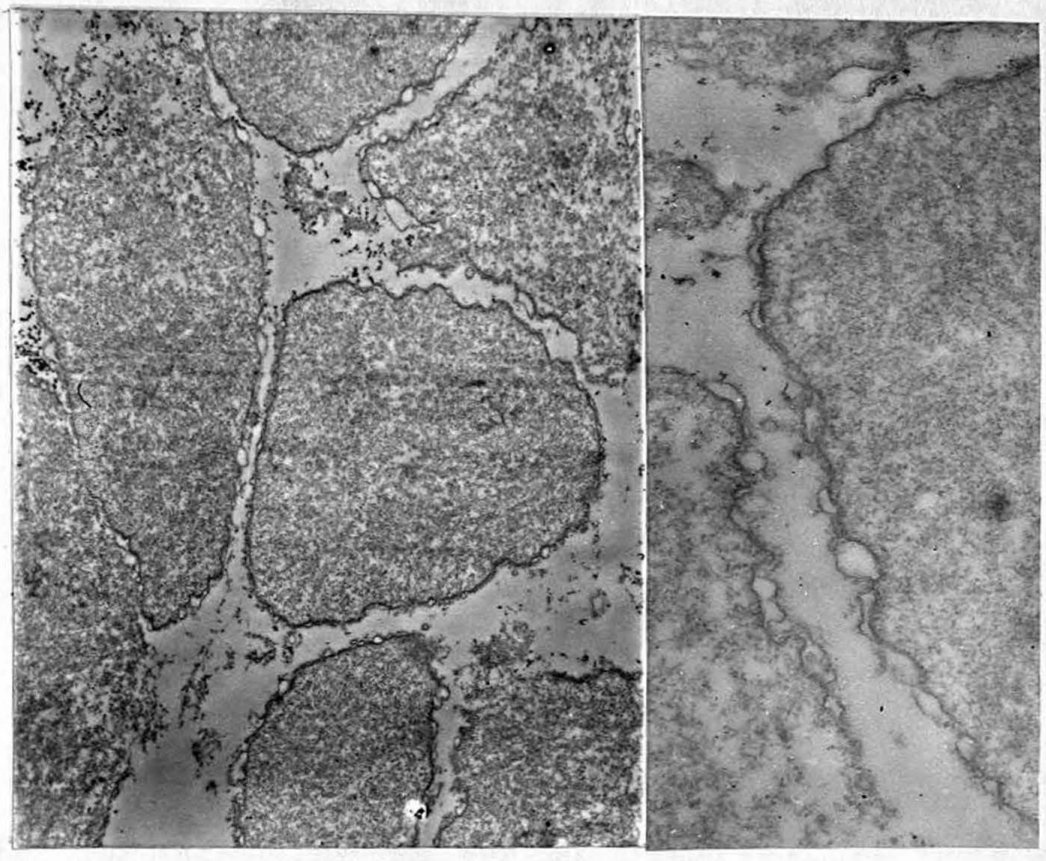


PLATE III IIIA.

Plate III: Rat liver nuclei isolated in 2.2M sucrose-0.002M $CaCl_2$-0.05M sodium glycerophosphate; OsO_4-fixed, araldite -embedded. Magnification 7,500 times.

IIIA: Portion of the membrane. Magnification: 20,000 times.

centrifugation was also examined and here again the nucleus could be observed in a "granular" form with a definite bounding surface, which in no case appeared as a double structure.

Nuclei isolated in 2.2M sucrose. (Plates III, IIIA)

With 2.2M sucrose nuclei, as in the case of 'citric acid' nuclei, little or no contamination was found. Some of the nuclei were broken and had their contents spilling out. In the interior of the nucleus there appeared to be a much greater concentration of stained material than was the case with 'citric acid' nuclei. The shape was also rather different; in general these nuclei were not circular but rather elongated. They had clumped together much more and this aggregation seemed to govern their shape. The appearance of the membrane was also very different. It was considerably thicker and in most cases its double structure could easily be seen. In some cases the outer layer of the double structure was 'peeling away' and appeared as a strand attached to the nucleus in only one or two places.

Enzymatic methods for the determination of purity

Since succinoxidase is almost exclusively a mitochondrial enzyme, its assay in other fractions can be

used as a measure of contamination by mitochondria. In the case of nuclei isolated in citric acid, the low pH of the medium destroyed succinoxidase (and rhodanese) activities in mitochondrial blanks, so that these methods could not be used.

Dr. P.J.O'Brien (148) has kindly given me data of enzyme assays carried out on his own preparations of nuclei in 2.2M sucrose. These showed zero succinoxidase and phosphomonoesterase activities and the nuclear preparation contained 0.44% of the total glucose-6-phosphatase activity of the homogenate.

Schneider fractionation of the nuclear sediment

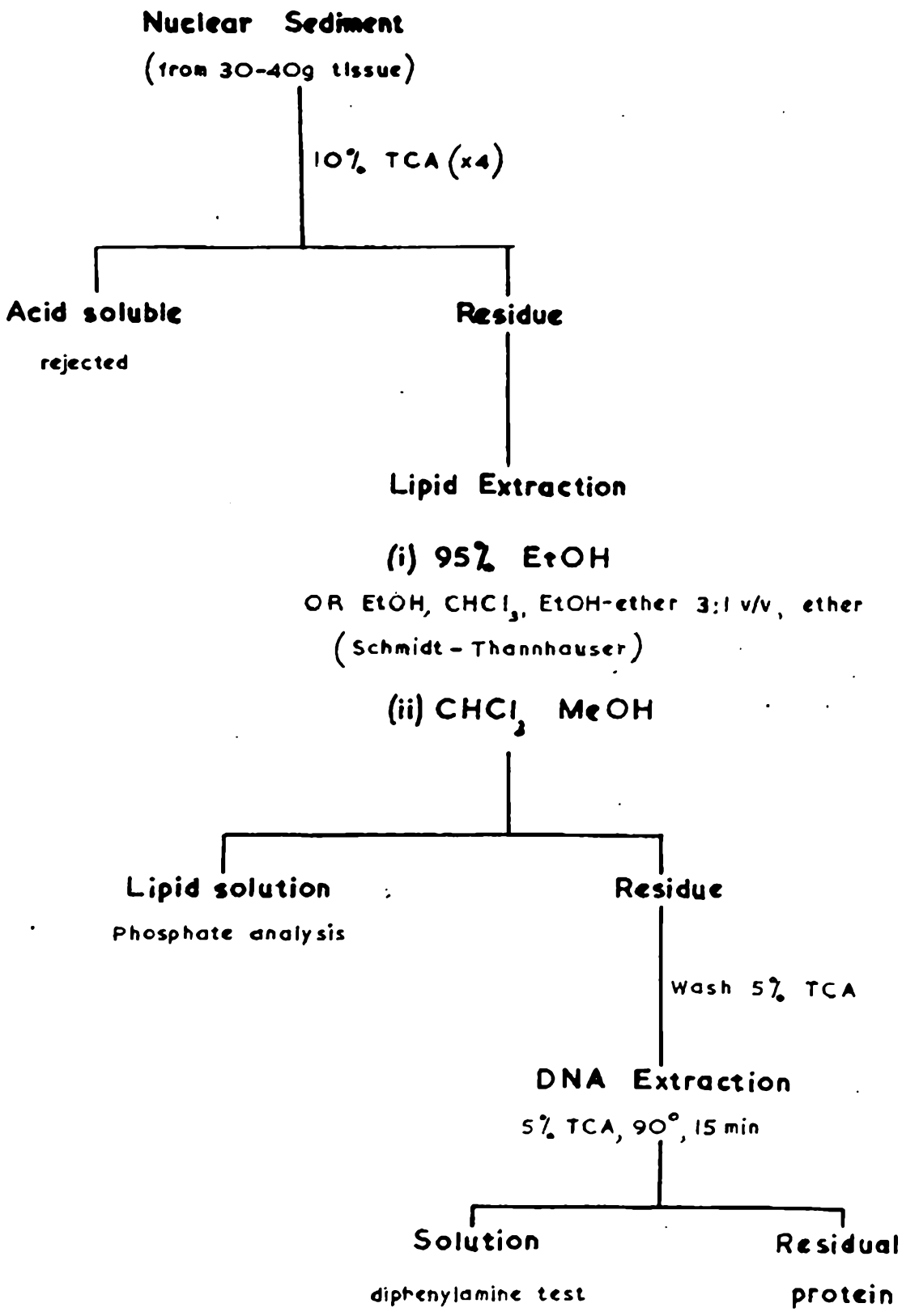
A series of nuclear preparations were made, and each sample was analysed for total phospholipid, DNA, and protein, using the Schneider (2) method of fractionation as follows.

The nuclear sediment from 30-40 g. of tissue was homogenised in 5 ml 10% TCA* to precipitate protein, nucleic acid and lipid material, which was separated out from the liquid phase using a small bench centrifuge. The sediment was washed 4 times with 5 ml. of 10% TCA to remove acid-soluble phosphorus compounds.

* See abbreviations.

fig 1.

FLOW SHEET SCHNEIDER EXTRACTION



The sediment was then washed with distilled water to bring the pH up to 6.

Extraction of lipids

Lipids were obtained by extracting 4 times with 10 ml 95% ethanol and then making two further extractions with the same volumes of chloroform-methanol, 2:1 v/v. It was found that the same amount of lipid was extracted by the Schmidt-Thannhauser (3) procedure (successive extractions with acetone, ethanol, chloroform, ethanol-ether 3:1, v/v, and ether), and also by simply extracting with chloroform-methanol. The amount of phospholipid was calculated from the phosphate analysis by the method of King (160). (E.J.King, Biochem.J.26 (1932) 292).

Extraction of DNA

The residue after lipid extraction was washed with 5 ml of cold 5% TCA, and extracted with 5 ml, 5% TCA at 90° for 15 minutes. Care needed to be taken in cases where small quantities of the lipid solvent remaining in the sediment caused vigorous frothing. The solution was cooled, filtered, and then made up to known volume for DNA analysis.

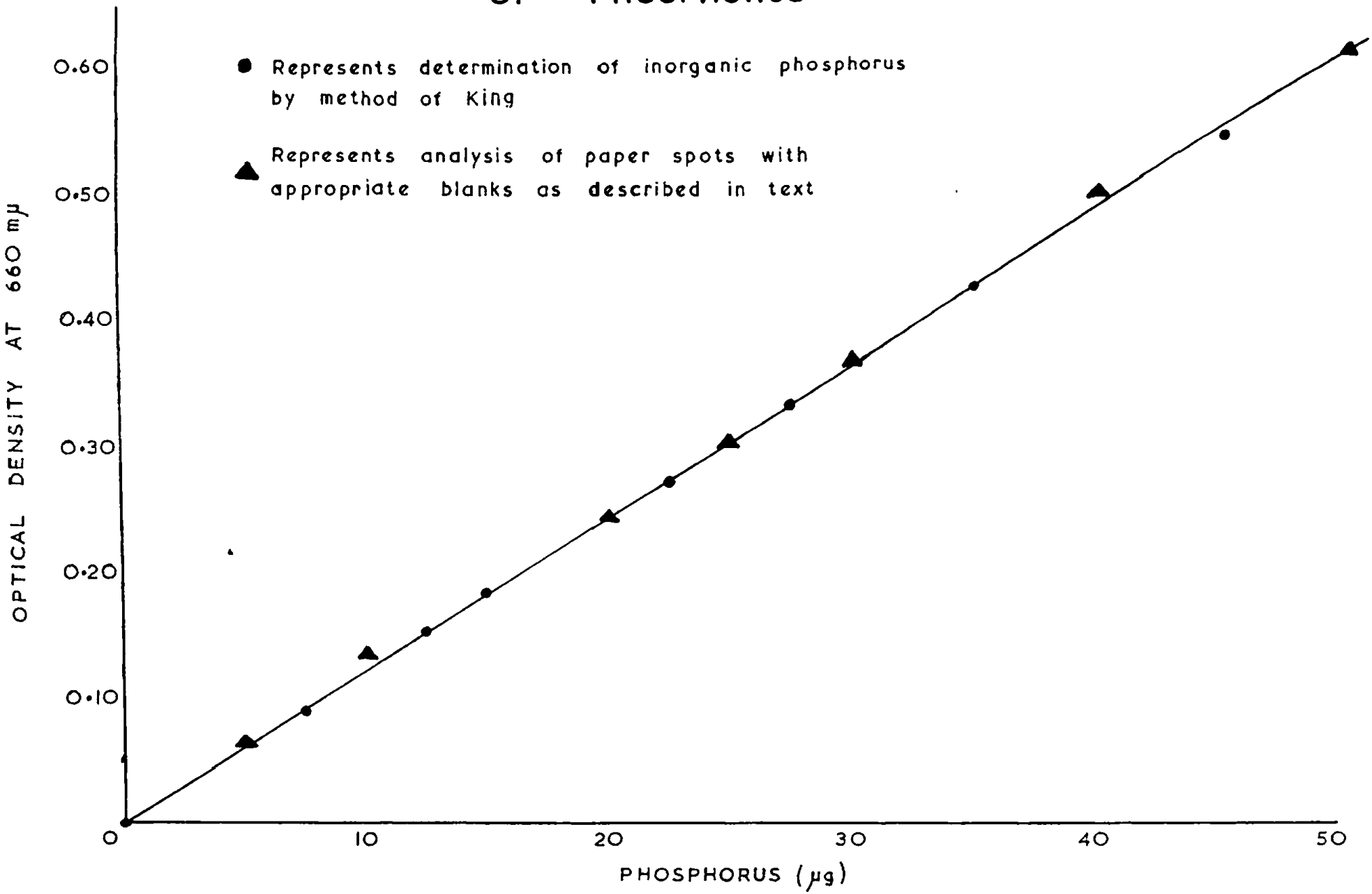
Chemical Analyses

Phosphate: Method of King (160). In some cases, a modification of King's method (161) was used for quantities of phosphorus from 0.5 - 3 μg. The digestion was carried out using 0.7 ml of 72% perchloric acid and the final volume of the test was made up to 5 ml instead of 10 ml. After addition of the reducing agent, tubes were heated at 100° for 10 minutes and after cooling the colour was read at 830 mμ. (1 μg of phosphorus gives an optical density of 0.15).

Phosphorus: analysis of chromatogram spots:

The method of Dawson was followed (15). Paper spots were digested with 1.2 ml 72% perchloric acid and volumes made to 10 ml. Suitable paper blanks were used and the colour read at 660 mμ. The standard curve, fig.2, shows that phosphate in paper spots can be estimated exactly as inorganic phosphate, the optical density being 0.012 per 10 μg phosphorus.

Fig 2 STANDARD CURVE FOR THE ANALYSIS OF PHOSPHORUS



DNA: Diphenylamine method (2). See fig.3 for standard curve.

Protein: Biuret reaction (162).

Cholesterol: Total cholesterol was determined by the Liebermann-Burchard reaction (163). See fig. 4 for standard curve.

Ester: Method of Stern and Shapiro (164).

Fatty acid:

Preparation of fatty acids from the lipid sample

A sample of the lipid containing approximately 1 mg of phosphorus (0.2 mg in the case of nuclear lipids), was taken to dryness in vacuo, dissolved in 5 ml 80% ethanol, and refluxed with 1 ml 40% aqueous potassium hydroxide at 65° for 2½ hours. After cooling, 3 ml of water and 2 ml of 10N sulphuric acid were added and the fatty acids extracted with 5 x 4 ml of petroleum ether, (boiling range 40-60°). The combined extracts were dried over a 4:1 w/w mixture of anhydrous sodium sulphate and sodium bicarbonate.

Methylation of fatty acids

Freshly prepared diazomethane in ether was added to

Fig 3 STANDARD CURVE FOR THE ANALYSIS OF DNA BY THE DIPHENYLAMINE REACTION

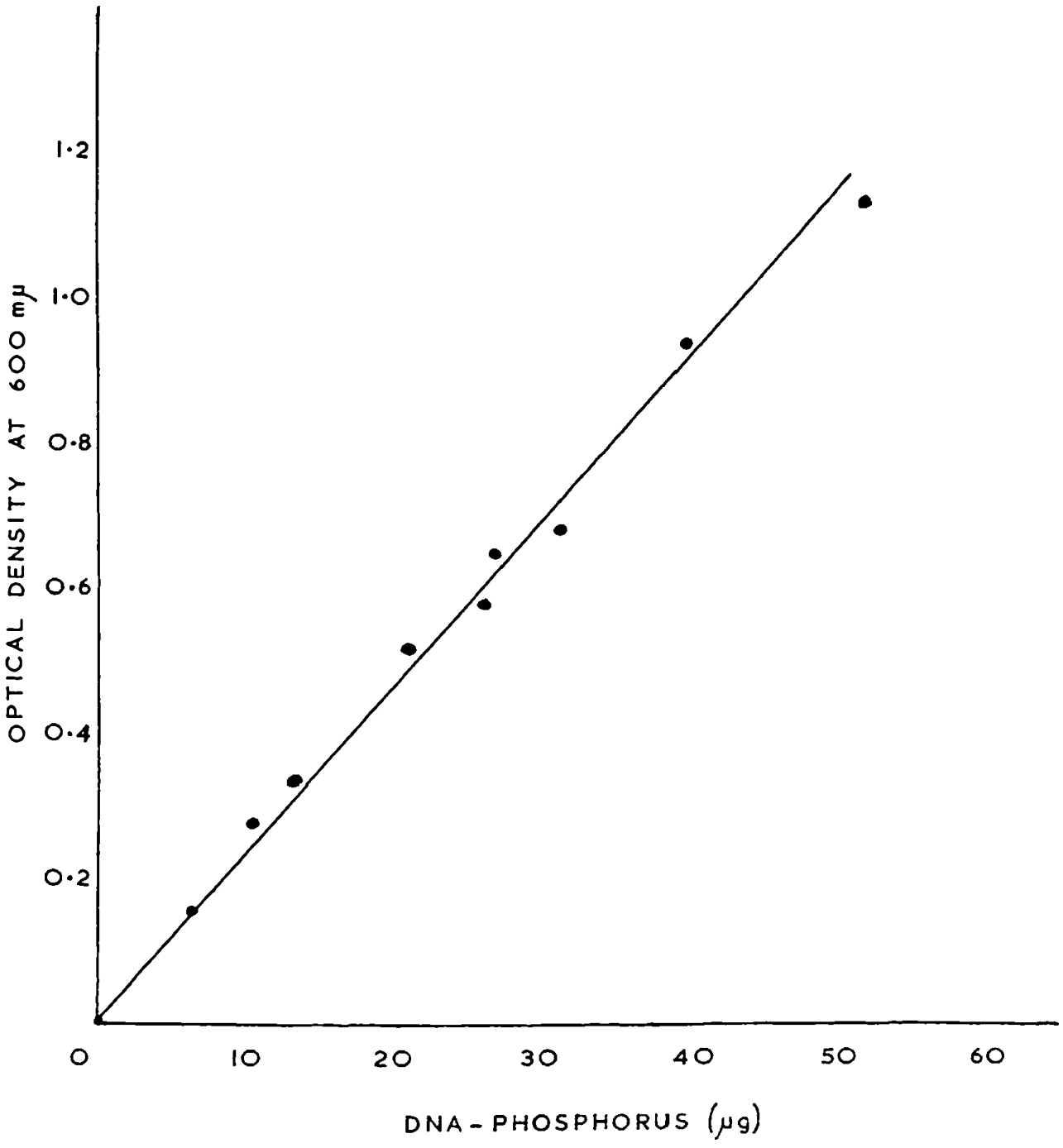
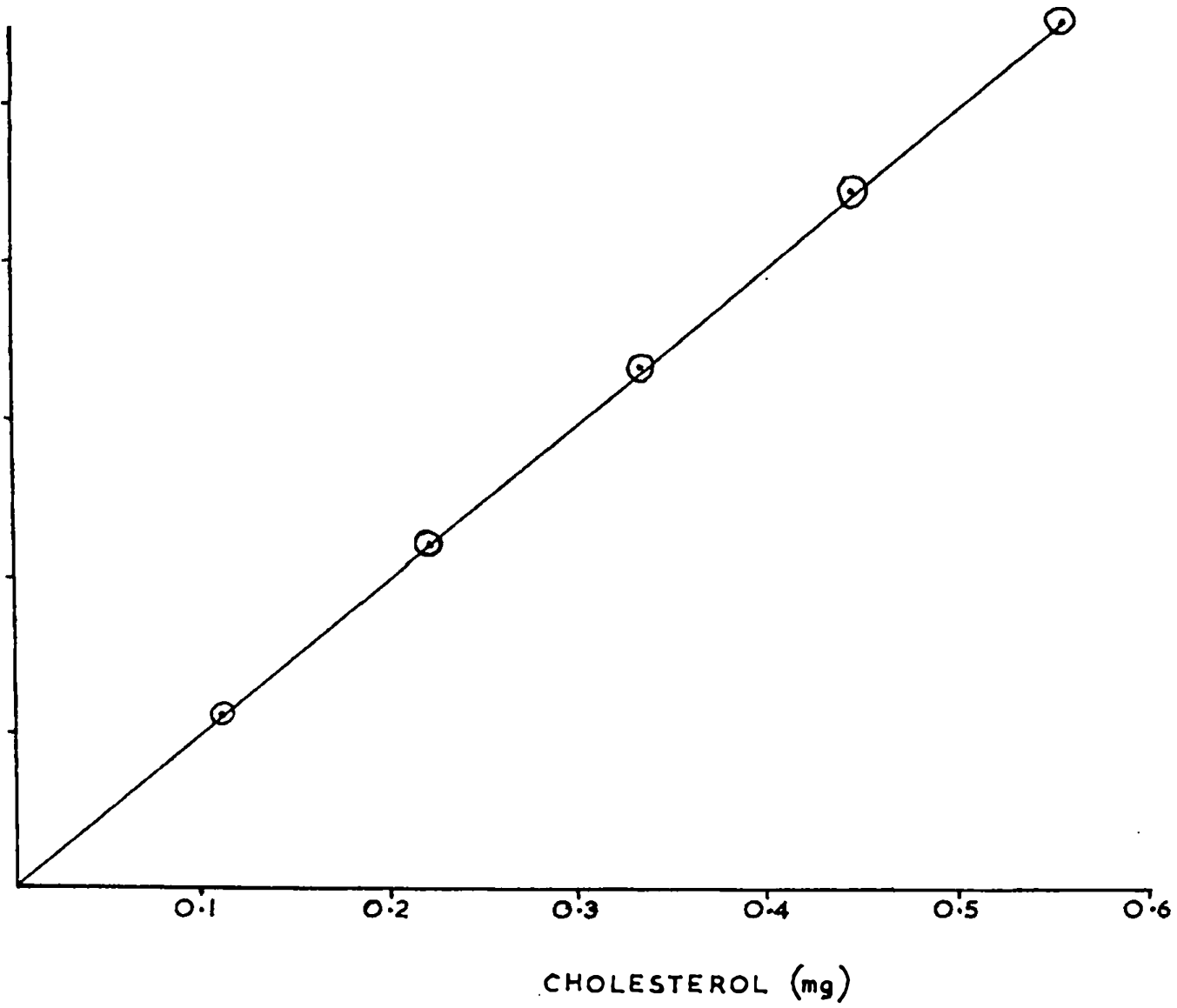


Fig 4 STANDARD CURVE FOR ANALYSIS OF BOUND AND FREE CHOLESTEROL (LIEBERMANN - BURCHARD REACTION)



the chilled petroleum ether extracts of fatty acids until the solution remained permanently yellow. After 30 minutes the solution was warmed to remove excess diazomethane (all operations up to this point were done in a fume cupboard with an efficient extraction fan) and stored at 0° until required. The ester values were determined (164).

Gas chromatography*

All samples were chromatographed on the Pye Argon Chromatograph, (Pye Ltd., Cambridge, England) using an APL column. The column was run at 200° with gas at 15 lbs/sq. in. pressure, for approximately 6 hours.

Extraction of lipids

Lipids were prepared for chromatography by the following procedure. The whole liver tissue or cell fraction was homogenised with about 10 vol. 10% TCA, and the precipitate separated off using a small bench centrifuge. The residue was washed 2-3 times with 5-10 ml of 10% TCA and then twice with the same volume of water. The sediment was then homogenised with 15-20 vols. of chloroform-methanol, 2:1, v/v, and allowed to stand for several hours at room temperature. (In some cases the whole tissue or fractions

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* footnote: the assistance of Dr. C.E.Rowe is acknowledged.

were homogenised directly in chloroform-methanol, 2:1).

After filtering through glass wool, the chloroform-methanol solution was shaken with 0.2 vol. of 0.9% sodium chloride and the liquid allowed to stand until the layers separated. The cloudy lower layer, together with any interfacial fluff was separated off in a tap funnel and taken to dryness *in vacuo* (bath temperature $<40^{\circ}$). The residue was dried off with successive evaporations with acetone. Chloroform was added and after extracting for some hours the residual protein was filtered off on glass wool.

Analysis of Phospholipids

Individual phospholipids were estimated as their water-soluble esters by the chromatographic method of Dawson (15.).

Mild alkaline hydrolysis

A sample of the lipid extract containing 500 µg of phosphorus, (450-550 µg) was taken to dryness *in vacuo* in a 50 ml round-bottomed flask. Carbon tetrachloride 0.8 ml, 7.5 ml ethanol, and 0.65 ml of water were added. At this stage the solution should be quite clear; in experiments with nuclear lipid a cloudiness developed and some of the lipid precipitated out. In these cases the carbon tetrachloride volume was increased to 5 ml

and only 3.3 ml of ethanol was used. Then 0.25 ml of 1N aqueous sodium hydroxide was added and the flask incubated at 37° in a water bath for 20 minutes. The excess of alkali was neutralised by one of two methods.

1. 0.4 ml of ethyl formate was added, and hydroxyl ion was removed by the following reaction :-

   ethyl formate + sodium hydroxide ⟶ ethanol + sodium formate.

   The advantage of this method was that the recovery of both the water soluble phosphate esters, and unsaponified lipids was very good; recoveries of total phosphorus were in the order of 90-100%. The water-soluble hydrolysate also gave quite a clean spot at the origin when put on to the paper. The disadvantage was that the sodium formate spot on the chromatograms ran to the same place as cyclic glycerophosphate and interfered with this spot, and also in many cases interfered with the glycerylphosphorylethanolamine spot, making it rather diffuse. When sprayed with the reagent of Hanes and Isherwood, (see below), the formate spot appeared a slate grey colour, but could be eliminated by the modified spray of Dawson (15). (see below).

2. Alkali was removed in many cases by passing the esters, cooled to 0°, through a small column, (dimensions 10 cm

x 0.7 cm) of Amberlite IRC-50 ion-exchange resin. The esters were eluted by two 5 ml washes with 80% ethanol. Such columns usually ran very slowly and the elution was speeded up by applying pressure from a nitrogen cylinder. Eluates from these columns had a pH of 5-6. Hydrolysates by this method gave a darker spot when applied to the paper but this did not necessarily interfere with the separations obtained. The recoveries of both water-soluble esters and plasmalogen were not so good as with formate but there was no interference with the spots on the chromatograms and separations were generally better.

The neutralised hydrolysates were taken to dryness in vacuo. Hydrolysed and unhydrolysed fractions were then partitioned between aqueous and organic layers of a solvent system prepared in the following way: Isobutanol, 2 vol., chloroform, 4 vol., water, 3 vol. 1 ml of the upper layer and 2 ml of the lower were added and the mixture shaken vigorously. The layers were separated in the bench centrifuge. The upper layer was removed with a Pasteur pipette and stored at 0^{o} for chromatography. (Fraction I).

Mild acid hydrolysis

About 80% of the lower layer was withdrawn, making sure that none of the water layer was present. 0.8 ml 10% TCA was added and the mixture shaken vigorously, by mechanical shaker in a water bath at 37° for 30 minutes. After cooling, 2 ml of 40-60° petroleum ether was added and the mixture again shaken vigorously. The top (organic solvent) layer contained unhydrolysed lipid (mainly sphingomyelin, fraction III) and the lower (water) layer, the acid-labile fraction (plasmalogen, fraction II).

Chromatography

Fraction I. The water-soluble esters were assayed for phosphorus and a sample giving about 80-100 μg phosphorus, (0.2-0.25 ml) was spotted on the paper. The papers used were Whatman number 1, 15" x 18", which had been washed with 2N acetic acid followed by water until the pH was 6-7. The paper was run for about 12 hours in the long direction in phenol saturated with water-acetic acid-ethanol, 100:10:12, v/v. The papers were dried in a stream of air and the last traces of phenol washed out in ether. The papers were then run in the second dimension in methanol-formicacid-water, 80:15:5, v/v for 3½ hours. After drying thoroughly in a stream of air overnight, the papers were sprayed with ninhydrin (0.2% in ethanol) to locate serine- and ethanolamine-containing spots and then

with either the phosphate spray of Hanes and Isherwood (165), or the modified spray of Dawson (15.).

Fraction II. About 5-10 μg of phosphorus from the fraction was spotted on to an acid washed Whatman number 1 paper and run in one dimension in phenol saturated with water-acetic acid-ethanol, 100:10:12 for 12 hours. The paper was washed in ether and sprayed as above.

Fraction III. The mild alkali and acid-stable fractions were examined by thin-layer chromatography. The solvent was chloroform-methanol-water, 95:35:6, run for 2 hours. Plates were sprayed with (i) Ninhydrin, (0.2% in ethanol) to locate amino-containing lipids, (ii) phosphomolybdic acid (2% in ethanol); after heating in the oven at 80° for several minutes, phospholipids appeared as dark blue spots on a pale yellow ground. Or, (iii) 50% sulphuric acid; after heating in a muffle furnace at 300° for a few minutes, organic compounds appeared as black spots on a white ground.

Identification of compounds on paper chromatograms

Initially, the spots obtained on two-dimensional paper chromatograms could be identified by their Rf values as given by Dawson (15.). These Rf values were found to vary considerably according to how well the papers had been washed, temperature of running, and whether much contaminating material was present with the esters.

The relative positions of the compounds were always the same, however, and after a little experience each spot on chromatograms of esters from a wide variety of sources could be readily identified.

As a more conclusive proof of the identity of each compound, the following tests were carried out.

1. GPE* and GPS*

These were initially picked out as amino-group containing esters by spraying with ninhydrin reagent. GPS gave a bluish-purple spot and usually appeared before GPE, which gave a more pink reaction.

A sample of ^{32}P-labelled phospholipids was hydrolysed and the esters separated by chromatography in the usual way. The compounds were located by radio-autography and those thought to be GPE and GPS were cut out and the compounds eluted from the paper with water. For this, the paper was cut into small squares, which were soaked in water overnight and then filtered. The eluates were taken to small volume and hydrolysed with 6N hydrochloric acid in a sealed tube at 110^{o} for 4 hours. After boiling to near dryness, adding water and boiling again to small volume, to remove HCl, the hydrolysates were subjected to

* See abbreviations

chromatography on Whatman No. 1 paper. Chromatograms were developed in isopropanol-acetic acid-water, 3:1:1 for 18 hours, using serine and ethanolamine markers (0.1 μ-mole per spot) (19).

2. GPC*

This compound is readily identifiable, being present in the largest quantity in the tissues examined, and since it runs with characteristic high R_f (0.86) in phenol-water-acetic acid-ethanol. The compound, after location by radio-autography, was eluted from the paper with water. The eluate was taken to small volume in vacuo, and rechromatographed on acid-washed, Whatman No. 1 paper in propanol-ammonia-water, 5:4:1, for 10 hours, or phenol saturated with water-acetic acid-ethanol, 100:10:12, for 10 hours, using a GPC marker prepared from pure lecithin (R.Michell and P.Kemp). Further confirmation of identity was obtained by spraying the chromatogram with Dragendorf reagent, (13) made up as follows:

Solution I: 1.7 g $Bi(NO_3)_3 \cdot 5H_2O$ in 100 ml 20% v/v glacial acetic acid.

* See abbreviations.

Solution II: 40 g KI in 100 ml water. The spray consisted of 4 ml of solution I, 1 ml solution II, 20 ml water. Free choline gave a purple coloration and bound choline, an orange.

3. GPI*

The compound was located and eluted as described above and the eluate subjected to hydrolysis in 6 N HCl in a sealed tube at 110° for 20 hours. Hydrochloric acid was removed by alternate addition of water and boiling to near dryness and the hydrolysate was then run through a small column of "Biodeminrolit" mixed-bed ion-exchange resin. (dimensions: 12 cm x 0.7 cm). After taking to a small volume, a suitable amount (10-15 μg inositol) was applied to a Whatman No. 1 paper and developed in isopropanol-acetic acid-water, 3:1:1, for 18 hours, using an inositol marker. Inositol was detected as a dark brown spot using the alkaline silver nitrate dip of Trevelyan et al (166).

4. GP* and GPGPG*

These were treated in the same way as GPC and re-chromatographed on acid-washed Whatman No. 1 paper, in propanol-ammonia-water, 5:4:1. The compounds were located

* See abbreviations

by spraying with the phosphate reagent described previously. GPGPG seemed to yield a considerable amount of GP. This applied to the compound taken from two-dimensional chromatograms and also to the GPGPG marker prepared from cardiolipin (donated by Dr. G.Hübscher).

EXPERIMENTS AND RESULTS

The results of a series of chemical analyses on different preparations of rat liver are set out in tables I - XIII. The results for nuclei isolated in citric acid are included in tables I - IX; those for nuclei isolated in 2.2M sucrose in tables X - XIII.

These experiments used both male and female rats fed on the standard laboratory diet up to the time of death; the weight of the animals was on average 250-350 g.

Perfusion: Citric acid is known to destroy erythrocytes, so that in general the perfusion of the tissue was not necessary. In one experiment, No. 6, table VII, the livers of two animals were perfused immediately before death to see whether the analysis was affected thereby. Each liver was perfused with 80 ml of ice-cold saline, containing sodium citrate, through the portal vein. The livers used for isolations in 2.2M sucrose were not perfused for reasons stated in the introduction.

Purity: The remarks about the purity of nuclei refer to examinations under the light microscope; routine checks were always made by this means. Samples of the nuclear sediment from experiments 7, 9 and 10 were taken for examination by the electron microscope. (See plates I - III and discussion).

Extractions: In experiment 1, a suspension of the nuclear sediment was divided into two equal portions and the lipid extracted by two procedures, Schneider (2), table I, and the Schmidt-Thannhauser (3), table II. Likewise, the effect of extracting solely with chloroform-methanol, 2:1, v/v, was investigated in experiments 7 and 8, tables VIII, IX. Otherwise the Schneider procedure was followed routinely.

I found that homogenisation with too high a concentration of TCA tended to produce a "rubbery" pellet which might be poorly extracted. Consequently care was taken to keep TCA concentrations to as low a value as was consistent with complete precipitation.

I experienced difficulty when analysing 2.2M sucrose homogenates; quite often low yields were obtained and it seemed that in this concentrated solution, TCA was not causing complete precipitation of the material. In

experiment 11, table XII, this was overcome by diluting the liver homogenate to the more normal 0.25 M concentration and analysing aliquots of this diluted homogenate. Since the homogenate analyses were on a sample different from that used for the nuclear isolation, I have had to base recovery figures on the amounts of material per gram of wet tissue. The figures in the last column represent the analyses for a homogenate containing 6 g of tissue.

The results of experiments 1 - 12 are summarised in table XIV. The figures for 100% recovery refer to the analyses which would be obtained if all the nuclei in the original homogenate were recovered; the calculations are based on the DNA analyses on the homogenate, assuming that all DNA is located in the nucleus.

In table XV the values published by a few authors are compared with my own. It is unfortunate that there should be other results in the literature which were difficult to convert to a form in which they would be readily comparable.

TABLE I

Experiment: 1	Isolation medium: Citric acid
Weight of tissue: 38 g.	Perfused or non-perfused: non-perfused

Comments on purity of Nuclei: Nuclear sediment was washed 6 times; very little debris was seen and little clumping or aggregation. Slight black deposit seen in sediment.

Extraction: Schneider Extraction on ½ of nuclear suspension.		NUCLEI		LIVER
		extracted	100% recovery	
Lipid Phosphorus (µg)	95% EtOH	33.8	-	-
	2:1 C/M	1.5	-	-
DNA-Phosphorus (µg)		725.0	-	-
Protein (mg)		21.1	-	-
% recovery of nuclei		-	-	-
Lipid-P / DNA-P		0.049	-	-
Lipid-P / Protein		0.0017	-	-
Nuclear lipid-P as % of liver lipid-P		-	-	-
Lipid-P per nucleus (µg x 10^8)		4.6	-	-

TABLE II

Experiment: 1	Isolation medium: Citric acid
Weight of tissue: 38 g.	Perfused or non-perfused: non-perfused
Comments on purity of Nuclei: As table I.	

Extraction: Schmidt-Thannhauser on ½ of nuclear suspension.	NUCLEI		LIVER
	extracted	Total for Experiment 1.	
Lipid Phosphorus (μg)	33.2	68.5	-
DNA-Phosphorus (μg)	750.0	1475.0	-
Protein (mg)	20.4	41.5	-
% recovery of nuclei	-	-	-
Lipid-P / DNA-P	0.044	0.046	-
Lipid-P / Protein	0.0016	0.0016	-
Nuclear lipid-P as % of liver lipid-P	-	-	-
Lipid-P per nucleus ($\mu g \times 10^8$)	4.2	4.4	-

TABLE III

Experiment: 2	Isolation medium: Citric acid
Weight of tissue: 42 g.	Perfused or non-perfused: non-perfused
Comments on purity of Nuclei: Nuclear sediment washed 8 times. After 6 washings small aggregates of nuclei and pieces of debris seen; negligible after 2 more washes.	

Extraction: Schneider		NUCLEI		LIVER
		extracted	100% recovery	
Lipid Phosphorus (μg)	95% EtOH	110.0	383.9	44094.0
	2:1 $CHCl_3$ MeOH	3.25		
DNA-Phosphorus (μg)		2260.0	7661.0	7661.0
Protein (mg)		94.4	320.0	3468.0
% recovery of nuclei		29.5	-	-
Lipid-P / DNA-P		0.050	-	-
Lipid-P / Protein		0.0012	-	-
Nuclear lipid-P as % of liver lipid-P		0.90	-	-
Lipid-P per nucleus ($\mu g \times 10^8$)		4.8	-	-

TABLE IV

Experiment: 3	Isolation medium: Citric acid
Weight of tissue: 40 g.	Perfused or non-perfused: non-perfused

Comments on purity of Nuclei: After 6 washes of sediment small strands of debris seen; 2 further washes gave clean nuclei.

Extraction: Schneider		NUCLEI		LIVER
		extracted	100% recovery	
Lipid Phosphorus (μg)	95% EtOH	120.0	120.0	36946.0
	2:1 $CHCl_3$ MeOH	-		
DNA-Phosphorus (μg)		2350.0	6825.0	6825.0
Protein (mg)		70.2	204.1	4236.0
% recovery of nuclei		34.4	-	-
Lipid-P / DNA-P		0.051	-	-
Lipid-P / Protein		0.0017	-	-
Nuclear lipid-P as % of liver lipid-P		0.94	-	-
Lipid-P per nucleus ($\mu g \times 10^8$)		4.9	-	-

TABLE V

Experiment: 4	Isolation medium: Citric acid
Weight of tissue: 23 g.	Perfused or non-perfused: non-perfused

Comments on purity of Nuclei: Sediment washed 6 times; very clean preparation; no large aggregates of nuclei.

Extraction: Schneider		NUCLEI		LIVER
		extracted	100% recovery	
Lipid Phosphorus (μg)	95% EtOH	68.6	72.5	-
	2:1 $CHCl_3$ MeOH	3.9		
DNA-Phosphorus (μg)		1929.0	3340.0	3340.0
Protein (mg)		63.3	126.1	1862.0
% recovery of nuclei		50.2	-	-
Lipid-P / DNA-P		0.037	-	-
Lipid-P / Protein		0.0011	-	-
Nuclear lipid-P as % of liver lipid-P		-	-	-
Lipid-P per nucleus ($\mu g \times 10^8$)		3.5	-	-

TABLE VI

Experiment: 5	Isolation medium: Citric acid.
Weight of tissue: 30 g.	Perfused or non-perfused: non-perfused
Comments on purity of Nuclei: Generally clean but few strands of membranous material.	

Extraction: Schneider		NUCLEI		LIVER
		extracted	100% recovery	
Lipid Phosphorus (µg)	95% EtOH	149.9	289.9	29542.0
	2:1 $CHCl_3$ MeOH	-		
DNA-Phosphorus (µg)		3000.0	5801.0	5801.0
Protein (mg)		104.7	202.9	1967.0
% recovery of nuclei		51.7	-	-
Lipid-P / DNA-P		0.050	-	-
Lipid-P / Protein		0.0014	-	-
Nuclear lipid-P as % of liver lipid-P		0.98	-	-
Lipid-P per nucleus (µg x 10^8)		4.8	-	-

TABLE VII

Experiment: 6	Isolation medium: Citric acid
Weight of tissue: 13 g.	Perfused or non-perfused: perfused; 80 ml saline/citrate
Comments on purity of Nuclei: Very clean appearance; one or two strands of debris seen; no aggregations of nuclei.	

Extraction: Schneider		NUCLEI		LIVER
		extracted	100% recovery	
Lipid Phosphorus (µg)	95% EtOH	102.0	106.0	16472.0
	2:1 CHCl3-MeOH	4.0		
DNA-Phosphorus (µg)		1440.0	2390.0	2390.0
Protein (mg)		59.6	98.8	1508.0
% recovery of nuclei		60.3	-	-
Lipid-P / DNA-P		0.074	-	-
Lipid-P / Protein		0.0018	-	-
Nuclear lipid-P as % of liver lipid-P		1.1	-	-
Lipid-P per nucleus (µg x 10^8)		7.0	-	-

TABLE VIII

Experiment: 7		Isolation medium: Citric acid		
Weight of tissue: 53 g.		Perfused or non-perfused: non-perfused		
Comments on purity of Nuclei: Sediment washed 6 times very clean preparation; little debris observed.				
Extraction: Schneider		NUCLEI		LIVER
		extracted	100% recovery	
Lipid Phosphorus (μg)	95% EtOH	Not used	-	-
	2:1 $CHCl_3$ -MeOH	87.8	234.8	31101.0
DNA-Phosphorus (μg)		2271.0	6001.9	6001.9
Protein (mg)		45.6	120.6	4365.0
% recovery of nuclei		37.8	-	-
Lipid-P / DNA-P		0.038	-	-
Lipid-P / Protein		0.0019	-	-
Nuclear lipid-P as % of liver lipid-P		0.8	-	-
Lipid-P per nucleus ($\mu g \times 10^8$)		3.6	-	-

TABLE IX

Experiment: 8	Isolation medium: Citric acid
Weight of tissue: 39 g.	Perfused or non-perfused: non-perfused
Comments on purity of Nuclei: Clean.	

Extraction: Schneider		NUCLEI		LIVER
		extracted	100% recovery	
Lipid Phosphorus (μg)	95% EtOH	Not used	-	-
	2:1 $CHCl_3$ -MeOH	153.9	-	-
DNA-Phosphorus (μg)		2784.0	-	-
Protein (mg)		-	-	-
% recovery of nuclei		-	-	-
Lipid-P / DNA-P		0.055	-	-
Lipid-P / Protein		-	-	-
Nuclear lipid-P as % of liver lipid-P		-	-	-
Lipid-P per nucleus ($\mu g \times 10^8$)		5.2	-	-

TABLE X

Experiment: 9	Isolation medium: 2.2M Sucrose
Weight of tissue: 32 g.	Perfused or non-perfused: non-perfused
Comments on purity of Nuclei: Clean and undamaged; very similar in appearance to nuclei isolated in citric acid.	

Extraction: Schneider		NUCLEI		LIVER
		extracted	100% recovery	
Lipid Phosphorus (μg)	95% EtOH	152.6	600.8	9228.3
	2:1 $CHCl_3$ -MeOH	-	-	-
DNA-Phosphorus (μg)		1271.3	4999.0	4999.0
Protein (mg)		68.8	270.9	2160.0
% recovery of nuclei		25.4	-	-
Lipid-P / DNA-P		0.12	-	-
Lipid-P / Protein		0.0022	-	-
Nuclear lipid-P as % of liver lipid-P		6.5	-	-
Lipid-P per nucleus ($\mu g \times 10^8$)		11.4	-	-

TABLE XI

Experiment: 10	Isolation medium: 2.2M Sucrose
Weight of tissue: 20 g.	Perfused or non-perfused: non-perfused
Comments on purity of Nuclei: Clean.	

Extraction: Schneider		NUCLEI		LIVER
		extracted	100% recovery	
Lipid Phosphorus (µg)	95% EtOH	53.3	160.5	6730.0
	2:1 C/M	-	-	-
DNA-Phosphorus (µg)		315.0	950.0	950.0
Protein (mg)		19.7	59.3	1136.2
% recovery of nuclei		33.2	-	-
Lipid-P / DNA-P		0.17	-	-
Lipid-P / Protein		0.0027	-	-
Nuclear lipid-P as % of liver lipid-P		2.4	-	-
Lipid-P per nucleus ($\mu g \times 10^8$)		16.1	-	-

TABLE XII

Experiment: 11	Isolation medium: 2.2M Sucrose
Weight of tissue: 6 g.	Perfused or non-perfused: non-perfused
Comments on purity of Nuclei: Clean.	

Extraction: Schneider		NUCLEI		LIVER
		extracted	100% recovery	
Lipid Phosphorus (μg)	95% EtOH	24.2	206.8	7080.0
	2:1 C/M	-	-	-
DNA-Phosphorus (μg)		155.0	1320.0	1320.0
Protein (mg)		12.5	106.8	1105.2
% recovery of nuclei		11.7	-	-
Lipid-P / DNA-P		0.16	-	-
Lipid-P / Protein		0.0018	-	-
Nuclear lipid-P as % of liver lipid-P		3.1	-	-
Lipid-P per nucleus ($\mu g \times 10^8$)		15.2	-	-

TABLE XIII

Experiment: 12	Isolation medium: 2.2M Sucrose
Weight of tissue: approx. 16 g.	Perfused or non-perfused: non-perfused
Comments on purity of Nuclei: Clean	

Extraction: Schneider		NUCLEI		LIVER
		extracted	100% recovery	
Lipid Phosphorus (µg)	95% EtOH	66	-	-
	2:1 C/M	-	-	-
DNA-Phosphorus (µg)		510	-	-
Protein (mg)		36.6	-	-
% recovery of nuclei		-	-	-
Lipid-P / DNA-P		0.13	-	-
Lipid-P / Protein		0.0018	-	-
Nuclear lipid-P as % of liver lipid-P		-	-	-
Lipid-P per nucleus (µg x 10^8)		12.3	-	-

Notes on table XIV

* The figures in these columns are in mg per g. of wet tissue; while the use of wet-weight values has been criticised, and the use of ratios is considered better, these figures have been included for completeness and to serve as a comparison with other work. The figures are an average of all results except those of experiments 7 and 8; in these cases the absolute values were abnormally low, although the ratios were comparable with those of other experiments.

** These figures are the mean values of nine experiments, with the standard deviation of the mean. In the case of analyses of sucrose-isolated nuclei too few data were collected to express the results in this way.

± This figure is calculated from the nuclear DNA-P content of 9.47×10^{-10}mg. (167)

TABLE XIV

The Chemical Composition of the Rat Liver Nucleus

Method of Isolation	WHOLE LIVER		NUCLEUS								
	lipid P*	Cholesterol mg per mg lipid P	lipid P*	DNA-P *	Protein *	Cholesterol mg per mg lipid P	lipid P as % of whole liver lipid P	lipid-P / DNA-P	lipid P / Protein	Protein / DNA	g lipid P per nucleus x 10^{+8} ±
1% Citric Acid	1.1	2.3	0.0095	0.18	6.5	2.5	0.9 ± 0.1**	0.049** + - 0.003	0.0015** ± 0.0001	3.6	4.7 ± 0.3
2.2M Sucrose	1.2	-	0.0299	0.23	18.6	-	2.5	0.130	0.0018	8.1	12.3

Notes on table XV

* Rees and Rowland give this figure as such in their paper; all other figures in this column are calculated on the assumption that each nucleus contains 9.47×10^{-10} mg. DNA-P. (167).

‡ Figures are for mouse liver.

‡ Dounce gives values as % lipid. Figures are worked out on the basis that 90% of this is phospholipid.

TABLE XV

Phospholipid composition of rat liver nuclei

AUTHOR	ISOLATION METHOD	µg lipid P* per nucleus x 10^9	$\frac{\text{lipid-P}}{\text{DNA-P}}$
(134.) Chauveau et al	2.2m Sucrose	104	0.11
(135.) Rees and Rowland	0.25M Sucrose/1.8 mM Ca^{++}	590	0.81
(139.) Barnum et al ±	Homogenisation in 0.85% NaCl; isolation in 2% citric acid	46	0.048
(137) Dounce	Citric acid; pH 3.8	83 ‡	0.088
(141.) McIndoe and Davidson	5%, 0.2% citric acid	35	0.037
Present work	1%, 0.2% citric acid	47	0.049
	2.2M sucrose, 0.002M $CaCl_2$, 0.05M sodium glycerophosphate	123	0.13

Chromatographic analysis of the phospholipids from nuclei and other cell fractions

Lipid extracts were hydrolysed by the method of Dawson (see page 78.) and the water-soluble esters separated by paper chromatography. The results of a series of such analyses are recorded in tables XVI - XXII.

Table I shows the distribution of lipid phosphorus between the three different lipid classes; (i) those labile to mild alkali, (glycerophosphatides), (ii) those stable to mild alkali, but labile to mild acid, (plasmalogens), and (iii) those stable to mild acid and alkali, (mainly sphingomyelins). All figures are in terms of μg of phosphorus.

Tables XVI - XVIII show the distribution of phosphorus among the different constituents of the alkali-labile fraction in rat liver cell fractions. The figures in column (a) are μg of phosphorus in each chromatogram spot; those in column (b) refer to the phosphorus in each spot as a percentage of the phosphorus applied to the paper. More analyses were done than are shown here, but in many cases there was a poor separation of the compounds, streaking, or incomplete analysis; only results from perfect chromat-

-ograms were recorded. Even so, the recovery from chromatograms of microsomal lipid hydrolysates were poorer than in other cases. It was found more accurate when dealing with spots containing less than 10 μg phosphorus to pool spots from identical chromatograms; therefore figures in the tables for the minor components will often be the same in two columns, and are really an <u>average</u> value for two chromatograms.

One of the difficulties encountered with this method of analysis arises from the fact that the breakdown of lecithin and monophosphoinositide is more complicated than the simple deacylation yielding fatty acids and the glycerylphosphoryl derivative, (see main introduction and discussion). The extra breakdown product is readily observable on most chromatograms as cyclic glycerophosphate (Rf in water-saturated phenol-acetic acid-ethanol, 100:10:12, 0.66,. See TableXXIII for Rf values of lipid deacylation products). In all the analyses of mitochondrial lipids recorded in Table XIX no cyclic glycerophosphate spot was observed and the remaining spots yielded quantitative recovery of phosphorus from the paper. In all other cases cyclic glycerophosphate was observed and consequently the correction factor applied by Dawson for breakdown of

TABLE XVI

Analysis of phospholipids of rat liver cell fractions

Fraction	Whole rat liver	Nucleus		Mitochondria		Microsomes	
Amount of phosphorus hydrolysed (µg)	504	417.6	574.0	539.5	539.5	530.2	530.2
Phosphorus recovered in each fraction (µg)							
Alkali-labile	403.2	338.0	538.5	486.2	464.4	503.8	500.5
Acid-labile	1.9		6.6	3.1	2.7	3.2	3.9
Alkali and Acid Stable	23.6	45.6	36.3	22.1	23.6	26.9	28.9
% of recovered P in each fraction:							
Alkali-labile	94.1	88.1	92.6	95.0	94.6	94.4	93.8
Acid-labile	0.4		1.1	0.7	0.6	0.6	0.8
Alkali and Acid stable	5.5	11.9	6.3	4.3	4.8	5.0	5.4

TABLE XVII

Whole rat liver

Chromatography of the alkali-labile fraction

Sample	1.		2.		3. paper 1.		3. paper 2.	
	a. µg	b. %	a. µg	b. %	a. µg	b. %	a. µg	b. %
Phosphorus applied to paper:	95.0	100	98.0	100	103.0	100	103.0	100
Phosphorus recovered in:								
GPC	53.3	56.1	59.7	60.9	58.7	57.0	60.0	58.3
GPE	24.6	25.9	24.3	24.8	25.4	24.7	26.3	25.5
GPS	4.5	4.7	4.5	4.6	4.3	4.2	4.3	4.2
GPI	5.2	5.5	5.2	5.3	5.3	5.2	5.3	5.2
GP	trace	-	trace	-	trace	-	trace	-
GPGPG	3.3	3.5	3.3	3.4	3.4	3.3	3.4	3.3
CYCLIC GP	2.7	2.8	2.7	2.8	2.9	2.8	2.9	2.8
Recovery:								
phosphorus (µg)	93.6		99.7		100.0		102.2	
% recovery from paper:	98.5	98.5	101.7	101.7	97.1	97.2	99.2	99.3

TABLE XVIII

Nucleus

Chromatography of the alkali-labile fraction

Sample:	1.paper 1.		1.paper 2.		1.paper 3.		2.paper 1.		2.paper 2.	
	a. μg	b. %	a. μg	b. %	a. μg	b. %	a. μg	b. %	a. μg	b. %
Phosphorus applied to paper:	91.0	100	91.0	100	91.0	100	96.9	100	96.9	100
Phosphorus recovered in:										
GPC	49.1	54.0	49.0	53.8	50.2	55.2	53.9	55.6	53.6	55.3
GPE	25.8	28.4	26.4	29.0	24.1	26.5	-	-	27.3	28.7
GPS	4.3	4.7	4.3	4.7	4.3	4.7	7.5	7.7	7.5	7.7
GPI	2.1	2.3	2.1	2.3	2.1	2.3	4.0	4.1	4.0	4.1
GP	Trace		Trace		Trace		Trace		Trace	
UNKNOWN AT ORIGIN	1.0	1.1	1.0	1.1	1.0	1.1	1.4	1.4	1.4	1.4
CYCLIC GP	3.2	3.5	3.2	3.5	3.2	3.5	2.9	3.0	2.9	3.0
Recovery:										
phosphorus (μg)	85.5		86.0		84.9		-		96.7	
% recovery from paper:	94.0	94.0	94.5	94.4	93.3	93.3	-	-	99.8	100.2

TABLE XIX

Mitochondria

Chromatography of the alkali-labile fraction

Sample:	1. paper 1.		1. paper 2.		2. paper 1.		2. paper 2.	
	a.	b.	a.	b.	a.	b.	a.	b.
	µg	%	µg	%	µg	%	µg	%
Phosphorus applied to Paper:	92.0	100	92.0	100	85.5	100	85.5	100
Phosphorus recovered in:								
GPC	50.8	55.2	50.8	55.2	46.2	54.0	45.7	53.3
GPE	32.2	35.0	28.3	30.8	27.4	32.0	26.2	30.6
GPS	1.7	1.8	1.7	1.8	3.3	3.9	3.3	3.9
GPI	5.6	6.1	5.6	6.1	5.6	6.5	5.6	6.5
GP	trace	-	trace	-	trace	-	trace	-
GPGPG	6.0	6.5	6.0	6.5	4.2	4.9	4.2	4.9
CYCLIC GP	-	-	-		-		-	
Recovery:								
phosphorus: (µg)	96.3		92.4		86.7		85.0	
% recovery from paper:	104.7	104.6	100.4	100.4	101.4	101.3	99.4	99.2

TABLE XX

Microsomal fraction

Chromatography of the alkali-labile fraction

Sample:	1. paper 1.		1. paper 2.		2. paper 1.		2. paper 2.	
	a.	b.	a.	b.	a.	b.	a.	b.
	µg.	%	µg	%	µg	%	µg	%
Phosphorus applied to paper:	87.0	100	87.0	100	102.8	100	102.8	100
Phosphorus recovered in:								
GPC	51.4	59.1	52.5	60.3	62.0	60.3	67.6	65.8
GPE	18.1	20.8	19.6	22.5	19.6	19.1	18.9	18.4
GPS	2.3	2.6	2.3	2.6	2.1	2.0	2.1	2.0
GPI	2.3	2.6	2.3	2.6	2.9	2.8	2.9	2.8
GP	trace	-	trace	-	trace	-	trace	-
GPGPG	trace	-	trace	-	nil	-	nil	-
CYCLIC GP	2.1	2.4	2.1	2.4	5.1	5.0	4.9	4.8
Recovery:								
phosphorus: (µg)	76.2		78.8		91.7		96.5	
% recovery from paper:	87.6	87.5	90.6	90.4	89.2	89.2	93.9	93.8

lecithin and monophosphoinositide (15.), was used in all cases except the mitochondrial analyses.

Investigation of the breakdown of phosphatidylinositol

In the course of studying the turnover of lipids in cell fractions a radioactive spot was observed on some chromatograms having an Rf value of 0.12 in water-saturated phenol-acetic acid-ethanol, 100:10:12. (GPI runs at an Rf of 0.18). Dawson notes in his 1960 paper (15.) that while the predominant product of monophosphoinositide breakdown is GPI (57%), the hydrolysate also contains 20% of cyclic glycerophosphate, 14% of phosphorylinositol, 3% of glycerophosphate, and 7% of an unidentified hydrolysis product. To investigate whether the product with Rf 0.12 corresponded to the unknown product of monophosphoinositide breakdown, the following experiment was done.

A solution of phosphatidylinositol containing 500 μg of phosphorus was hydrolysed under the exact conditions described in the methods section, page 78 . This substance had been purified by silicic acid chromatography and was known to contain 30% of phosphatidyl-serine from nitrogen and phosphorus analyses. One-dimensional chromatography in water-saturated phenol-acetic acid-water, 100:10:12, (v/v) revealed 31% of the phosphorus in GPS as

expected. The distribution of phosphorus from monophosphoinositide was:

GPI Phosphorylinositol	64.5%
cyclic-GP	22.9%
unknown compound with Rf 0.12	12.6%
	100.0%

A two-dimensional paper chromatogram revealed in addition, a slight trace of glycerophosphate.

Phospholipid composition of other tissues

In the course of the work, various other tissues were examined when they became available, namely rat pancreas*, rat heart muscle, and optic nerve. The work on intestinal absorption, described in part III led to the analysis of mucosal phospholipids, and similarly, rabbit liver homogenates and nuclei were analysed in course of the incorporation experiments described in part II. A comparison of these various results is found in tables XXI and XXII. Figures in all cases represent phosphorus as a percentage of the total lipid phosphorus.

Footnote: * The assistance of Mr. R.H.Michell is acknowledged.

TABLE XXI

Phospholipid Composition of Various Tissues of the Rat

Tissue or fraction	LIVER				Intestinal Mucosa	Pancreas	Heart Muscle	Nerve
	whole	Nucleus	Mitochondria	Microsomes				
Alkali-labile fraction	94.1	92.6	94.8	94.1		74.6	90.7	44.0
Acid-labile fraction	0.4	1.1	0.6	0.7		25.4	9.3	56.0
Acid and alkali stable fraction	5.5	6.3	4.6	5.2				
Phosphatidylcholine	57.9	53.7	51.6	61.3	53.5	46.8	42.2	17.5
Phosphatidyl-ethanolamine	23.7	26.1	30.4	19.0	25.4	9.9	35.3	8.4
Phosphatidylserine	4.1	5.5	2.7	2.2	5.2	5.3	2.5	9.5
Phosphatidylinositol	6.9	3.9	6.0	3.5	4.1	8.7	1.5	Not determined
Phosphatidic acid	trace	trace	trace	trace	3.2	trace	trace	
Polyglycerol Phospholipid	3.2	-	5.4	-	2.8	3.9	8.3	
No. of determinations	(4)	(5)	(4)	(4)	(5)	(1)	(2)	(1)

Figures represent Phosphorus as % of total lipid phosphorus

TABLE XXII

Phospholipid Composition of Various Other Tissues

Tissue or fraction	Whole Rabbit Liver	Rabbit Liver Nucleus	Fish Nerve
Alkali-labile fraction	86.9	79.7	57.0
Acid-labile fraction	13.1	20.3	43.0
Acid and alkali stable fraction			
Phosphatidylcholine	43.1	41.2	32.1
Phosphatidyl-ethanolamine	24.0	18.3	12.8
Phosphatidylserine	5.6	8.0	3.7
Phosphatidylinositol	7.3	4.8	Not determined
Phosphatidic acid	2.4	3.4	
Polyglycerol phospholipid	2.7	-	

Figures represent phosphorus as % of total lipid phosphorus

TABLE XXIII

Some Typical Rf Values for Lipid Deacylation Products

Deacylation Product	Parent Phospholipid	Rf value in Phenol, water -Acetic acid -Ethanol 100:10:12	Rf value in Methanol -formic acid - water 80:15:5
Glycerylphosphorylcholine (GPC)	Phosphatidylcholine	0.86	0.66
Glycerylphosphoryl-Ethanolamine (GPE)	Phosphatidyl-ethanolamine	0.70	0.49
Glycerylphosphorylserine (GPS)	Phosphatidylserine	0.37	0.49
Glycerylphosphorylinositol (GPI) Phosphorylinositol	Phosphatidylinositol	0.18	0.35
L-3-Glycerophosphate (GP)	Phosphatidic acid	0.42	0.70
Di-glycerylphosphoryl-glycerol (GPGPG)	Diphosphatidylglycerol	0.33	0.58
Cyclic-glycerophosphate	Phosphatidylcholine / Phosphatidylinositol	0.66	0.58
Unknown	Phosphatidylinositol	0.12	0.35
Glycerylphosphorylinositol phosphate (GPIP)	Phosphatidylinositol phosphate	0.09	0.51
Glycerylphosphorylinositol diphosphate (GPIPP)	Phosphatidylinositol diphosphate	0.09	0.42

The fatty acid composition of rat liver cell fractions

The fatty acid analyses of the lipids from nuclear, mitochondrial, and microsomal fractions of rat liver are shown in table XXIV. Figure 5 is the gas chromatographic trace of the fatty acid methyl esters of the nuclear fraction; the traces for the other fractions are remarkably similar in appearance.

The figures in table XXIV represent the amount of each component as a percentage of the total amount of palmitic + stearic + oleic + linoleic + arachidonic acids present in the fraction. These acids are the major components and no other acid is present in quantity greater than 6% of the total fatty acids. It was necessary to express the result in this way as the minor components could not be determined accurately from the mitochondrial or microsomal traces. The percentages have been calculated by measuring the area beneath each peak.

Fig 5. THE FATTY ACIDS OF THE TOTAL LIPID FRACTION OF RAT LIVER NUCLEI

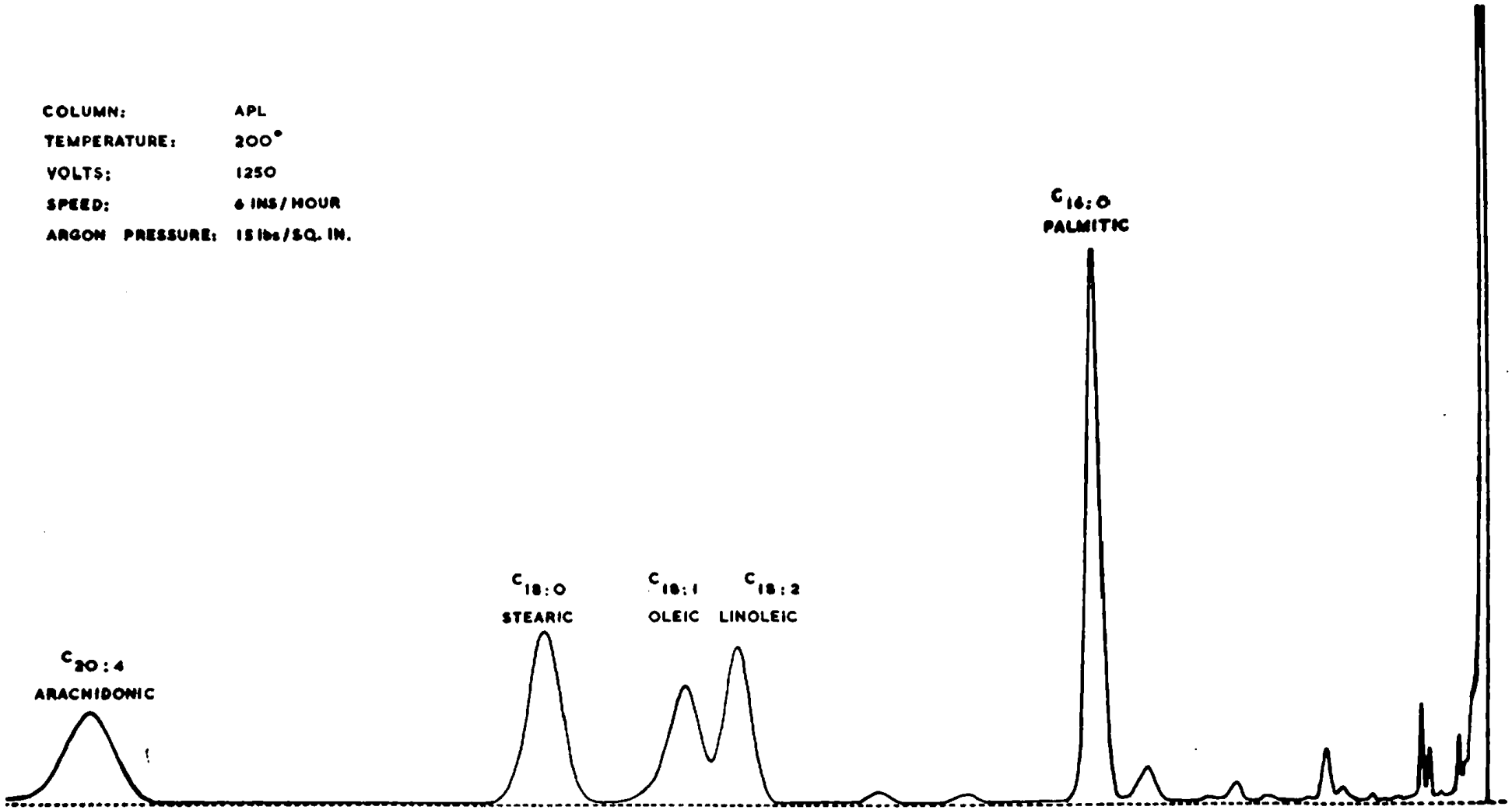


TABLE XXIV

Fatty acid composition of rat liver cell fractions

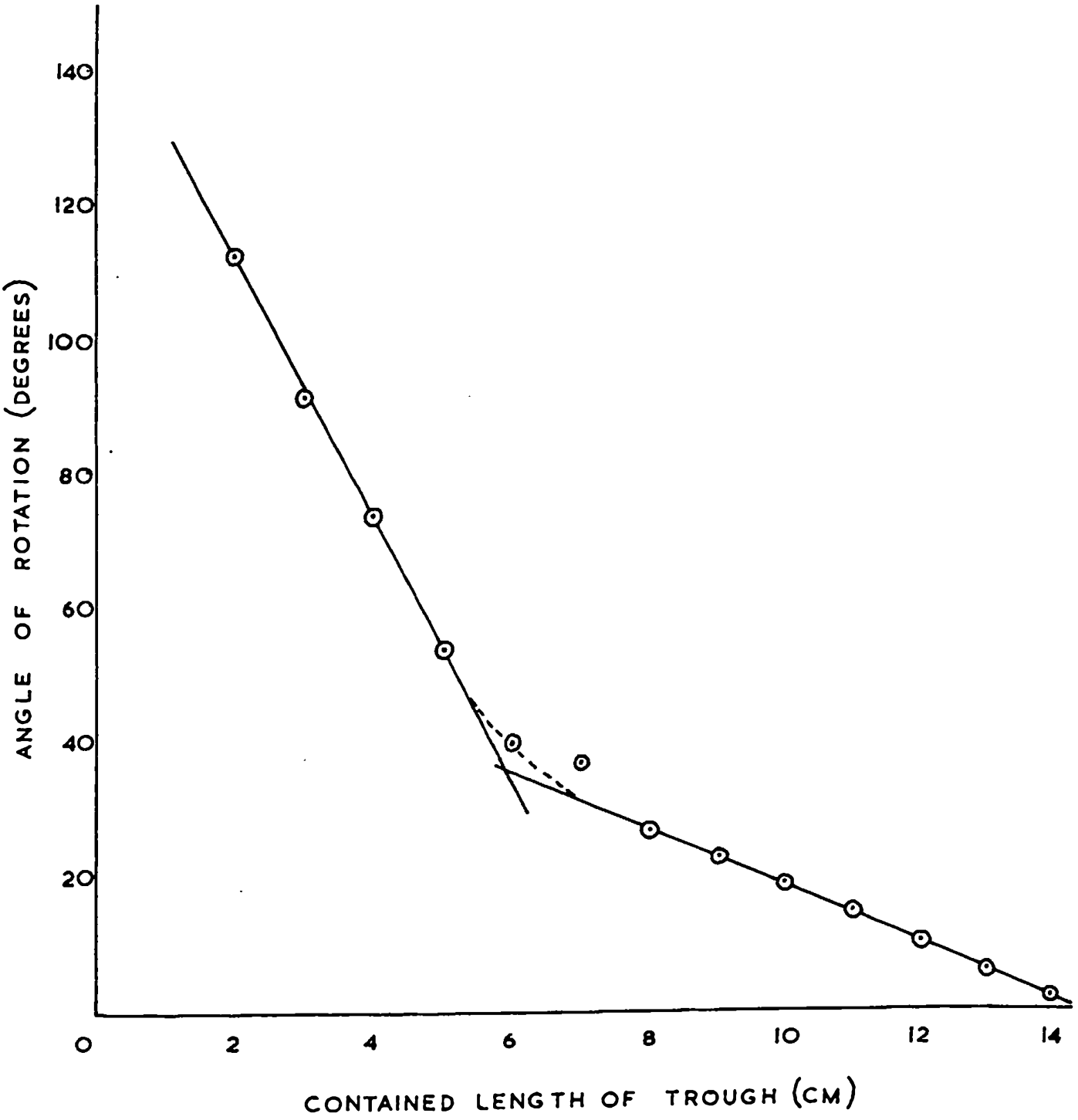
No. of C atoms and double bonds	Fatty acid	Nuclear Fraction	Mitochondrial Fraction	Microsomal Fraction
16-0	Palmitic	25.8	20.8	22.2
18-0	Stearic	22.7	22.5	22.2
18-1	Oleic	16.4	14.0	20.9
18-2	Linoleic	16.8	25.0	21.1
20-4	Arachidonic	18.3	17.7	13.6
Saturated acids		48.5	43.3	44.4
Unsaturated acids		51.5	56.7	55.6

The behaviour of nuclear lipid films: investigation of the surface properties of nuclear lipid extracts

The finding that so little phospholipid was contained in the nuclear fraction compared with other subcellular fractions, and later the discovery that there was regularly more phospholipid extracted from sucrose-isolated nuclei per unit of DNA, than from citric acid-isolated nuclei, led to the question of the location of lipid within the nucleus. It was of interest to know whether the lipid was situated entirely in the membrane and whether the amount of lipid found in the two types of nuclei could be correlated with the obvious differences in appearance of these nuclei, especially regarding the membrane. The Langmuir trough was used to determine the area covered by a given quantity of lipid and this was related to the known size of an individual nucleus.

Weighed quantities of nuclear lipids in petroleum ether-chloroform solutions (they were insoluble in pure petroleum ether) were introduced on the water surface of the trough with an Agla micrometer syringe. Force area curves were then plotted and a typical curve is shown on figure 6.

Fig 6 BEHAVIOUR OF NUCLEAR LIPID ON A LANGMUIR TROUGH



Calculation of the area occupied by nuclear lipid films, and correlation of this area with the size of the nucleus

Data from trough experiment:*

Limiting area of 0.01596 mg lipid is 89.68 sq. cm.

Citric acid-isolated nuclei

If the nuclei are assumed to be approximately spherical and of average diameter 11.0×10^{-4} cm (168), then:

Surface area of a single nucleus = 3.8×10^{-6} sq.cm.

Amount of DNA contained in a single nucleus (167) = 9.47×10^{-13} g.

Amount of lipid (Phospholipid + cholesterol) per nucleus, from table XIV = 1.3×10^{-12} g.

This neglects small amounts of triglyceride.

Assuming the lipid component of the nuclear membrane to form a layer similar to that obtained by spreading on the Langmuir trough; then the limiting area covered by 1.3×10^{-12} g. of lipid is: 7.96×10^{-6} sq.cm.

Relating this to the surface area of the nucleus:

Number of monolayers = $\frac{7.96}{3.8} = 2.1$

---

* The assistance of Mr.K.Parkes is acknowledged.

Sucrose-isolated nuclei

Lipid content of sucrose-isolated nuclei is 2.65 times that of citric acid-isolated nuclei, therefore:

Number of monolayers = 2.65 x 2.1 = 5.6

DISCUSSION

Summary of results

Nuclei isolated in citric acid and those isolated in 2.2M sucrose have been shown by both light microscopy and electron microscopy to be free from cytoplasmic contamination. Enzymatic methods, although not applicable to citric acid-isolated nuclei, confirmed the purity of sucrose-isolated nuclei. The relative constancy of the phospholipid-phosphorus/DNA-phosphorus ratio over nine experiments is thought to be additional evidence that nuclei are free from contamination with other cell particles. Such contamination is unlikely to occur always to the same extent, hence the amount of lipid present compared to DNA would be expected to vary considerably. The amount of phospholipid is low - being only about 1% of the whole cell phospholipid phosphorus in the case of citric acid-isolated nuclei, or 2.5% in the case of sucrose-isolated nuclei. A comparison of the protein figures in each type of nucleus confirms the view that large amounts of protein are lost from nuclei isolated in citric acid at low pH.

The electron micrographs of nuclei isolated in citric acid show a definite, single boundary line around the nucleus, suggesting a unit membrane or bimolecular lipid layer, whereas in the case of sucrose-isolated nuclei, a double structure is visible, which might be interpreted as two unit membranes or four lipid monolayers.

Measurements with the Langmuir trough showed that lipid extracted from the citric acid-isolated nuclei would be capable of providing 2.1 lipid monolayers while the analysis of sucrose-isolated nuclei yielded a figure of 5.6 monolayers.

Analysis of the phospholipids of the various cell fractions - homogenate, nuclear, mitochondrial and microsomal fractions - showed that the largest phospholipid group in each case consisted of those phospholipids labile to mild alkali, (glycerophosphatides), comprising about 94.5% of the total phospholipid phosphorus, with sphingomyelins 5% and plasmalogens only 0.5%. In the case of the nuclear phospholipids there appeared to be a very slightly higher proportion of plasmalogens and sphingomyelins.

Chromatography of the water-soluble hydrolysis products of the glycerophosphatides showed amazing similarity in the composition of these lipids in all cell fractions. The

major component was always phosphatidylcholine (+ lysophosphatidylcholine) at about 58%, with phosphatidylethanolamine, 24%; phosphatidylserine, 4%; phosphatidylinositol, 7%; cardiolipin, 5½% in mitochondria. A trace of phosphatidic acid was almost always seen. The mitochondrial phosphatidylcholine tended to be lower and the phosphatidylethanolamine higher than the whole tissue figures, while the microsomal fraction has the highest concentration of phosphatidylcholine with rather less phosphatidyl-ethanolamine. All the minor components, too, occur in all cell compartments, with the exception of cardiolipin which was only observed in the mitochondrial fraction; sometimes traces were found in the microsomal fraction, but never in the nuclear fraction. The phospholipids of rabbit liver homogenate and nuclei followed a similar, though not identical, pattern.

Table XXI which summarises the phospholipid composition in various other tissues of the rat, shows that phosphatidylcholine is always the major component, and that in fact the pattern is remarkably similar throughout all these tissues. Intestinal mucosa illustrates this point well - the composition of the phospholipids is almost exactly the same as in liver.

There is a higher proportion of phosphatidic acid, and while it is probable that glycerophosphate measured on chromatograms did not all originate from phosphatidic acid, evidence from column chromatography given in part III indicates that a substantial proportion originated in this lipid. Heart muscle had a larger proportion of cardiolipin than other tissues and pancreas seems to be slightly richer in inositide; however it should be pointed out that very few analyses were done on these tissues.

The similarities in fatty acid composition of the total phospholipid fraction from each cell compartment are no less striking. Only the five major acids were measured quantitatively, but gas-phase chromatograms showed that the pattern of minor acids was almost identical in each fraction. The proportion of total unsaturated acids was slightly higher in the nucleus than in the other fractions.

Isolation of nuclei: loss of material by extraction

There seems little doubt that the use of citric acid as a medium for the isolation of nuclei is acceptable for lipid studies. Dounce, (137) thinks that there is little probability of lipid being lost from nuclei isolated in

citric acid at pH 3.0-4.0. For such statements to be meaningful one must have a reliable reference standard. There is good evidence (127) that DNA is firmly bound and not lost from nuclei isolated at pH 4.0 or lower, and hence is a valid "standard". At high pH values DNA seems to be less firmly bound and loss can be caused by breakage of nuclei in a high speed mixer. Since I have used a much more gentle homogenisation procedure and lower pH (about 3) it is certain that the DNA standard is reliable.

As mentioned in the introductory remarks, loss of protein is much more uncertain. Dounce (137) thinks it probable that little material of high molecular weight is lost from nuclei isolated at pH 3.8-4.0. Protein of high molecular weight is undoubtedly denatured at this pH and thereby rendered insoluble. Nucleohistone, for instance, is near its isoelectric point at this pH and is quite insoluble. However, at pH less than 3, nuclei lose much or all of their histone. Allfrey (131) states that nuclei may lose as much as 55% of their protein at this pH. This raises the question: "Is some of the lipid removed as lipo-protein?". If this were so, it would mean that values for nuclear lipid using the DNA standard would be too low. Hence the low values

achieved in this work need not be due solely to high nuclear purity, but to loss of lipoprotein.

There is good evidence that nuclear lipid is associated with protein in the form of a lipoprotein. Wang et al.(169) claim that a lipoprotein constitutes 50% of the dry weight of nuclei prepared by the method of Dounce (170) (isolated at pH 6.0-6.2). The protein, which was insoluble in water or salt solutions, but extracted with alkali and reprecipitated with acetic acid, contained 30% of "fatty substances". The lipid was firmly bound and was not extractable with ether but by hot alcohol. This lipoprotein described by Wang is essentially the same as the substance which the Stedmans (171) called "chromosomin" and the same material as the "residual chromosomes" of Mirsky and Ris (172).

Dallam (173) also extracted a similar lipoprotein from the nuclear, mitochondrial and microsomal fractions of dog kidney. The lipid was not extracted by ether, but was removed by alcohol. After electrophoresis, analysis revealed the same percentage of lipid attached to the protein as before. Wang and Dallam regarded this lipoprotein as probably part of the nuclear membrane system. The evidence for this was rather scant, but Wang observed that after extraction of the nucleohistone with salt solution, intact "ghost" nuclei were seen;

these could not be observed after extraction of the lipoprotein.

Certainly large differences have been reported in the literature in regard to the lipid content of nuclei and not all these variations are due to cytoplasmic contamination. Dounce (137) observed differences in the lipid content of nuclei in rats of different strains, but more important, at different pH values. A higher lipid content occurred at pH 6 than at pH 4. This may have been partly due to cytoplasmic contamination but not entirely. Little protein was absorbed at pH 6 as was shown by the fact that the percentage content of DNA was much the same in each case. The reason for the difference in lipid content is unknown.

Total nuclear lipid was found by Williams et al. (138) to be 14-18% of the dry weight of nuclei (isolated at pH 6). This figure is likely to be too high as it was obtained by addition of the figures for various lipid classes. A figure of 11% was obtained by Levine and Chargaff (43) for nuclei isolated in 5% citric acid. Barnum et al. (139) found a total of 3.4% phospholipid in mouse liver nuclei isolated in 2% citric acid. Various results have been compared in table XV.

There is good evidence (148) that nuclei isolated in

2.2M sucrose retain most of their protein and this method is becoming widely used for protein work. Hence it is not likely that such nuclei will have lost lipid in the form of lipoprotein. The electron micrographs (Plates I - III) show a definite boundary line around citric acid-isolated nuclei and this is consistent with the Langmuir trough data that a single unit membrane is present. The lipid analyses for sucrose-isolated nuclei indicate the presence of enough lipid for a double membrane, such as is known to be a feature of the nuclear membrane in intact tissue. (62,174-176). It seems reasonable to conclude that the outer membrane of the nucleus is less closely associated with the nucleus and is removed by citric acid. Since lipid and protein are intimately bound together in membrane systems, it may be that removal of this membrane is synonymous with the removal of lipoprotein of a type described by Wang et al. (169) and Dallam (173) and earlier demonstrated with the electron microscope by Callan and Tomlin (175). It is doubtful, however, whether this could account for some 50% of the weight of the nucleus as claimed by Wang. That much material is removed from the interior of the nucleus is clear from the vacuous appearance of the citric acid-isolated nucleus as compared with the sucrose-isolated nucleus. (see micrographs).

The problem of whether the chromosomal material contains phospholipid cannot be considered from my data. Chayen et al. (177) have published a series of papers showing that a phospholipid is a constituent of the chromosomes. Their evidence is based mainly on histological staining reactions, with little backing from biochemical analyses. The present results, while strongly suggesting that lipid is confined to the membrane, cannot entirely rule out the possibility that some may be located in the chromosomes.

In view of the assumptions made in the calculations from Langmuir trough data, an accurate figure for the number of lipid monolayers which would be present at the nuclear surface would not be expected. The figure of 5.6 layers in the case of sucrose-isolated nuclei might be partly due to the fact that parts of the endoplasmic reticulum are still attached to sucrose nuclei, which might be expected if the nuclear membrane and endoplasmic reticulum are continuous structures in the living cell (64). On the other hand it might be due to the situation of lipid at other sites in the nucleus.

Extraction of the lipids

Another source of error in the quantitative analysis of nuclear lipids might be incomplete extraction of lipid from the nuclear sediment. The variety of solvent systems used in this work seems to indicate little difference in lipid content when these different methods are used. Getz et al. (4), however, found differences in the lipid content of cell fractions according to the treatment of the fraction before extraction with lipid solvents. Freeze-drying or TCA precipitation could result in low recoveries of lipid.

Differences in lipid content could also be due to the use of rats of different strains. As far as possible, rats of one strain were used but this was not always possible. No striking differences were ever noticed.

Conditions of feeding could be important. For the isolation of nuclei in citric acid, rats were fed on the standard laboratory diet until the time of death. Rats used for isolating nuclei in sucrose were starved for 16 hours before death, for reasons outlined in the introductory section. Weber and Cantero (178) showed that the liver phospholipid content (per average cell) of fasted rats decreased slowly until at 6 days it was 50% of the original; after 24 hours it had decreased to only about

90%. If most of the liver phospholipid is located in mitochondrial and microsomal membranes it is difficult to see why this should occur without serious decrease in the numbers of mitochondria. In any case such differences are unlikely to be important in these experiments.

Comments on Table XIV

Before progressing from the discussion of the phospholipid content of nuclei to the pattern of the individual phospholipids, some remarks should be made about table XIV. Figures for lipid phosphorus of whole liver (42b) and cholesterol content of liver (42c) are within the range quoted in the literature. The DNA content agrees with the values given by Schweigert et al. (179). The protein value for citric acid-isolated nuclei is, as expected, low; but that for sucrose-isolated nuclei is somewhat higher than the value quoted by Chauveau (134). It falls, however, in the range quoted in the literature. (42d,179). As pointed out in the experimental section, several analyses were done with sucrose nuclei before it was realised that DNA analyses were often too low, possibly due to inefficient precipitation by TCA from the viscous medium. Also, some experience is required with the technique before very pure samples can be

obtained. Hence results for sucrose nuclei in table XIV are those from the preparation which was thought to be the best.

Isolation of mitochondrial and microsomal fractions

Little need be said about the method used for the isolation of these particles. It is essentially the method of Schneider and Hogeboom (126) and is used widely. In this laboratory, 0.3M sucrose is thought to preserve the structure of the mitochondria rather better than the more usual isotonic medium; EDTA was added to retard the action of digestive enzymes. Care has been taken to refer to the products after centrifugation as mitochondrial "fraction" and microsomal "fraction". These are defined purely by the centrifugation data quoted in the "Methods" section; they are undoubtedly heterogeneous and may well contain lysosomes, fluffy layer and other cytoplasmic particles. No attempt has been made to acquire fractions of homogeneous particles or to establish the "purity" or "composition" of the fractions.

Phospholipid composition of each cell fraction

Most investigators have found that nuclear lipid and the lipid of other cell particles was rich in phospholipid and poor in neutral lipid. The proportion of neutral

lipid has not been investigated in this work.

Early workers could only fractionate the phospholipids into classes - "lecithin", "cephalin" and sphingomyelin. Williams et al. (138) found 70% lecithin, 27.1% cephalin and 2.9% sphingomyelin in rat liver nuclei. Levine and Chargaff (43) found a 1:1 ratio of choline:ethanolamine in lipid from rat liver nuclei.

More recently, the intracellular distribution of phospholipids in rat liver has been studied by Getz et al. (47). The mitochondrial fraction contained 8.8% of the liver lipid phosphorus and this included 12.4% of the liver phosphatidylethanolamine but only 7.5% of the liver lecithin; microsomes contained about 44% of the liver lipid phosphorus and 58.9% of the liver lecithin. Their mitochondrial fraction contained almost all the cell cardiolipin (3.5 times the concentration in the whole cell), only trace amounts being found in the microsomes. They did not study the nuclear fraction. The figures of Getz et al. (47) and those of Marinetti et al. (46) are shown in table XXV on the next page. The most recent survey by Biezenski et al. (49) is reproduced in table XXVI. Figures given by Dawson (15) for sheep liver, obtained by the method of mild alkaline hydrolysis, while not strictly comparable with my results, nevertheless

show a strikingly similar pattern.

Some comments should be made about the data in table XXI. The figures for the phospholipid composition of homogenate should be expected to be an average over all cell fractions, weighted towards the microsomal fraction since this fraction provides the major part of the liver phospholipid. This is not the case for phosphatidylserine or phosphatidylinositol. These minor components, are of course, subject to greater inaccuracies in measurement than the major ones; some discrepancy may be expected due to the greater inhomogeneity of the whole tissue - it will be expected to contain blood, connective tissue etc. Figures for phosphatidylinositol also will be subject to inaccuracies due to breakdown during hydrolysis. My figures for the breakdown of monophosphoinositide are similar to Dawson's. (15). A constant correction factor has been applied, based on the known breakdown of monophosphoinositide under specified conditions. I have been careful to try to maintain the same conditions for all hydrolyses, but slight differences in <u>time</u> of hydrolysis or <u>bath temperature</u> might cause variations in the analyses.

In general, it seems that <u>phosphatidylcholine</u> is the major component of the phospholipid mixture irrespective of the cell fraction. The only serious disagreement seems

to be found in the results of Biezenski et al (49)who found a low concentration of phosphatidylcholine in the nuclear fraction. The finding, in this present work, that the microsomal fraction has a very high proportion of phosphatidylcholine with rather less phosphatidylethanolamine than in the whole cell, and that these two lipids are present in more nearly equal quantity.in the mitochondrial fraction

TABLE XXV

Author	Phospho-lipid	Whole Liver	Mitochondrial Fraction	Microsomal Fraction
Getz et al. (47)	PC		37.5	59.5
	PS + PE		39.0	26.0
	PI		9.5	9.5
	CL		10.0	1.0
	OTHERS		4.0	4.0
Marinetti et al (46)	PC	58.1 ± 2.4		
	PE	20.4 ± 1.2		
	LYSO-PC	3.4 ± 0.6		
	PS	2.0 ± 0.9		
	PI	1.9 ± 0.7		
	CL	3.4 ± 0.5		
	SPHINGO-MYELIN	8.2 ± 0.7		

Figures represent phosphorus as % of total phospholipid phosphorus

is in good agreement with the results of Getz et al. Figures for phosphatidylserine, too, are generally agreed to be of the order of 2-6% of the lipid phosphorus. Here again there is serious disagreement with the work of Biezenski et al. who find 30% of the lipid phosphorus in the nucleus to be in phosphatidylserine.

There are less data on the proportions of inositides in liver cell fractions. The work of Getz shows 9.5% in the mitochondria and microsomes, rather higher than in the present work, while the figure given by Marinetti (46) is very low, 1.9%. It should be pointed out that Getz et al. paid more attention to the homogeneity of their fractions, and treated the fluffy layer as a separate fraction. The data of Biezenski et al. are extremely difficult to understand; the inositide would be expected to be included in the fraction they have called "other lipids". This fraction has a value of 0 in the case of mitochondria, hence one assumes that they find no inositide (or cardiolipin) in the mitochondria; this is quite contrary to the findings of all other workers, as is the finding that "other lipids" comprise 48.0% of the nuclear phospholipid fraction.

My figures for cardiolipin, though rather lower than those of most other workers, are not too different from

TABLE XXVI : Results of Biezenski *et al* (49)

Phospholipid	Whole Liver	Nuclei	Mito-chondria	Micro-somes	Super-natant
Total choline	54.0	14.5	77.5	45.0	70.0
Ethanolamine	24.5	7.5	15.0	16.0	10.0
Serine	6.0	30.0	7.5	11.0	12.0
Sphingomyelin	9.0	5.0	5.0	8.0	2.0
Others	15.5	48.0	0.0	28.0	8.0

Figures are moles/100 moles phospholipid phosphorus "other phospholipids" are obtained by subtracting sum of serine + ethanolamine + choline from 100.

the figures of Getz *et al*. (47), Strickland and Benson (48), and Macfarlane *et al*. (50) and agree in the respect that this lipid is confined almost entirely to the mitochondrial fraction.

Fatty acid composition

The fatty acid composition of rat liver cell fractions has been studied by Getz *et al*. (4), Macfarlane *et al*. (50), and Veerkamp *et al*. (51). A comparison of my results with those of Getz (see tables XXIV, XXVII.) shows no great differences.

These authors have pointed out the striking similarity in the fatty acid pattern of all cell fractions, and to a

large extent this is true of the phospholipids themselves.

TABLE XXVII

The fatty acid composition of rat liver cell fractions
Getz et al. (4)

Fatty Acid	Nucleus	Mitochondria	Microsomes
Palmitic	29.6	24.5	26.4
Stearic	18.9	20.1	22.5
Oleic	16.1	14.0	15.5
Linoleic	19.0	23.6	18.1
Arachidonic	16.4	17.8	17.5
Total saturated acids	48.5	44.6	48.9
Total unsaturated acids	51.5	55.4	51.1

The solubility properties of phospholipids

The fatty acid analyses discussed above were originally undertaken to discover whether the peculiar solubility properties of nuclear lipids were due to some differences in fatty acid composition.

It has been mentioned earlier that addition of alcohol to carbon tetrachloride solutions of nuclear lipids produced a white precipitate (presumably "cephalin"), whereas this effect did not occur with lipids of other

cell fractions, or of whole liver. Several possible reasons suggested themselves: 1) differences in phospholipid composition from fraction to fraction, 2) solubilising influence of triglycerides, and 3) differences in fatty acid composition. Since the phospholipid composition of all fractions is so similar it would not be likely to cause differences in solubility; likewise, since all fractions contain so little neutral fat, any influence it might exert on the solubility of the phospholipid fraction would not cause the differences noted here. Such solubility differences might therefore have been thought due to a greater saturation of the fatty acids of nuclear lipids; the ratio : total saturated acids/total unsaturated acids is slightly higher in the case of the nucleus but the difference would seem to be insignificant in this respect.

It is interesting to note that solubility differences have been observed before in nuclear lipids. Dounce (127) points out in his review that Stoneburg (143) and Levine and Chargaff (43) were unable to completely dissolve their nuclear lipids in petroleum ether, whereas he himself had no difficulty. Dounce had isolated nuclei at pH 6, whereas the pH used by Stoneburg and Levine and Chargaff was very much lower. Dounce suggested that

changes brought about by the pH differences were responsible for the solubility changes. I, too, have observed only partial solubility of nuclear lipids in petroleum ether. The problem still remains unsolved.

Insight into the function of phospholipids in the different sites in the cell, and the way in which their properties are connected with the working of the cell, should be gained not only from a study of their composition and distribution, but also of their dynamic activity. In the next chapter some observations on this aspect of phospholipid biochemistry are recorded.

PART II

THE INCORPORATION OF ^{32}P INTO PHOSPHOLIPIDS OF LIVER CELL FRACTIONS

INTRODUCTION

Radioactive tracers are now regarded as indispensable tools in all branches of biochemistry; their increasing use over the years is due mainly to two things - the pioneer work of George Hevesy in the 30's and the fact that the high neutron flux produced in nuclear reactors, has, since the war, provided a cheap means of manufacturing radioisotopes.

The first isotopes used in biochemistry were the stable isotopes of hydrogen (^{2}H) and nitrogen (^{15}N); these were used by Schoenheimer and his colleagues (180) to demonstrate the hitherto unsuspected activity of the turnover of the fats and proteins of the body - findings which revolutionised our concepts of metabolism.

Very soon after the production of the artificial isotope of phosphorus, ^{32}P, by Joliot and Curie in 1934 (181), Chiewitz and Hevesy (182) used it to trace the absorption, tissue distribution, and excretion of inorganic orthophosphate. They were able to demonstrate phospholipid synthesis and to show the very significant turnover of phosphorus in the bone material. Since this early work,

the variety of reactions in which ^{32}P has been employed seems to have increased exponentially.

Requirements of a good isotopic tracer and the limitations of radioactive indicators

In general, the biochemist is interested in solving such problems as the fate of a particular molecule in a given metabolic process, the manner in which component atomic groupings in such a molecule are mobilised as energy sources in the synthesis of organic material, the importance of a molecule as a contributor to the structural elements of nuclear and cytoplasmic constituents of the living cell, or determining the general level of dynamic activity of a cellular constituent.

For a tracer to be useful in biological experiments, it must fulfil certain requirements, and certain assumptions must be made about the nature of the isotope and the processes in which it is involved.

1. The labelled material should behave in exactly the same manner as the naturally occurring element or compound. Effects due to differences in isotope mass have been discussed by Bigeleisen (183). Isotopes of lighter mass tend to form less stable bonds, so that rate constants for processes involving the rupture of these bonds will be

increased. For isotopes of higher mass, such as phosphorus, this effect is negligible; for ^{12}C, ^{13}C, and ^{14}C, the effect is small but demonstrable. Only in the case of hydrogen, where the isotopes differ from each other by factors of 2 and 3 does the problem become serious. The rate constants involving bonds of hydrogen and tritium may differ up to 60 times.

2. In experiments with stable tracers it is assumed that elements exhibit constancy of isotopic composition. Minor fluctuations have been noted in some elements and these determine the ultimate precision of work with samples enriched in rare stable isotopes. As far as natural radioactive isotopes are concerned, ^{40}K is the only isotope of importance in biology.

3. The initial concentration of the tracer must be sufficient to withstand dilution during metabolism. On the other hand it is important to use an indicator of negligible weight, (in other words - very high specific activity), as a sudden increase in, say, the phosphate concentration of the plasma may have very disturbing effects. With phosphorus, a fair amount of latitude is possible, since Fries and Chaikoff found that there was no difference in ^{32}P recovery in such tissues as brain and blood, when

such widely differing amounts of labelled phosphorus as 6.0 and 0.3 mg were injected into 200g rats (184).

4. The label must adhere to the particular molecule or portion of molecule with which it was originally incorporated. Processes involving removal of tracer by "exchange reactions" must be minimised, or obviated by suitable control experiments.

5. Caution should be taken to determine that the level of radiation produced by the isotope does not create abnormalities in the metabolism of the animal under investigation. Low-Beer (185), for example, has estimated the threshold for radiation effects on bone marrow due to ^{32}P to be 6-9 μc/Kg body weight.

The concept of the metabolic pool

This concept, based primarily on researches made possible by the tracer method, refers to the existence of a circulating body of chemical substances in partial or total equilibrium with similar substances derived by continual release and uptake from cellular tissues. It is a mixture of components, derived from diet, or tissue breakdown, which the organism uses for synthesis of new tissue constituents and its existence is a consequence of

Schoenheimer's "Concept of the dynamic state of cell constituents".

Precursor-product relationships

In a study of metabolism in the growing animal, it is possible to demonstrate relationships between metabolites by non-tracer feeding experiments; in the steady state, when growth has stopped, the only available method is the tracer method. By such means it is possible to demonstrate that substance B (product) is derived from substance A (precursor) and criteria for the establishment of such a precursor-product relationship have been given by Zilversmit et al (36), in cases where a steady-state condition exists.

Steady state

In the steady state, the amount of product is constant with time, the rate of appearance equalling the rate of disappearance.

Turnover

Turnover is the continual renewal of a given substance without change in the overall amount present. The process of renewal refers to a given component not necessarily a whole molecule. It is only the component that is being labelled whose turnover is measured. From a labelling

standpoint, a substance can be renewed in one or both of the following ways:

(i) The labelling atoms may be incorporated by synthesis or exchange.

(ii) The labelled substance may replace unlabelled substance by transport into the tissue site under consideration.

The turnover time is the time required to renew completely the amount of substance initially present in the tissue.

The turnover rate of a substance in a tissue is the amount of the substance that is turned over by that tissue per unit of time.

The rate-determining step should be known if calculations of turnover based on specific activity are to be valid. For example, if the reaction:

$$\text{Plasma } P_i \rightleftharpoons \begin{matrix}\text{phospholipid} \\ \text{precursor}\end{matrix}$$

is a simultaneous reaction, then a calculation of turnover based on the specific acitivity of P_i will be valid. Consider the following reactions, where P_i refers to plasma inorganic phosphate, and XP is an intermediate in phospholipid biosynthesis:

(a) $P_i \xrightarrow[\text{fast}]{} XP \xrightarrow[\text{fast}]{} \text{phospholipid}$

(b) $P_i \xrightarrow[\text{fast}]{} XP \xrightarrow[\text{slow}]{} \text{phospholipid}$

(c) $P_i \xrightarrow[\text{slow}]{} XP \xrightarrow[\text{slow}]{} \text{phospholipid}$

In cases (a) and (b), a calculation of turnover based on the specific activity of P_i will be valid, since the specific activity of XP nearly equals that of P_i; in (c), however, only information about the specific activity of XP will yield good results. In general, if there is a series of slow steps, the specific activity of the immediate phospholipid precursor must be known.

Data should be taken from the ascending part of the time curve since here synthesis rather than breakdown predominates. Another important factor influencing the specific activity of a component is the rate of transport of the labelling agent to the site of formation of the product molecules.

Calculation of the precursor-product relationship by the method of Zilversmit et al (36).

If one assumes that all molecules of substance, whether newly formed or old, are equally available for synthesis or breakdown, one may arrive at a simple criterion

for the precursor-product relationship as follows:

let p = constant rate of conversion of precursor A to product B.

r = amount of B present in tissue (constant).

x = amount of labelled B in tissue, at time t

f(t) = specific activity (s.a.) of A

$$\left(\frac{\text{no. of labelled molecules}}{\text{no. of unlabelled molecules}}\right)$$

which is a function of time t.

The amount of labelled material converted into B per unit time is p. f(t).

The amount of labelled material lost per unit time is p. $\frac{x}{r}$.

Since at steady state, p = rate at which B is destroyed (lost), the change in specific activity (or labelled content) of B with time is then:

$$\frac{dx}{dt} = p.\ f(t) - p.\ \frac{x}{r} \qquad (1)$$

since r is constant:

$$r\ \left(\frac{dx}{r}/dt\right) = p\left[f(t) - \frac{x}{r}\right] \qquad (2)$$

therefore:

$$\frac{d\left(\frac{x}{r}\right)/dt}{\left[f(t) - \frac{x}{r}\right]} = \frac{p}{r} = \text{constant} \qquad (3)$$

The <u>numerator</u> in equation (3) measures the slope of the specific activity - time curve of product B.
The <u>denominator</u> is the difference at any given time between the specific activities of precursor A, f(t), and of product B, $\frac{x}{r}$.

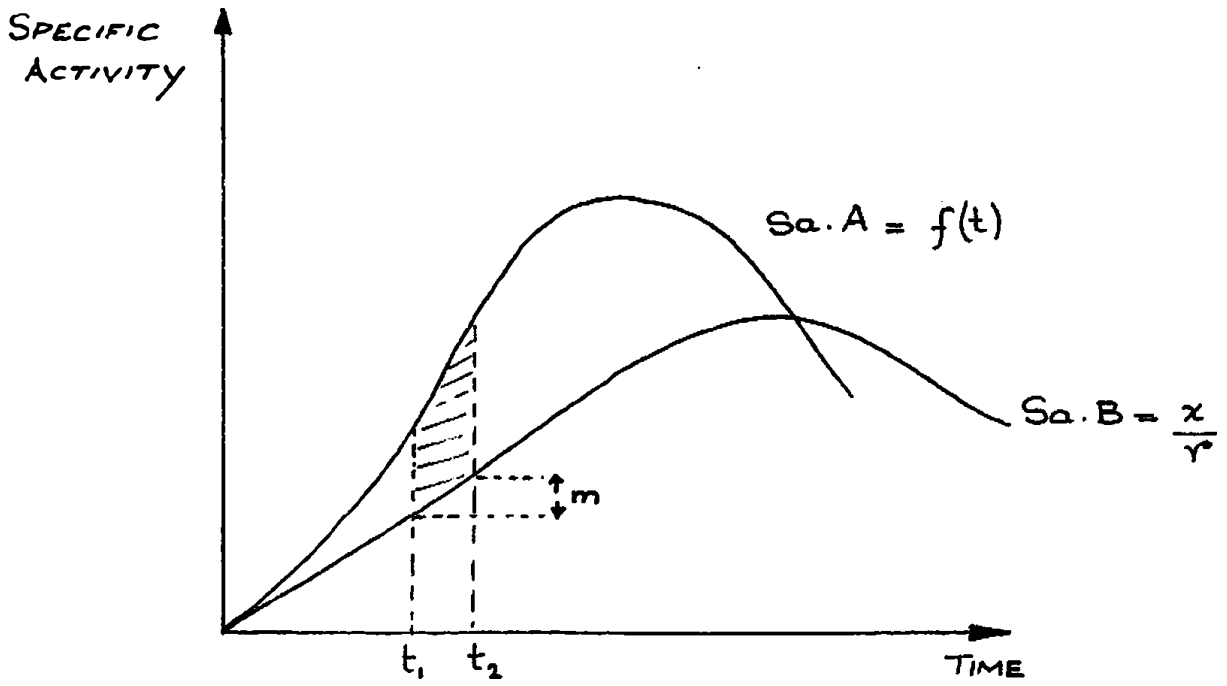


Figure 1. <u>Specific activity-time relationships of precursor, A, and product, B.</u>
<u>(Zilversmit et al)</u> (36)

From equation (3), it can be seen that when the specific activity of B is increasing (slope s.a.B is positive) the specific activity of A must at all times be

greater than that of B until the specific activity of B reaches a maximum. After B reaches its maximum specific activity, the relationships are reversed. At the maximum specific activity of B ($(\frac{dx}{r})/dt = 0$) the two specific activities are equal.

The <u>turnover time</u> $(\frac{r}{p})$ can be obtained by dividing the area between the specific activity curves for any two times t_1, t_2, by the increase in specific activity of B between times t_1-t_2.

The <u>turnover rate</u> is most easily determined from the rate of disappearance of label after the steady state has been established. Under these conditions, the nature of the immediate precursor and its labelling content do not need to be known.

<u>Incorporation of ^{32}P into phospholipids of animal tissues</u>

Large numbers of publications can be found describing experiments to measure the incorporation of radiophosphorus into phospholipids of a wide variety of tissues. It seems better here to concentrate on reviewing the relevent work with liver and the sub-cellular fractions of liver, rather than to try to deal with all aspects of this wide field. Much of the work on intestinal phospholipids is covered in Part III, and quite a number of experiments

not touched on in this section, especially the "in vitro" work have been described in the introductory section on the biosynthesis and function of phospholipids.

In vivo incorporation of ^{32}P into phospholipids of liver cell fractions

Some of the earliest experiments were Hevesey's. He studied the uptake of ^{32}P by the tissue and nuclei of rat liver (and of sarcoma) (186a). The nuclei were isolated by the method of Dounce (137) and the exchange period for labelling was two hours. The ratios of specific activity of the phosphatide phosphorus of the fraction to the specific activity of the inorganic phosphorus of the organ were: 15.3 and 9.9 for liver and nuclei respectively. In another study (187) Hevesey determined these values in control rats and those which had been irradiated with 1200 r. Specific activities of controls were 10.3, 8.2 and 11.3 for nuclei, and 20.4, 11.3, and 14.3 in whole tissue in three separate experiments; these values were decreased by 59% and 36% respectively immediately after exposure to X-rays.

Other early workers in the field were Chaikoff and his group; they studied the turnover of the whole phospholipid fraction in various tissues of the rat. In the

liver the feeding of choline (188) was found to increase the phospholipid turnover, and in the intestine, feeding of fat likewise increased the turnover (189).

A thorough study of rabbit liver cell fractions has been made by Ada (190). Nuclei were prepared in 1% citric acid and mitochondria and microsomes by the Schneider-Hogeboom technique. Figures for the specific activities of the whole phospholipid fraction relative to the liver inorganic phosphorus at 1000, four hours after injection of the isotope were: 25.6, 29.2, 33.3 and 18.7 for nuclei, mitochondria, microsomes and supernatant, respectively. Using the criteria of Zilversmit et al (36), Ada was able to show that the lipid component in each cell fraction could not act as a precursor in respect to the lipid in any other fraction.

Barnum's group has studied the same phenomena in mouse liver (191). In contrast to Ada they found that at all time intervals, the specific activity of the microsomal phosphatides were the same as those of the supernatant phosphatides, but in agreement with Ada and Hevesy, they showed that nuclear phospholipids had a significantly lower specific activity, varying over a twelve hour period, from 59 - 75% of the microsomal lipid specific activity. At

six hours after injection of the isotope, specific activities relative to tissue inorganic phosphorus at 1000 were; 519 and 687 for nuclear and microsomal fractions respectively. A point of interest: they found that in non-fasted animals, the specific activity of each phosphate fraction, (RNA, DNA, phospholipid) was markedly lower than in the case of fasted animals and depended on whether the injection was in the morning or at night.

The studies of Davidson and his group (141), like many others, were directed mainly toward nucleic acids, and included the phospholipid fraction for the sake of completeness. Nuclei were isolated by the method of Hirsky and Pollister (131), and phosphorus compounds extracted by either the Schneider (2) or Schmidt-Thannhauser (3) procedures; values for specific activity of the phospholipid fraction of the nucleus, relative to total acid-soluble phosphorus at 1000 (exchange period 2 hours) were: 196;391; and 725 for rabbit, unfasted rat and fasted rat, respectively. In another paper (192) their figures for each cell fraction of rat liver after a 4-hour exchange were: whole cytoplasm, 18.6; nuclei, 14.8; mitochondria, 14.3; microsomal fraction, 17.1; and cell sap, 17.1 (relative to blood inorganic phosphorus at 1000).

The detailed series of investigations of Johnson and Albert (193-196) are of more interest from the point of view of ^{32}P uptake in regenerating liver (see later section), but it is relevant to report their results for normal rat liver at this stage. In whole liver the uptake of isotope by total lipid, "cephalins" lecithins and sphingomyelins were respectively, 215, 305, 108, and 13, 1 hour after injection (194). These figures are expressed as:

$$\frac{\text{counts/min/}\mu\text{g P in fraction}}{\text{counts/min/}\mu\text{g body wt injected}} \times 10^{-1}$$

In subcellular fractions (196), figures were:

	Nuclei	Mitochondria	Microsomes	Supernatant
Lecithin	619	646	635	833
Cephalin	758	733	977	664
Sphingomyelin	90	122	114	82

As I have already pointed out, the nuclear fraction in these experiments was highly contaminated; the lipid fractions, too, were heterogeneous in the extreme.

Having described a number of experiments on the subject of ^{32}P uptake into phospholipids of liver cell nuclei, with a fair amount of agreement between the results, it is probably pertinent to mention the work of Harth et al (197)

with brain nuclei. They studied the distribution of lipid phosphorus in, and the uptake of radiophosphorus by, the various parts of the rat central nervous system. The phosphatides of the nucleus had the highest uptake of radioactivity, and the greatest difference in activity between this and other fractions was after 24 hours. Their relative values for cerebral cortex are as follows: nuclei, 67; heavy mitochondria, 16; light mitochondria, 19; endoplasmic reticulum, 15 and cytoplasmic fluid 23. This seems to be a complete reversal of the situation in liver.

With the advent of better lipid separation methods, a closer look has been taken at the behaviour of individual phospholipids. Marinetti et al (198) have used silica-impregnated paper to separate phospholipids and claim to have isolated fifteen different phosphatides from rat liver and other tissues by this means. Eighteen hours after injection of 1 mC of ^{32}P they found 1110 c/m/μgP in the total liver phospholipids. Phosphatidylcholine, phosphatidyl-ethanolamine and phosphatidylinositol were labelled most; they could find no labelled phosphatidic acid in vivo. This situation contrasts markedly with the picture in vitro (10), where they found a large incorporation into phosphatidic acids with very little labelling of lecithin or

phosphatidylethanolamine. The overall labelling *in vitro* was quite small and was dependant on a supply of metabolic energy. The figures quoted by Marinetti for liver (above) compare with other tissues as follows: intestine, 938; kidney, 938; spleen, 618; lung, 618; heart, 321; and brain, very low.

Dawson (37) used his method of mild alkaline hydrolysis of phospholipids and paper chromatography of the water-soluble phosphate esters to investigate the specific activity-time relationships of phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine and an inositol-containing phospholipid, (almost certainly phosphatidyl-inositol). At all times up to 20 hours, phosphatidyl-ethanolamine had the highest specific activity and phospha-tidylserine, the lowest. In no instance did any two curves obey the precursor-product criteria of Zilversmit *et al* (36). While Stetten (199) showed that in the intact rat, labelled serine could be decarboxylated to form ethanolamine, and that this base could then be methylated to form choline, Dawson's results indicate that these processes could not occur to any appreciable extent while these bases were combined in phospholipid molecules. In a previous study, Dawson (200) had shown

by plotting specific radioactivity-time curves for GPC and GPE (obtained in aqueous extracts of liver tissue) and for phosphatidylcholine and phosphatidylethanolamine, that neither GPC or GPE can act as a major phosphorus-containing precursor of the phospholipids; on the contrary, the curves showed that the specific radioactivities of phosphatidylcholine and phosphatidylethanolamine were consistent with the assumption that they were the precursors of GPC and GPE respectively. This suggests that *in vivo* such esters are the products of the catabolism of liver phospholipids. The striking difference between the metabolism of phosphoglycerides *in vivo* and *in vitro* has been brought out by Dawson's work as well as by that of Marinetti. Dawson was able to show a rapid synthesis of diphosphoinositide and (probably) phosphatidic acid in guinea pig brain dispersions, whereas incorporation of ^{32}P into lecithins and other cephalins was negligible (14).

Van Deenen (51), too, has investigated individual phospholipids in different cell fractions of rat liver and tumour *in vitro*. A similar ^{32}P pattern of the phosphatides from all cell fractions was found after incubation of slices with ^{32}P.

Phospholipid metabolism and turnover in regenerating liver

Polynucleotides have received by far the greatest amount of attention from the point of view of phosphorus turnover, especially in the case of regenerating liver. Phospholipids have been studied in a few cases, but only really thoroughly by Johnson and Albert (193-196).

In 1940, Marshak (201) had shown that tumour nuclei incorporated a larger proportion of the total (not lipid) ^{32}P of the tissue, than liver nuclei. By performing partial hepatectomies, he was able to show that this increase was not characteristic of the tumour *per se*, but was attributable to greater mitotic activity. Later, Smellie *et al* (192) studied the effect of partial hepatectomy on the various phosphorus compounds of rat liver. Their figures for the phospholipid fraction were:

Whole Cytoplasm		Nuclei		Mitochondria		Microsomes		Cell Sap	
R	C	R	C	R	C	R	C	R	C
402	207	370	211	366	213	422	242	396	278

Figures refer to specific activity (counts/min/100μgP) relative to the tissue inorganic P as 1000. R represents regenerating liver, C, sham-operated control. The rats were killed 26 hours after operation, 4 hours after injection of ^{32}P.

The results of Albert and Johnson are the most comprehensive in this field. They studied the phosphorus concentrations and turnover of acid-soluble, RNA, DNA, phospholipid and phosphoprotein fractions in regenerating rat liver at different times after partial hepatectomy. Later they extended their lipid studies to the behaviour of the lecithins, "cephalins", and sphingomyelins of cell fractions, and the effect on these fractions of inhibiting mitosis with colchicine (193-196).

The effect of partial hepatectomy on the phosphorus concentration of liver was to produce a rise in the inorganic phosphorus, RNA, DNA, and phospholipid fractions and no change in phosphoprotein and acid-soluble organic phosphorus. Phospholipid concentration was at a maximum one day after partial hepatectomy; colchinine seemed to produce an increase in the amount of lecithin after three days.

The effect of partial hepatectomy was to produce an immediate increase in the turnover of all phosphorus fractions; after 24 hours the ^{32}P uptake of all but the lipid fractions declined, the lipid reaching a maximum at three days after partial hepatectomy, at a time concurrent with maximum mitotic activity. Detailed figures are

reproduced in the discussion section. The effect of colchicine was to produce a 50% increase in "cephalin" turnover three days after partial hepatectomy, but no effect on any other lipids.

The uptake of ^{32}P in the lecithins of all fractions was increased 18 hours after partial hepatectomy and was highest at three days. Similarly maximum uptake occurred on the third day in the cephalins of the nucleus. In the "cephalins" elsewhere, however, ^{32}P uptake at the time of highest mitotic activity was no greater than at the time associated with premitotic processes (18 hours).

The authors speculate on the possible causes of these phenomena and how they might be correlated with the function of phospholipids in the cell. They suggest that "cephalins" of the cytoplasm are concerned in building block (?) formation during premitotic cell metabolism; the lecithins, and the cephalins of the nucleus, the authors suggest, might be concerned primarily in the formation of structural materials, e.g. lipoproteins, that are required in the formation of new cells. They also suggest that phospholipids are involved in changes in cell permeability during processes of cell division.

Aims of this work

Although, as the foregoing review has pointed out, there have been many studies of phospholipid turnover in liver fractions, there remain several gaps in our knowledge. In few cases has there been a careful study of individual phospholipids in different cell fractions, *in vivo*. For instance, although Davidson studied turnover in all cell fractions, he did not separate his lipids. Johnson and Albert studied incorporation in different cell fractions, but only studied groups of phosphatides; their nuclear fraction was rather crude. Marinetti and Dawson have studied individual lipids but not in cell fractions.

I have pointed out in Part I that I was interested in comparing the phospholipids of nuclear and microsomal fractions, since there is evidence that they may be part of the same membrane system, in which phospholipids play an important part; these considerations apply to the turnover of the phospholipids of these fractions as well as the composition. It is also of interest to see whether any large differences in turnover between individual lipids could be observed and if so, whether such differences are characteristic of any particular fraction. Johnson and Albert's results were also of interest and I thought that

that now that better techniques were becoming available for lipid separations, and by using careful techniques for the isolation of uncontaminated nuclei, new information might be obtained.

At the outset, quite severe problems were encountered. Firstly, the fact that so little lipid could be recovered from pure nuclei meant that each experiment (each point on the time curve) involved using a large number of rats, (at least 10 animals to obtain enough lipid for a hydrolysis). This in turn created problems of isotope handling: large quantities would be needed for so large a number of animals, and the radiation hazard would increase proportionately, since the injections would need to be spaced over a considerable period. After considering all aspects of the problem, I decided to use rabbits as the experimental animal until a time curve had been constructed and I had acquired sufficient experience with the methods. The rabbit, too, brought its difficulties; owing to its large size, quite a considerable amount of label had to be injected in order to recover a reasonable amount of counts from the small quantities of nuclear lipid. Such quantities provided comparatively little radiation hazard, however; the isotope could be injected quickly and radiation to the

hands during injection was reduced by keeping the syringe in a perspex radiation shield. As pointed out in an earlier section, the radiation effects to the animal's metabolism can be serious if large amounts of isotope are injected; this problem is treated further in the discussion section.

Regenerating liver studies

I have already discussed my reasons for repeating the work of Johnson and Albert. The technique of partial hepatectomy is described in the section on methods. Experiments using this technique are beset with difficulties and it is easy to introduce variations large enough to make the interpretation of results somewhat hazardous. These and other difficulties are treated in the discussion of results.

METHODS

1. Injection of $NaH_2{}^{32}PO_4$

The isotope was purchased as carrier-free inorganic orthophosphate, $NaH_2{}^{32}PO_4$ in HCl solution at pH 2-3, from the Radiochemical Centre, Amersham. This was neutralised with sodium bicarbonate and made isotonic by the addition of the calculated quantity of sodium chloride. The injections were made in several ways.

Rats were injected either,

(i) intraperitoneally,

or (ii) via the tail vein; the animals were lightly anaesthetised in an ether chamber and the tail washed in warm water. The tail was then dried and rubbed with xylene. This caused the vein to stand out very clearly, facilitating location with the syringe needle. By withdrawing the plunger after insertion of the needle, thus drawing blood into the syringe, location of the vein could be confirmed. Rats were injected with quantities up to 1 mC.

Rabbits were injected either,

(i) intraperitoneally,

or (ii) via the ear vein; the ear was rubbed with

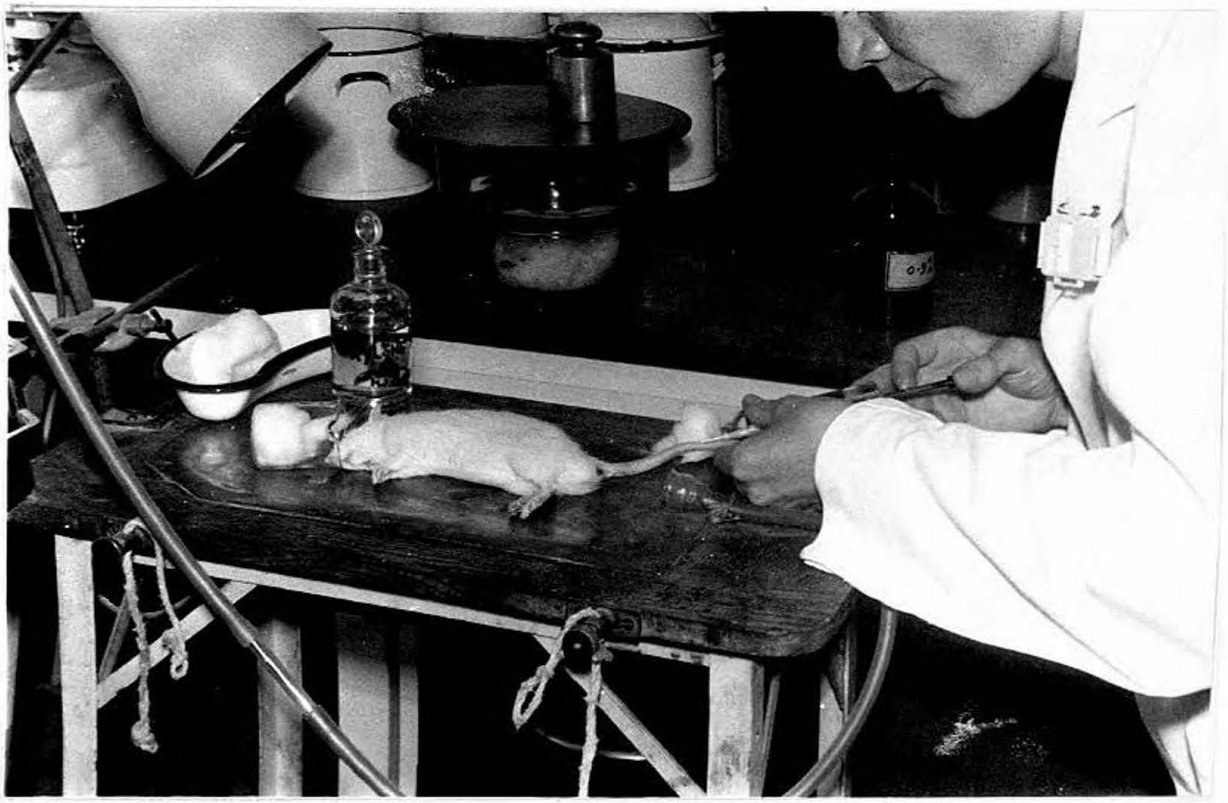


Plate I.

Intravenous Injection.

xylene to cause the vein to stand out. Rabbits were given doses between 1 and 3 mC.

For the injection of such large doses the syringe was protected by a shield of clear perspex through which the graduations could be read quite easily.

2. Assay of samples labelled with ^{32}P

Samples were assayed for radioactivity in a 10 ml Geiger-Muller liquid counter coupled with the Echo Scaler. Three counts of 100 sec. were made for each sample and the mean taken. The efficiency of the counter was about 8%.

3. Radio-autography of chromatograms

Radio-autograms were prepared from chromatograms and thin layer plates of labelled compounds, by exposing to Kodak "Blue Brand" X-ray film enclosed in X-ray cassettes for several days.

4. The expression of results: definitions of specific activity and relative specific activity.

Specific activity is defined in this work as:

counts per 100 seconds per microgram phosphorus.

Relative specific activity. In all experiments involving the incorporation of ^{32}P, the specific activity of the lipid of lipid hydrolysis product was related to

Plate II.

Counting Apparatus for ^{32}P.

the specific activity of the acid-soluble phosphorus fraction to allow for differences in labelling of the phosphate pool from animal to animal. (See discussion of Part II) The tissue or cell fraction was treated with 10% TCA before lipid extraction (see Part I) and after centrifuging, the filtered supernatant was taken as the acid-soluble fraction.

Therefore,

$$\text{relative specific activity} = \frac{\text{counts/100s/}\mu\text{g P in lipid fraction}}{\text{counts/100s/}\mu\text{g P in acid-soluble fraction}} \times 1000$$

Corrections

Dead time of the counter

The 'dead-time' of the counter was 400 μs per count. The error is thus approximately 5% at 12,000 counts per 100s. and corrections were made to all counts over 12,000 per 100s.

If C_o is the real count,

and, C_r is the recorded count,

$$C_o = \frac{Cr}{100 - (\frac{4.Cr}{10^4})} \times 100$$

Decay

The half-life of ^{32}P is 14.3 days; the date of injection of the isotope was always recorded, and all figures were corrected to this date.

5. The technique of partial hepatectomy*

The method is basically that of Higgins and Anderson (202). Animals used were albino males of the university strain weighing approximately 250g. They were kept on the normal laboratory diet before and after the operation. All operations were performed between the hours of 8.30 and 11.00 a.m.

The animal was placed in the ether chamber until it became unconscious. One minute later it was taken out and placed on the operating table. While the animal is in the ether chamber, three definite stages of breathing can be seen. The initial normal breathing becomes rapid and after a while this rapidity subsides and the breathing becomes slow and regular. When on the table, the animal should be relaxed before operation is attempted. After attachment of ties to the paws to maintain the animal in a suitable position an incision is made in the fur and the fur cut for about 1" - 1½" over the liver. The peltwas pushed aside to clear the abdominal wall. The wall was then cut as far as the xiphisternal cartilage. By pressure of the fingers on the body to either side of the incision, the right and left central lobes, (1 single and 1 double) could be squeezed out in turn, ligatured with stout thread,

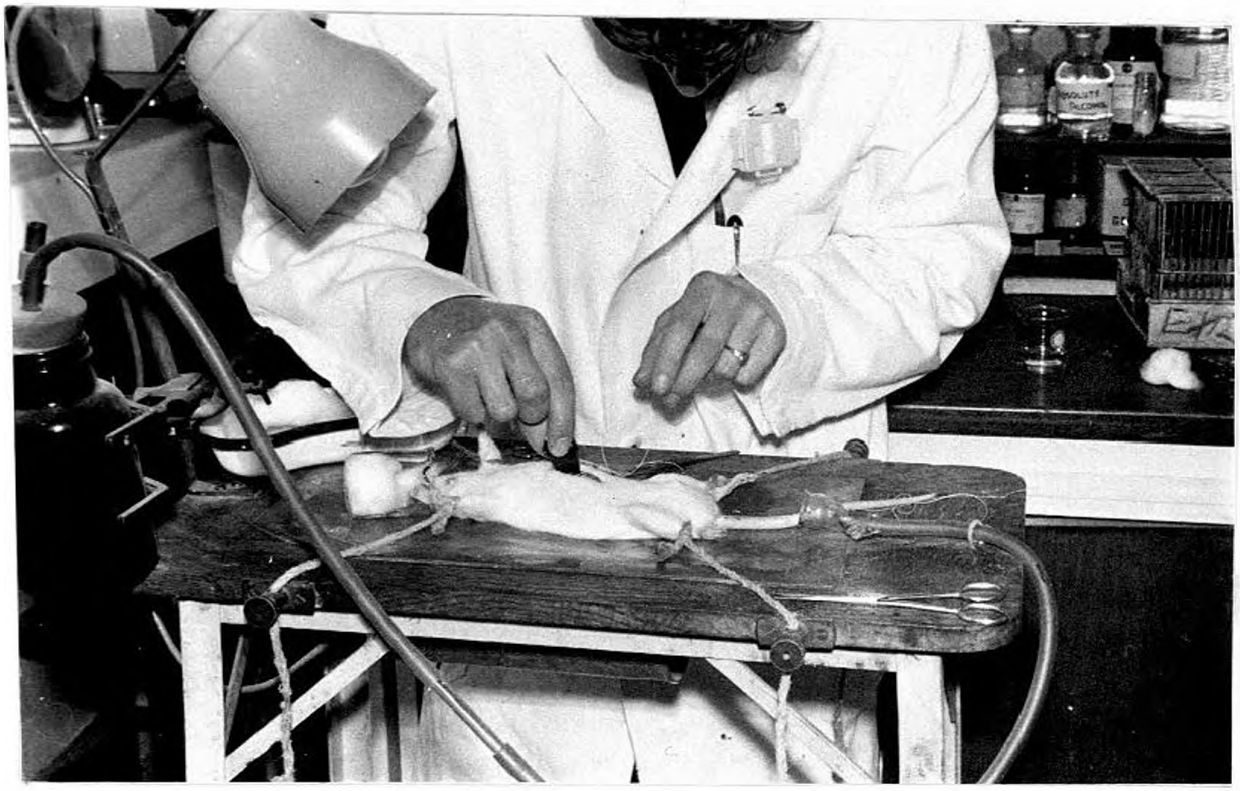


Plate III.

Partial Hepatectomy.

and as much of the lobe as possible removed with sharp scissors. The peritoneum and skin were then sewn separately. The animal was ideally maintained just unconscious by careful application of ether anaesthetic and should have regained consciousness at the time stitching was completed.

As controls, animals were sham-operated by making the incision and disturbing the liver, without removing any part of it.

* Footnote:

I acknowledge the assistance of Dr. P.J. O'Brien and Mr. R. Jones.

EXPERIMENTS AND RESULTS

1. ^{32}P incorporation into the phospholipids of homogenate and nuclei of rabbit liver at different times in vivo

Adult rabbits of either sex, weighing approximately 2 kg. were injected with 3 mC (^{32}P)-inorganic orthophosphate. Injections, the isolation of nuclei and the extraction of lipid and acid-soluble fractions are described in the "Methods" sections of Parts I and II. The results, expressed as the specific activities of the lipid fractions relative to the cellular acid-soluble fraction are recorded in Table I and shown graphically in figure 1. Relative specific activities were determined at 3, 6 and 9 hour exchange periods. The results are the mean for 2-4 animals. Full details of the phosphorus recoveries, counts recorded, and specific activities in each individual experiment are recorded in the appendix. (pp.254-258).

TABLE I

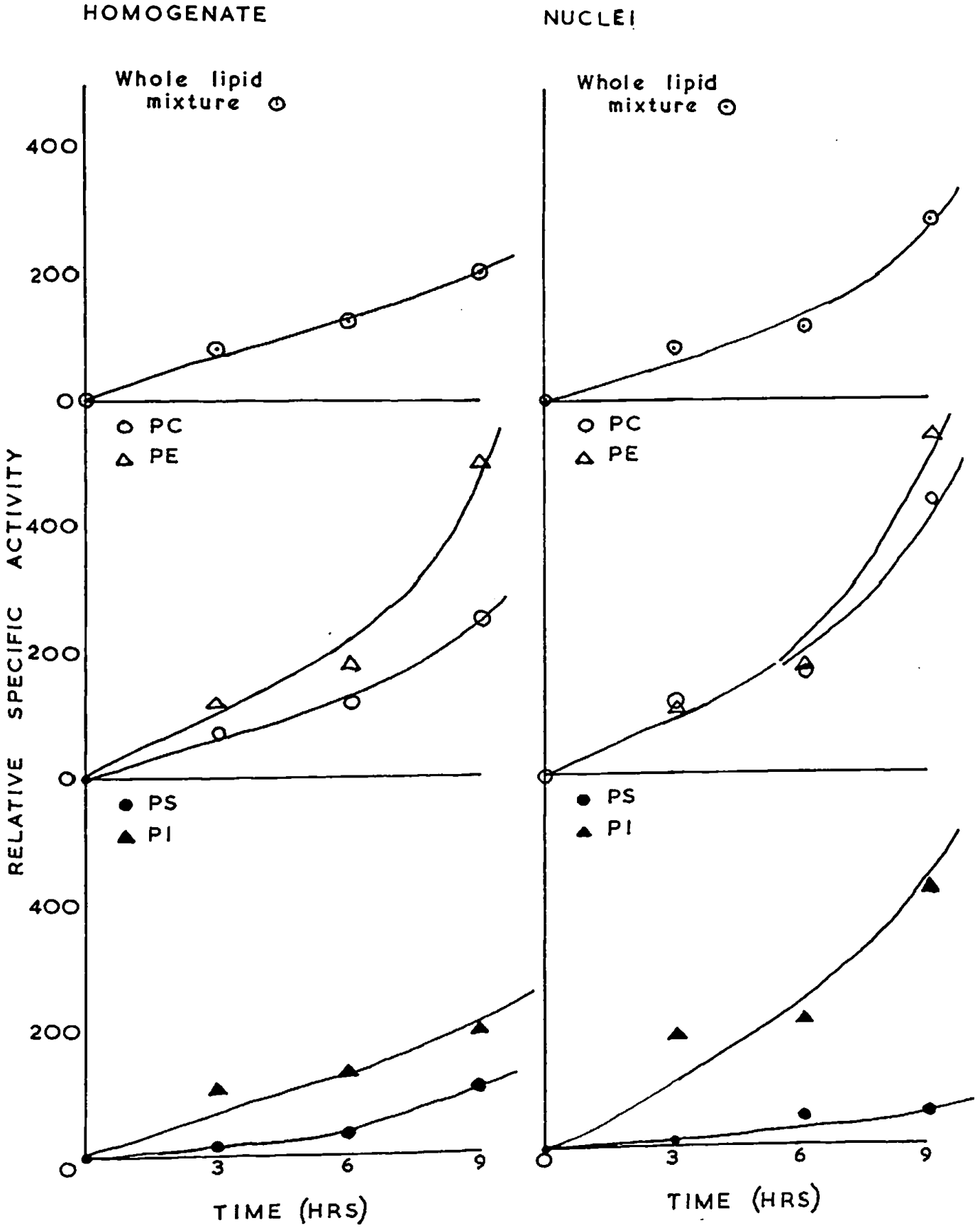
Relative specific activities of phospholipids of rabbit liver

Homogenate and Nuclei

HOMOGENATE				NUCLEI			
LIPID	EXCHANGE PERIOD (hours)			LIPID	EXCHANGE PERIOD (hours)		
-	3	6	9	-	3	6	9
Whole lipid mixture	83	126,120	206	Whole lipid mixture	84	95,144	296
PC	71	99,136	249	PC	118	156,182	446
PE	118	228,125	500	PE	115	188,153	550
PS	21	46,25	110	PS	12	57,520	59
PI	108	140,121	198	PI	183	206,212	422
PA				PA			
CL				CL			

FIGURE 1

The incorporation of ^{32}P into the phospholipids of whole rabbit liver and nuclei at different times *in vivo*.


HOMOGENATE
NUCLEI
Whole lipid mixture
Whole lipid mixture
400
200
0
PC
PE
400
200
0
PS
PI
400
200
0
3
6
9
RELATIVE SPECIFIC ACTIVITY
TIME (HRS)
TIME (HRS)

2. ^{32}P incorporation into the phospholipids of the mitochondrial and microsomal fractions of rat liver at different times 'in vivo'

Adult male albino rats* weighing approximately 250 g. were lightly anaesthetised with ether and injected with 0.25, 0.50, 0.75, or 1.0 mC of (^{32}P)-inorganic orthophosphate. Injections, the isolation of cell fractions, and the extraction of lipid and acid-soluble fractions are described in the "Methods" sections of Parts I and II. The results, expressed as the specific activities of the phospholipids of each cell fraction, relative to the acid-soluble phosphorus of the respective cell fraction are recorded in Table II and graphically in figure 2. Relative specific activities were determined at 3, 6, 9, 12 and 19 hours exchange periods. Full details of the phosphorus recoveries, counts recorded and specific activities in each individual experiment are recorded in the appendix. (pp.259-267).

* Footnote:

All rats were male albinos of the department strain weighing approximately 250 g. and fed on the standard laboratory diet up to the time of death.

TABLE II

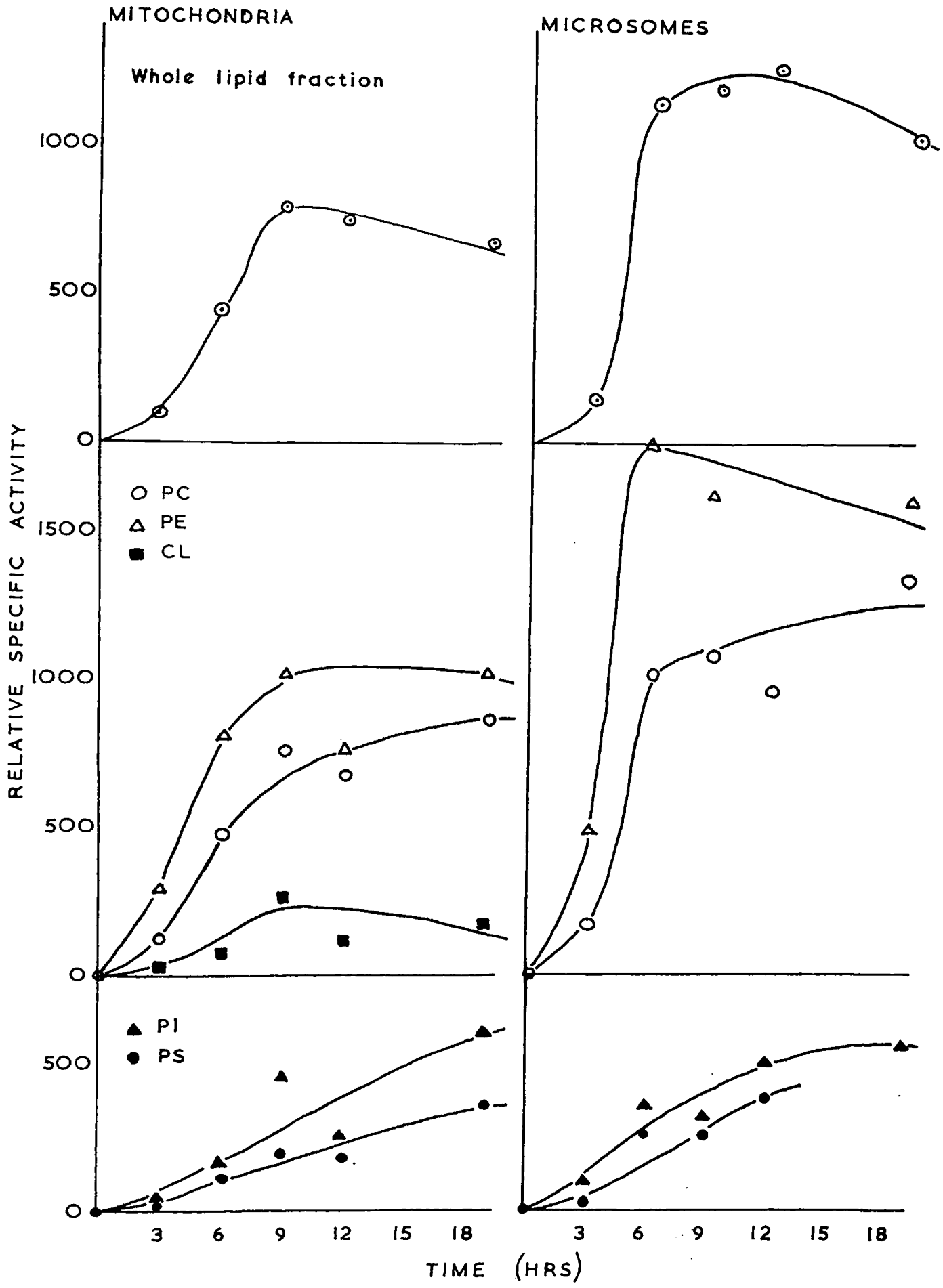
Relative specific activities of phospholipids of rat liver mitochondria and microsomal fractions

LIPID	MITOCHONDRIAL FRACTION					MICROSOMAL FRACTION				
	EXCHANGE PERIOD (hours)					EXCHANGE PERIOD (hours)				
	3	6	9	12	19	3	6	9	12	19
Whole Lipid Mixture	102	281 608	733 865	744	672 680	141	489 1820	1250 1160	1275	1090 980
	(102)	(445)	(799)	(744)	(676)	(141)	(1155)	(1205)	(1275)	(1035)
PC	123 121	288 266 666 684	663 665 828 880	687 658	818 900	177 183	528 480 1510 1520	1000 1025 1120 1115	949 920	1335 1345
	(122)	(476)	(759)	(673)	(859)	(180)	(1010)	(1065)	(935)	(1340)
PE	288 273	506 500 1095 1140	1022 945 1100 990	718 800	1000 1042	492 479	1161 1000 2500 2550	1780 1840 1430 1410	930 960	1540 1650
	(281)	(810)	(1014)	(759)	(1021)	(486)	(1803)	(1615)	(945)	(1595)
PS	15	92 140	194	176	360	24	164 347	233	384	905 1050
	(15)	(116)	(194)	(176)	(360)	(24)	(256)	(233)	(384)	(978)
PI	43	55 279	462	248	602	84	270 432	303	491	467 645
	(43)	(167)	(462)	(248)	(602)	(84)	(351)	(303)	(491)	(556)
PA	>280	>1620	>296 >257	>1240		>204	>211	>990 >1740 >780	>1450	>1200
CL	33	76	105 429	116	170					
	(33)	(76)	(267)	(116)	(170)					

Mean values in brackets.

FIGURE 2

The incorporation of ^{32}P into the phospholipids of rat liver mitochondrial and microsomal fractions at different times *in vivo*.


MITOCHONDRIA
Whole lipid fraction
MICROSOMES
RELATIVE SPECIFIC ACTIVITY
1000
500
0
1500
1000
500
0
500
0
PC
PE
CL
PI
PS
3
6
9
12
15
18
TIME (HRS)

3. Experiments to study the possible "in vitro" incorporation of ^{32}P into phospholipids of cell fractions during the fractionation of the tissue in the course of an "in vivo" experiment

It has been suggested (203) that cell fractions, mitochondria and microsomes, incorporate ^{32}P *in vitro* during their isolation by differential centrifugation after an *in vivo* experiment. Since the isolation medium (0.3 M sucrose) contains EDTA, it is unlikely that any enzyme system performing the *in vitro* synthesis of phospholipids would be very efficient under these conditions but this experiment was performed as a check.

Two rats were injected simultaneously with 0.5 mC of (^{32}P)-inorganic phosphate and killed simultaneously five hours later. The killing and all subsequent operations were performed in the cold room at 0° - 4°. Immediately on removal of the livers, a portion of each (approximately 10%) was homogenised in 20 ml 10% TCA. This was taken for the "zero-time" results. The rest of the liver was homogenised in 160 ml 0.3 M sucrose-0.002M EDTA, pH 7.4. Twenty-millilitre portions of this homogenate were allowed to stand and 20 ml of 10% TCA was added to each tube after periods of $\frac{1}{2}$, 1, $1\frac{1}{2}$, and 2 hours. The remainder of the

homogenate was used for the isolation of cell fractions and after various times these were also homogenised in TCA. Specific activities were determined on the lipid and acid soluble phosphorus of each fraction. The figures from two such experiments are recorded in Table III and plotted graphically in figure 3.

TABLE III

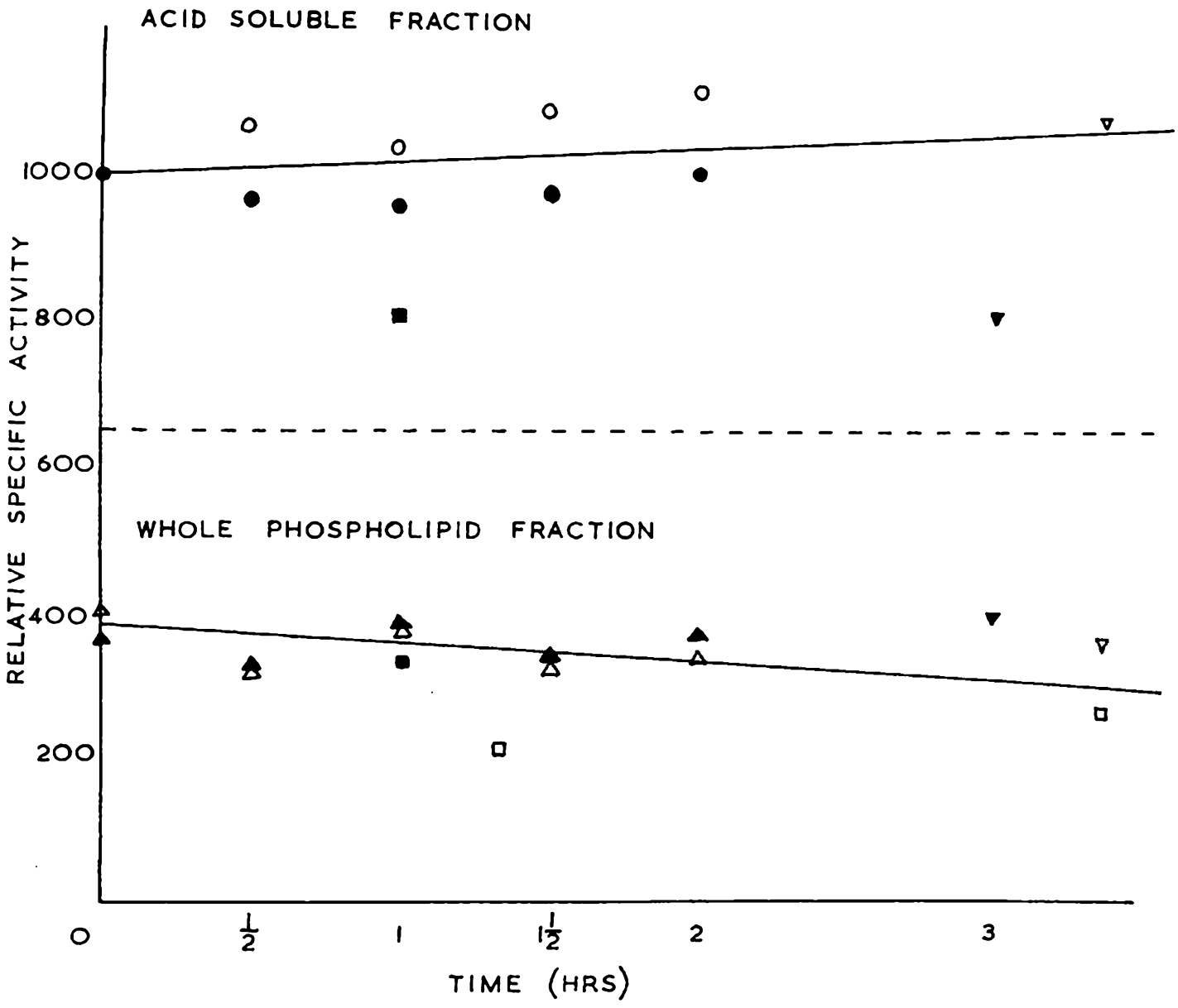
Investigation of the "in vitro" effect during preparation of cell fractions

EXPERIMENT 1			EXPERIMENT 2		
Fraction	S.A.	R.S.A.	Fraction	S.A.	R.S.A.
Acid-soluble			Acid-soluble		
0.	1070	1000	0.	917	1000
½ h.	1150	1070	½ h.	890	970
1 h.	1120	1040	1 h.	877	960
1½ h.	1170	1090	1½ h.	900	980
2 h.	1200	1120	2 h.	925	1010
mitochondria			mitochondria		
1. (1.3 h.)	2370	2210	(1 h.)	737	810
2. (3.4 h.)	1395	1300			
microsomes			microsomes		
(3.4 h.)	1157	1080	(2 h.)	735	807 ?
Phospholipid			Phospholipid		
0.			0.		
whole lipid	429	400	whole lipid	334	364
			PC	240	262
			PE	485	529
			PS	25	27
			PI	102	111
			CL	31	34
½ h.			½ h.		
whole lipid	371	322	whole lipid	288	323

TABLE III (Continued)

EXPERIMENT 1			EXPERIMENT 2		
Fraction	S.A.	R.S.A.	Fraction	S.A.	R.S.A.
Phospholipid			Phospholipid		
1 h.			1 h.		
whole lipid	415	370	whole lipid	252	388
			PC	193	211
			PE	400	436
			PS	29	32
1½ h.			1½ h.		
whole lipid	370	316	whole lipid	306	340
2 h.			2 h.		
whole lipid	403	336	whole lipid	342	370
			PC	197	215
			PE	411	449
			PS	27	29
			PI	143	156
mitochondria			mitochondria		
whole lipid			whole lipid		
1. (1.3 h.)	495	209	(1 h.)	246	335
2. (3.4 h.)	360	258	PC	182	198
			PE	402	439
			PS	52	57
			PI	81	88
			CL	43	47
microsomes			microsomes		
(3.4 h.)			(3 h.)		
whole lipid	409	354	whole lipid	280	390
			PC	201	219
			PE	475	519
			PS	22	24

Fig 3 Investigation of 'in-vitro' incorporation during preparation of cell fractions



LEGEND

- ○ Acid-soluble: experiment 1
- ● Acid-soluble: experiment 2
- △ Phospholipid: experiment 1
- ▲ Phospholipid: experiment 2
- } homogenate
- □ Mitochondria: experiment 1
- ■ Mitochondria: experiment 2
- ▽ Microsomes: experiment 1
- ▼ Microsomes: experiment 2

4. ^{32}P incorporation into phospholipids of nuclear, mitochondrial, and microsomal fractions of normal and regenerating rat liver in vivo

For this series of experiments, a six-hour exchange period was chosen, since this time interval was on the ascending limb of the specific activity-time curve, and represented a large incorporation of radioactivity into each fraction.

Two sets of controls were used; firstly, normal, unoperated rats, and secondly sham-operated rats. The technique of partial hepatectomy has been described in the "Methods" section. The animals were killed 36 hours after the operation, because the evidence of several investigators is that this is the time when the greatest number of cells are in mitosis (148,202,204). (See discussion)

The results are recorded in Table IV and displayed in the form of a histogram in figure 4. The results for the mitochondrial and microsomal fractions are the mean of figures from six animals. In the case of nuclei, only non-operated controls were used; the results are the mean from ten animals. The results for regenerating liver are the mean of the figures from twenty operated animals.

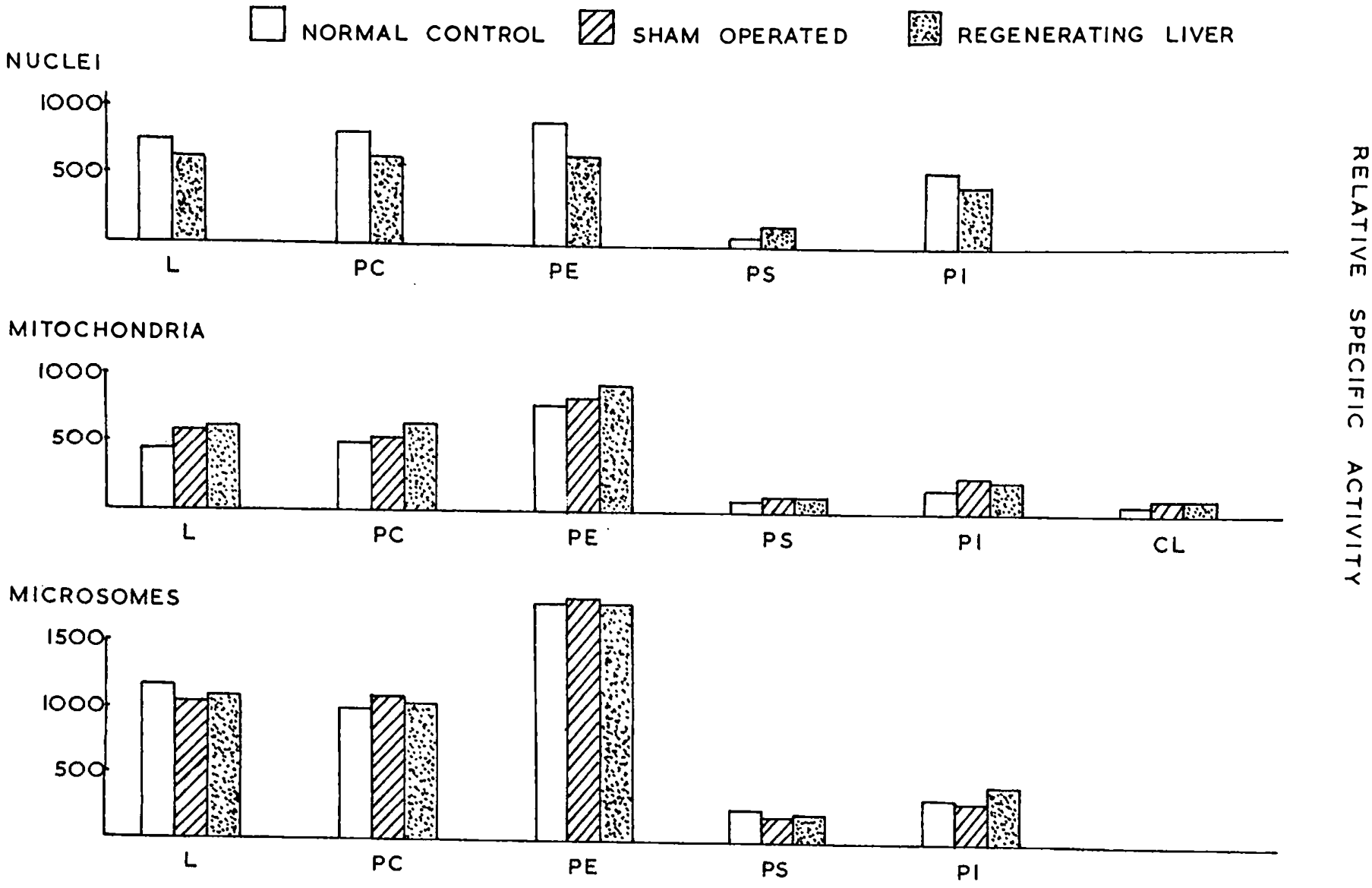
Full details of phosphorus recoveries, counts recorded, and specific activities in each individual experiment are recorded in the appendix (pp.268-281).

TABLE IV

Relative specific activities of phospholipids of cell fractions of normal, sham-operated and regenerating rat liver

	NUCLEAR FRACTION		MITOCHONDRIAL FRACTION			MICROSOMAL FRACTION		
	N O R M A L C O N T R O L S							
lipid	Mean RSA	No. of values	Mean RSA	No. of values		Mean RSA	No. of values	
whole lipid	770	)Results	445	2	281-608	1155	2	489-1820
PC	839	)from	476	4	266-684	1010	4	480-1520
PE	890	)10	810	4	500-1140	1803	4	1000-2550
PS	54	)animals	116	2	92-140	256	2	164-347
PI	546	)(pooled)	167	2	55-279	351	2	270-432
CL	-		76	1		-		
	S H A M - O P E R A T E D C O N T R O L S							
whole lipid			587	3	486-775	1067	3	842-1785
PC			541	6	419-642	1079	6	729-1675
PE			870	6	781-928	1852	6	1440-2590
PS			116	3	97-133	168	3	76-290
PI			266	2	249-282	308	3	181-482
CL			118	1		-		
	R E G E N E R A T I N G L I V E R							
whole lipid	612	)Results	627	5	474-895	1072	5	644-1865
PC	623	)from	652	10	446-1120	1036	10	710-1850
PE	653	)19	975	10	555-1930	1810	9	1020-3060
PS	144	)animals	137	5	71-341	218	6	114-418
PI	466	)(pooled)	271	6	219-416	454	6	296-980
CL	-		131	4	53-282	-		

Fig 4 ^{32}P incorporation into phospholipids of cell fractions of normal, sham-operated and regenerating livers



5. ^{32}P incorporation into phospholipids of whole rat liver "in vivo", using an exchange period of 30 minutes

The purpose of this experiment was to compare the metabolic activities of each individual phospholipid of whole rat liver with those in the intestinal mucosa (see Part III) after an exchange period of only 30 minutes.

The livers were removed 30 minutes after intraperitoneal injection of 0.25 mC (^{32}P)-inorganic orthophosphate and immediately homogenised in 200 ml 10% TCA. Lipid and acid-soluble fractions were extracted as described previously. Both rats which had been starved for 20 hours previous to death and which had been fed normally were used. No significant difference in turnover of the lipids was observed between these groups. The mean values for three animals are compared in Table V with the figures for intestinal mucosa.

TABLE V

Relative specific activities of rat liver and intestinal phospholipids after 30 minutes exchange period

Phospholipid	Liver	Intestinal Mucosa
whole lipid mixture	36	17
phosphatidylcholine	24	8
phosphatidylethanolamine	30	8
phosphatidylserine	-	5
phosphatidylinositol	40	73
phosphatidic acid	>1270	143
cardiolipin	10	11

6. The turnovers of different glycerophosphatides in other rat tissues

During the course of the work on the incorporation of ^{32}P into phospholipids of rat liver cell fractions, some other tissues were investigated. Unfortunately the exchange periods were different and figures are therefore not readily comparable, but they are included here for the sake of completeness.

(i) Phospholipids of rat heart muscle

The alkali-labile phospholipid composition of rat heart muscle has been given in Part I. The relative specific activity of the whole phospholipid fraction was measured at a 12 hour exchange period; this was 73. The specific activities of the individual lipids were not measured, but radioautograms were prepared from the chromatograms after 17 days exposure to the film. GPC and GPE were highly labelled. No label could be detected in GPS or GPGPG (in spite of the relatively large amount of phosphorus in this latter compound). Small amounts of activity could be detected in spots corresponding to GP and GPI. Judging from the radioautograms, it is probable that the order of specific activities is:

$$\text{GPE} \succ \text{GPC} \succ \begin{cases}\text{GPI}\\ \text{GP}\end{cases} \succ \begin{cases}\text{GPS}\\ \text{GPGPG}\end{cases}$$

(ii) Phospholipids of rat pancreas

The pancreas was dissected from two rats, 20 hours after intravenous injection of 0.5 mC of (^{32}P)-inorganic phosphate. Extracts from the glands were pooled. The counts in the acid-soluble fraction were not recorded; specific activities are therefore related to the specific activity of the whole lipid mixture at 1.0 (Table VI). The phosphorus composition has been recorded in Part I.

TABLE VI

Specific activities of the phospholipids of rat pancreas

Lipid	Phosphorus μg	Specific activity	Specific activity relative to whole lipid mixture
whole lipid	356	26	1.0
PC	40.6	40	1.51
PE	9.1	21	0.81
PS	4.9	5	0.20
PI	5.7	45	1.70
PA	trace <1.0	>54	>2.06
CL	3.6	3	0.11

Errors involved in radioisotope experiments

The errors expected in results from *in vivo* radioisotope experiments will be compounded from a number of errors of different kinds.

1. Statistical errors due to biological variation

Proper allowance for errors of this type can only be made by using an extremely large number of experimental animals. It is realised that the number of animals in each group in these experiments is far from adequate for a proper assessment of the degree of significance of the results.

2. Errors in counting due to the random nature of radioactive decay

The probable error in counts associated with a total number of counts in a given period is given by the equation:

$$E_n = 0.67\sqrt{N}$$

where N is the total number of counts observed.

3. Error in analysis of phosphate

The probable error in the phosphate determination is $\pm$ 5%. Much of this is probably due to a sampling error and since most of the determination in this work was done on paper spots from chromatograms, the error may well be

less due to elimination of the sampling error.

A typical background count during this work was 30 counts/100s.

Therefore:

$$E_{bg} = 0.67 \sqrt{30}$$
$$= 3.7 \ (12.2\%)$$

The error in background count is therefore:

$$30 \pm 3.7$$

Low activity samples

For low activity samples such as phosphatidylserine, a typical count was 150 counts/100s.

$$E = 0.67 \sqrt{150}$$
$$= 8.2 \ (5.5\%)$$

$$\text{Net count} = 120 \pm \sqrt{(8.2)^2 + (3.7)^2}$$
$$= 120 \pm 9 \ (7.5\%)$$

In cases where the count was as low as 50 counts/100s:

$$\text{Net count} = 20 \pm \sqrt{(4.7)^2 + (3.7)^2}$$
$$= 20 \pm 5.8 \ (29\%)$$

High activity samples

For high activity samples such as phosphatidylethanolamine, a typical count was 5,000 counts/100s.

$$E = 0.67 \sqrt{5,000}$$
$$= 47.5 \ (0.95\%)$$

Probable errors in specific activity:

$$E_{50} = \sqrt{(29)^2 + (5)^2}$$

$$= \pm 29.5\%$$

$$E_{150} = \sqrt{(7.5)^2 + (5)^2}$$

$$= \pm 9\%$$

$$E_{5000} = \sqrt{(1)^2 + (5)^2}$$

$$= \pm 5.1\%$$

DISCUSSION

Summary of the results

A study of the uptake of ^{32}P by the phospholipids of whole rabbit liver *in vivo* showed a steady increase in relative specific activity during the nine hours after injection of the isotope; the relative specific activity had not reached a peak during this time period. The uptake in nuclei was very slightly, but not significantly, higher than in whole liver. In each case the relative specific activities were in the order phosphatidylethanolamine > phosphatidylcholine > phosphatidylinositol > phosphatidylserine at all time intervals. The increased uptake into nuclear lipids as compared with homogenate was slightly more marked in the case of phosphatidylinositol.

The phospholipids of rat liver mitochondrial and microsomal fractions incorporated radioactivity rapidly in the first nine hours after injection, reaching a peak between 9 and 12 hours. After 12 hours, the specific activity compared with that of the acid-soluble fraction tailed off extremely slowly. The major contributor to radioactive uptake in each fraction was phosphatidyl-

ethanolamine. Phosphatidylcholine also had a large and rapid uptake but reached its peak somewhat later than phosphatidylethanolamine. The incorporation of isotope by phosphatidylinositol was considerably lower in each fraction and the labelling of phosphatidylserine was very low; the relative specific activity of these lipids, however, continued to rise after that of the major phospholipids had reached a peak. The labelling of the mitochondrial cardiolipin was extremely poor and comparable with that of phosphatidylserine. The relative specific activities of the whole phospholipid mixture, phosphatidylcholine and phosphatidylethanolamine of the microsomal fraction exceeded the corresponding figures for the mitochondrial fraction by 50-100%, whereas the labelling of phosphatidylserine and phosphatidylinositol was similar in both fractions.

A comparison of the ^{32}P uptake into phospholipids of rat liver when extractions were made quickly after the death of the animal, with the uptake when the homogenates had been allowed to "stand" for different periods, revealed little <u>in vitro</u> labelling of homogenates on "standing" or of cell fractions during centrifugation; hence values recorded are true <u>in vivo</u> values.

A comparison of the radioactivity incorporated by phospholipids of rat liver nuclei, mitochondria and microsomes after a 6-hour exchange period shows microsomes to have the highest labelling and mitochondria the lowest, with nuclei intermediate. Due to the degree of variation, however, differences between cell fractions are not very striking. The relative specific activity of the phosphatidyl-ethanolamine of the nuclear fraction is not so much greater than that of the phosphatidylcholine, as in the microsomal fraction.

Difficulties in measuring the phosphorus content of the phosphatidic acid (or more correctly glycerophosphate) on chromatograms means that specific activities of the lipid were always uncertain and have been expressed in each case as $> x$. In general, phosphatidic acid turnover is not much different from those of phosphatidylcholine and phosphatidylethanolamine at times after 3 hours, but at short times its turnover greatly exceeds all other lipids.

Figures for phospholipid turnover in liver at a 30 minute exchange period make interesting comparison with those for intestinal mucosa. The pattern is similar, but liver lipids seem to have a slightly higher turnover as measured at this time interval. The predominance of

phosphatidic acid and phosphatidylinositol labelling at this time is noteworthy.

The phospholipids of rat heart muscle had a much lower turnover than liver lipids after a 12-hour exchange period, (7%), but the order of labelling of the lipids was the same. In the case of rat pancreas (20 hours exchange) phosphatidylinositol and phosphatidic acid had rather greater specific activities than the other lipids.

The results from regenerating livers and sham-operated animals show very little difference from those of normal animals under the conditions chosen for the experiments.

Phospholipid turnover in normal liver: comparison of results with those of other investigators

A detailed survey of the relevant results of others in this field has been presented in the introduction to this chapter. My results differ essentially from those of Hevesy (186a) and Ada (190), for rat and rabbit liver nuclei respectively, who found that the turnover of nuclear phospholipids is significantly less than that of whole tissue. The figures of Barnum et al (191) (see page 155) for mouse liver are comparable to mine, bearing in mind that the results are related to inorganic phosphorus and not total acid-soluble phosphorus. Unlike Barnum (191)

or Davidson (141) I have been unable to show a significantly different turnover of the lipids of fed and fasted rats. The turnover of phospholipids in rabbit liver is rather lower than in the rat; this is in agreement with the findings of Hevesy (186b).

Smellie et al (192) also found the degree of labelling of phospholipids of the different cell fractions to be in the order: microsomes > nuclei > mitochondria, but the absolute figures are difficult to compare since they are related to blood inorganic phosphorus. Levin, Johnson and Albert (196) found that for "cephalins", the order of labelling in the cell fractions was: microsomes > nuclei > mitochondria, and for lecithins, mitochondria > microsomes > nuclei. In the case of lecithins, however, there was very little difference between the cell fractions. Care must be exercised in the interpretation of these data, since the order depends on the way in which the results are expressed. Johnson et al (193) used the method of concentration coefficients, where:

$$\text{concentration coefficient} = \frac{\text{counts/min/}\mu\text{g P of fraction}}{\text{counts/min injected/}\mu\text{g body weight}} \times 10^{-1}$$

If relative specific activities are computed from their data, relating lipid specific activity to that of the

acid-soluble organic phosphorus fraction, then the order becomes microsomes > mitochondria > nuclei for both types of lipid.

Quite a different picture is seen in brain. The results of Harth et al (197) show an incorporation into nuclei four times greater than into mitochondria, with microsomes lower still.

As far as the specific radioactivity of individual phospholipids is concerned, my results are in agreement with those of Dawson (37) for whole liver, where he found phosphatidylethanolamine to have the highest specific activity at all times up to 20 hours, with lecithin > phosphatidylinositol > phosphatidylserine. Marinetti et al (198) also found lecithin and phosphatidylethanolamine to be most highly labelled after 18 hours exchange, but could find no labelling at all in phosphatidic acid in vivo. According to these workers, liver phospholipids had a slightly higher incorporation than those of intestine, and lipids in both these tissues were more highly labelled than in heart. My own results seem to be in qualitative agreement with these, but the fact that measurements were made at different times makes exact comparison impossible. Johnson and Albert's (196) results are in line with the

general finding that the "cephalin" fraction incorporates radiophosphorus faster than the lecithin fraction.

We are left with the general picture, then, that the phospholipid specific activity in all cell fractions of rat liver, at all times up to 20 hours after administration of the isotope, is of the same order, with microsomes having rather higher activity than the other fractions. Of the individual lipids, phosphatidylethanolamine almost always has a higher incorporation than phosphatidylcholine; phosphatidylinositol has a rather lower turnover than these two phospholipids at times after 3 hours, but at short time intervals its turnover is higher. Phosphatidylserine always has the lowest turnover, but the mitochondrial lipid, cardiolipin, also has a low incorporation, of the same order as phosphatidylserine. Phosphatidic acid, like the inositide, has an activity not very much different from lecithin and phosphatidylethanolamine at times after 3 hours, but at short times its turnover is significantly higher. This pattern is the same for all cell fractions studied.

Van Deenen (51) has remarked on the similarity of the phospholipid labelling pattern in all cell fractions of liver in vitro.

Discussion of some factors influencing liver phospholipid turnover in vivo

To gain a proper understanding of the metabolism of important biochemical compounds, results from both *in vivo* and *in vitro* experiments should be compared. Each type of experiment has its own particular advantages and difficulties. Whereas the *in vitro* system has the advantage of simplicity and is not subject to the influence of indirect factors, its simplicity is in a sense its drawback, since one can never get a picture of what really takes place in the living body. From the outset, the *in vivo* experiment is subject to a great many disturbing factors which should be critically considered before assessing results. The very act of injecting or handling the animal may, under certain circumstances, affect the balance of the animal's metabolism. If the animal is 'calmed' by anaesthesia before even so simple an operation as an injection, the effects due to shock will be lessened, but the anaesthetic itself may have a disturbing effect on the processes to be investigated.

Also to be considered in experiments involving the use of radioisotopes, are the possible harmful effects of radiation, the errors involved in counting radioactivity, and not least, the factor of biological variation.

Administration of the isotope

Many routes of administration have been described. The intravenous route provides the most rapid means of distribution of labelled phosphate to most body tissues. Its disadvantages lie in the greater skill required for the injection and handling of the animal. For the rat, the tail vein is a convenient site, being fairly large and accessible.

The intraperitoneal or intramuscular routes may be somewhat slower but seem to have no major disadvantages. If the amount of phosphorus injected is rather small, it passes through the intraperitoneal cavity rather slowly and it has been suggested that the intramuscular route is more direct; injection by this route can sometimes reduce the wide variation in specific activity of liver inorganic phosphate from animal to animal (148). This variation is often greater than would be expected if the size of the inorganic phosphate pool were the only factor.

Effect of anaesthesia

For the intravenous, and even intraperitoneal, injection of radioisotopes into the rat, light ether anaesthesia was found desirable. There is a certain amount of evidence to show that ether may affect the general

metabolism of the animal and hence there may be a real danger that the measured phospholipid activities are not those of the normal animal.

There is no firm evidence about the mechanism of ether anaesthesia. Among the many theories (205), the Meyer-Overton, or "lipid" theory of anaesthesia holds that there is direct parallelism between the affinity of the anaesthetic for lipid and its depressant action. The substance is thought to gain access to nerve tissue through the membrane by virtue of its lipid solubility and cause changes in membrane permeability and polarisation. Such changes would bring about energy changes which might depress the reception or discharge of impulses. Ether has a large affinity for lipid and therefore (according to the theory) is a good anaesthetic. Thus, if the theory is true, ether might be expected to interfere with lipid metabolism to some degree.

Some of the metabolic effects of ether are briefly: a rise in the blood sugar, coincident fall in liver glycogen, and a degree of acidosis. The fatty degeneration of liver associated with chloroform does not seem to occur with ether, (205). According to Goodman and Gilman (205), even when ether is employed for deep surgical anaesthesia

for several hours, it seems that hepatic disturbance is mild and transient, and there is no cytological liver damage. Such small effects as there are, would seem to be not directly attributable to ether, but are more likely due to post-operative shock. Ether is not oxidised or hydrolysed in the body and over 90% of the dose is recoverable in exhaled air.

Views are at variance, however. Ansell and Dohmen (206) found a large decrease in phospholipid synthesis in rat brain during thiopentone anaesthesia. Barbiturates are known to uncouple oxidative phosphorylation in nervous tissue in vitro and these authors think that anaesthetics in general might have this property. Hulme and Krantz (207) found a similar effect on brain mitochondrial preparations. Furthermore, Ansell and Dohmen speculated that anaesthetics may possibly inhibit some more specific reactions necessary for the biosynthesis of phospholipids, e.g. the reaction between CTP and phosphorylcholine.

Not enough is known about the effects of anaesthesia on general metabolism. However, it is likely that with care, light ether anaesthesia should produce less serious effects than those caused by shock in the handling of non-anaesthetised animals for purposes of injection.

The radiation of phosphorus-32 as a health hazard to the animal

Another question to be asked is whether the dose of the isotope administered is sufficiently large to cause general metabolic disturbance while the experiment is in progress.

^{32}Phosphorus has a physical half-life of 14.3 days and emits beta-rays of energy 1.7 MeV, which have a maximum range in the tissue of 0.7 cm. A single intra-peritoneal dose of 3 μC/g in rats represented the lethal dose of 20% of a group of 15 days (LD 20/15), while 6 μC/g caused 7% mortality in 10 days or 80% mortality in 21 days (208). The main points of attack by ^{32}P are bone marrow and lymphoid tissue; liver is a strongly radio-resistent tissue.

Doses administered to rats in these experiments were normally 500 μC per 250g rat, or 2 μC/g. In a limited number of cases it was 4 μC/g. Rabbits received 3 mC per $2\frac{1}{2}$ - 3 kg animal, or 1 - 1.2 μC/g body weight. Calculating from figures quoted by Hevesy (186c), 2 μC/g represents a dose to the liver of approximately 4.5 rads per hour; a figure which would probably not be expected to produce significant adverse effects during the time periods studied.

Amount of isotope injected

It has been shown (184) that the injection of such widely differing amounts of labelled phosphorus as 6.0 mg and 0.3 mg into 200g rats made no difference to the recovery of ^{32}P. The isotope used in these present experiments was carrier-free inorganic phosphate in neutral isotonic saline. Thus the phosphorus injected represented a minute proportion of the phosphorus of the metabolic pool and could have no disturbing effect on phosphorus metabolism.

Method of expressing results

To make useful comparisons between one animal and another, measures of ^{32}P incorporated into tissue phospholipids should be based on the specific radioactivity of a precursor phosphate in the metabolic pool. Workers in this field have used many different standards: blood inorganic phosphate, tissue inorganic phosphate, tissue acid-soluble phosphate and in other cases figures have been related to the activity of the substance injected, (concentration coefficient).

Tissue acid-soluble phosphate has the advantage that it is easily extracted, but has the disadvantage when compared with inorganic phosphate that one is measuring the

specific activity of a large number of substances of unknown composition. Hence fluctuations are more likely in this case and it may well be that some of the large fluctuations encountered in this work originated from this source. Extraction of inorganic phosphorus into butanol by the method of Martin and Doty (209) would possibly have been more useful, although there are indications that a great deal of fluctuation can occur with this method also (210). Thirty minutes after injection, the specific activity of the plasma acid-soluble phosphorus is sufficiently higher than that of the liver for the presence of plasma in the isolated liver to make a significant contribution to the specific activity of the liver acid-soluble phosphorus pool. Blood in isolated liver may account for some of the variation in levels of acid-soluble phosphate specific activity among different animals, since the amount of blood removed with each liver will be variable. Johnson and Albert (193) claim that perfusion of the livers reduces the degree of variation considerably.

Chemical exchange reactions

In experiments involving the use of radiophosphorus, (or other radioisotopes), the interpretation of the results

depends on the assumption that no exchange other than that brought about metabolically, occurs between phosphate esters and inorganic phosphate. The validity of this assumption has been demonstrated by a number of workers (211, 212). Marshak (201) has investigated the problem in relation to the ^{32}P uptake by cell nuclei. He showed that liver nuclei shaken at room temperature in isotonic (^{32}P) sodium orthophosphate take up less than 1/100th of the *in vivo* concentration of ^{32}P and concluded that the measured uptake of ^{32}P could not be attributed to simple chemical exchange.

Errors due to the "invitro" effect

It has been said (203) that cell fractions incorporate ^{32}P *in vitro* while they are being isolated by differential centrifugation in sucrose solutions. Since the isolation of the washed mitochondrial fraction takes ½ - 1 hour after homogenisation of the liver and the microsomal fraction, 2 - 3 hours, it is conceivable that *in vivo* results would be altered appreciably by such an *in vitro* incorporation. However, since the medium contains 0.002 M EDTA, it would be surprising if the enzymes responsible for further phosphorylation of phospholipid precursors were very active. The experiments performed here, though not very extensive or elaborate, suggest that this factor is negligible.

Regenerating liver studies

The work which prompted the studies described in this chapter was that of Johnson, Albert et al (193 - 196). An outline of these experiments was sketched in the introductory section.

Before attempting to interpret data from liver regeneration experiments, some of the events following partial hepatectomy should be outlined and the many factors influencing these events, examined.

Events following partial hepatectomy: time of maximum mitotic activity

The partial hepatectomies were performed by the method of Higgins and Anderson (202). In their original paper, the authors described the events following the operation. The first event is known as cell hypertrophy; the liver increases in size without increase in the number of cells. This is later followed by hyperplasia, in which the amount of liver tissue increases due to active cell division. According to Brues et al (213), after 70% of the liver has been removed, the remaining portion doubles in size within 72 hours. The time at which the greatest number of cells are in mitosis and the rate of liver regeneration depend on a number of factors:

1. Age of the animal. Age dependence of the time of maximum mitotic division was demonstrated by Marshak and Byron (214). For rats 1, 5 and 16 months old, mitosis was a maximum at 1, 2 and 3 days after operation respectively. Brues et al (215) found mitosis at a maximum at 24 hours (2.13% of cells in division), while Higgins and Anderson (202) observed mitotic divisions in the first day but not at a maximum until the 2nd or 3rd day after the operation.

2. Species differences.

3. Amount of liver removed in operation. MacDonald et al (216) showed that there was a threshold amount of liver to be removed in order to stimulate liver regeneration. Above a certain amount removed, the number of nuclei synthesising DNA was proportional to the amount of liver removed. When 70% of the liver was removed, the amount actually removed varied by 12-14%; this variation was sufficient to v ary the number of cells exhibiting DNA synthesis by a factor of 3-7.

4. Necrotic liver tissue. Teir (217) found that necrotic liver tissue stimulated mitosis and suggested that the necrotic liver releases an active principle.

As the liver is not removed close to the ligation in case the ligature slips off, the amount of necrotic liver tissue left inside the animal after partial hepatectomy probably varies considerably, and may account for some of the experimental variation in the rate of regeneration between animals.

5. Time of operation. The time at which maximum mitotic division occurs is dependent on the time at which the operation is performed since rats of different strains have a marked diurnal variation in activity. The mitotic rate seems to be high in the morning, irrespective of the time of day at which the operation was carried out. Jaffe et al (204) showed that for operations performed at 9 - 10 a.m. the peak of mitosis occurred at 21 - 25 hours, whereas for operations done between 9 - 10 p.m., the peak was at 33 - 37 hours.

The use of sham-operated rats as controls

The control normally used for regenerating liver studies, is that of laparotomy (making an incision in the abdominal wall) followed by handling of the liver. Such procedures have been shown by Bitensky and Gahan (218) to cause profound changes in normal rat liver. Gahan (219)

has shown histochemically that handling of the liver is important in sham operations; the changes he described were: (a) appearance of neutral fat droplets, (b) increase in availability to staining of cytoplasmic phospholipid and (c) increase in liver lipid content.

I have also noticed that during centrifugation of cell fractions from regenerating or sham-operated livers, the floating fat layer is much larger than in normal livers.

In spite of such noticeable changes in sham-operated livers, the control leaves much to be desired, since the stress caused by removal of 2/3 of the liver and the ligature shock must entail far more stress.

In my experiments, as in thoseof Johnson and Albert, no significant difference was observed between the phospholipid turnover in normal and sham-operated controls.

Counting of mitoses

The tissue sections proved disappointing from the point of view of counting mitoses. Dr. P.J. O'Brien (148), however, has determined the peak of mitosis for rats of the departmental strain, under the conditions described in this thesis. The mitotic counts rise sharply from 0 - 0.2% at 30 hours to 3.9% at 32½ hours. Mitotic counts are still quite high at 42 hours and then decrease. For

this reason a period of 36 hours after partial hepatectomy was chosen for these studies. This interval differs radically from that quoted by Johnson and Albert (193); this may be due to one or a combination of factors outlined above. Nevertheless, most other authors have quoted times of from 24 - 48 hours.

Results of the experiments

The results of Johnson and Albert's (193,196) experiments show a maximum synthesis of phospholipids at a time coincident with maximum mitotic activity. This increased synthesis was attributable to all phospholipid groups studied, but the "cephalins" of the nucleus were the only lipids to reach a maximum rate of synthesis before the time of maximum mitosis. Johnson and Albert's results were expressed in concentration coefficients and the increases observed from control to operated animals ranged from 200 - 300% in all lipid classes of all cell fractions. If these are converted to relative specific activities to compare more nearly with my own results, the increases are still consistent but much less in terms of percentage change (approximately 100% change for lecithins and 50% for cephalins). This is due to an increase specific activity of the acid-soluble fraction over the controls.

TABLE VII. Comparison of results.

Johnson and Albert (196)					Present work				
Fraction		Nuclei	Mit.	Mic.	Fraction		Nuclei	Mit.	Mic.
lecithin	C				PC	C	839	476	1010
	S	232	268	339		S		541	1079
	R	550	544	740		R	623	652	1036
					PE	C	890	810	1803
						S	-	870	1852
						R	653	975	1810
"Cephalin"	S	284	305	520	PS	C	54	116	256
	R	476	413	695		S	-	116	168
						R	144	137	218
					PI	C	546	167	351
						S	-	266	308
						R	466	271	454

C = control, S = sham-operated control, R = regenerating liver.

My failure to repeat the results of Johnson and Albert may be due to a combination of the factors outlined above. The differences in my results are certainly not significant. The use of detailed statistical methods when there are only a maximum of six animals in a group is out of the question. The use of the Friedman ranking method (220) indicated that there was no statistical difference between results.

The difference in time after the operation could be a reason for the disagreement with Johnson and Albert's results; these authors did not quote any figures for any time between 24 and 72 hours after the operation, either for mitotic counts or specific activities. The different behaviour of the lipids in their nuclear fraction is difficult to explain and in view of the negative nature of my results and large variation between animals, further speculation is probably foolhardy.

PART III

PHOSPHOLIPIDS AND FAT ABSORPTION

INTRODUCTION

The history of the role of phospholipids in the mechanism of fat absorption has been sketched in the main introduction. From this, several points stand out.

(1) After nearly fifty years of experimentation on these lines, the question of the exact role of phospholipids is still unsolved; many of the results are conflicting and ideas opposed.

(2) Quite often, experiments have been inadequate to draw definite conclusions; in many experiments conditions have been such that a much wider range of metabolism has been affected than that involving merely the participation of phospholipid in the resynthesis of fat. Hence measurements may be invalidated because of interference with processes quite other than those studied. Such criticism may be levelled against the phlorizin poisoning methods of Verzar or the protein-deficiency experiments of Artom. On the whole, too broad a conclusion has often been drawn from too little evidence.

(3) Labelling experiments have their limitations. Sinclair's experiments are difficult to interpret in the

light of recent evidence that unsaturated fatty acids are preferentially incorporated into phospholipids anyway. Likewise, labelling experiments with radiophosphorus or (^{14}C)-fatty acids only gave information about one particular moiety of the more complex phospholipid molecule, so that generalisations about turnover should be treated with caution.

(4) Most experimenters have treated the complex lipid mixture as a whole and not fractionated the individual lipids. This is mainly due to lack of refined separative methods in the past.

One of the few experiments reported in which individual phospholipids have been separated was that of Johnston and Bearden (113) and this used an *in vitro* system. Consequently, the present work stemmed from the need to reinvestigate the problem *in vivo*, exploiting modern chromatographic techniques to study the behaviour of single phospholipids.

Olive oil was chosen as a suitable source of triglyceride; it also seems to have been widely used by others, so comparisons with other results can the more readily be made. Administration by stomach tube is a means of applying a measured quantity, at a given place, at one time.

It should be noted, however, that such quantities as were administered (0.75 ml) were highly unphysiological and as such, open to criticism. Absorption was thought to be proceeding at a reasonable rate after the two hour period allowed in each experiment, although Schmidt-Nielsen (106) claims maximum absorption of similar doses at five hours.

The incorporation of label should be on the ascending limb of the time curve when analyses are made (see Discussion of Part II) and if the incorporation time is small, any differences in the uptake of label by different phospholipids should then be most marked. Hence thirty minutes was judged to be a reasonable time; this was based on the knowledge of the time curves for liver; no time curve for intestine was constructed.

The controls were treated exactly as the experimentals except that they were given glucose solution. It was thought that the mucosal cells of control animals should be actively absorbing some substance other than fat, to avoid measuring an increase in turnover which might be caused by general increase in absorptive activity of the cells. Schmidt-Nielsen also made this point. The concentration of glucose (25%) has no significance other

than that it was thought that the volume was the important factor and that it should be applied in large concentration which indeed was the case with the oil.

It was natural, after the publication of Johnston and Bearden's paper, to be most interested in the behaviour of phosphatidic acid. This compound has, in most cases, chromatographic properties similar to diphosphatidyl-glycerol, and although their respective de-acylation products, GP and GPGPG are quite well separated by Dawson's method, nevertheless they run fairly close together. Moreover, phosphatidic acid is usually present in tissues in extremely small quantities so that there is always the likelihood of these two compounds being confused. As a check to paper chromatographic identification of glycero-phosphate, an experiment employing column chromatography was included in the series. It has been shown that 5% methanol in chloroform will elute phosphatidic acids and diphosphatidylglycerols from silicic acid columns. These two compounds are separable as their water-soluble hydrolysis products GP and GPGPG*, on a column of Nalcite SAR ion-exchange resin. The scrapings from one rat intestine yield insufficient lipid for a column separation, so that six animals were used for each analysis.

* Footnote: abbreviations see p. 283

MATERIALS AND METHODS

Materials

In addition to those used in Parts I and II:

Mallinkrodt silicic acid was obtained from Savory and Moore Ltd., London.

Hyflo-super-cel (Johns-Manville, U.S.A.) was supplied by L. Light and Co. Ltd., Colnbrook, Bucks.

Nalcite SAR x 10 was supplied by the National Aluminate Co., Chigago, Illinois, U.S.A.

Methods

Methods of extraction of lipids, chromatography of lipids, and assays of phosphorus and radioactivity have been described in Part I, section (i), and Part II, section (i). The identity of compounds on two-dimensional paper chromatograms were also examined in the same way.

Separation of lipids by column chromatography

Silicic acid chromatography

Lipid extracts from the pooled mucosa of six rat intestines were applied to columns of silicic acid mixed

with hyflo-super-cel, using 2 g silicic acid and 1 g hyflo-super-cel per mg of lipid phosphorus. Column sizes were 13 x 2 cm in one experiment and 25 cm x 1.5 cm in another, using about 6 mg of lipid phosphorus. The triglyceride of the lipid mixture was washed through the column with 200 ml of pure chloroform and the eluate checked to show that no phosphorus had been eluted, by reading in the Geiger counter. Phospholipids were eluted as groups of compounds by a sequence of solvents (9):

1. 5% methanol in chloroform,
2. 20% methanol in chloroform,
3. 30% methanol in chloroform, and
4. 40% methanol in chloroform.

Fractions of 25 ml were collected, using a Towers automatic fraction collector. The elution of the peaks was tracked by counting 10 ml samples in a liquid Geiger counter. Fractions from each peak were pooled (see section (ii)) and taken to dryness. After phosphate assay, these were subjected to hydrolysis and further chromatography as below.

Chromatography of the phosphate esters

The first peak eluted with 5% methanol in chloroform was subjected to mild alkaline hydrolysis following the method of Dawson (15). The hydrolysate was brown and

dirty in appearance (since the pigments present in the lipid mixture were also eluted with 5% methanol in chloroform). The hydrolysate was made to 20 ml and rendered 0.005 M with respect to sodium borate. This solution was loaded on a column of Nalcite SAR x 10 which measured 8 cm x 1 cm, and which had been previously washed with ammonium formate and water.

Elution of the phosphate esters was carried out with 0.12 M formate/ 0.005 M borate followed by 0.15 M formate/ 0.005 M borate, after the method of Hübscher (221). Again, 25 ml fractions were collected and 10 ml samples counted in the liquid Geiger counter.

Later peaks eluted from silicic acid columns were subjected to mild alkaline hydrolysis following the method of Hübscher et al (18). Hydrolysates were made 0.005 M with respect to sodium borate and loaded on Dowex 1 x 10 columns which had previously been washed with: (i) 2 N hydrochloric acid, (ii) water until neutral, (iii) 2N ammonium formate, and finally with water. The columns were washed with 0.005 M sodium borate until the eluate gave only a background count; the GPC fraction was by then completely removed from the column. Remaining compounds were eluted with 0.06 M ammonium formate/ 0.005 M sodium borate.

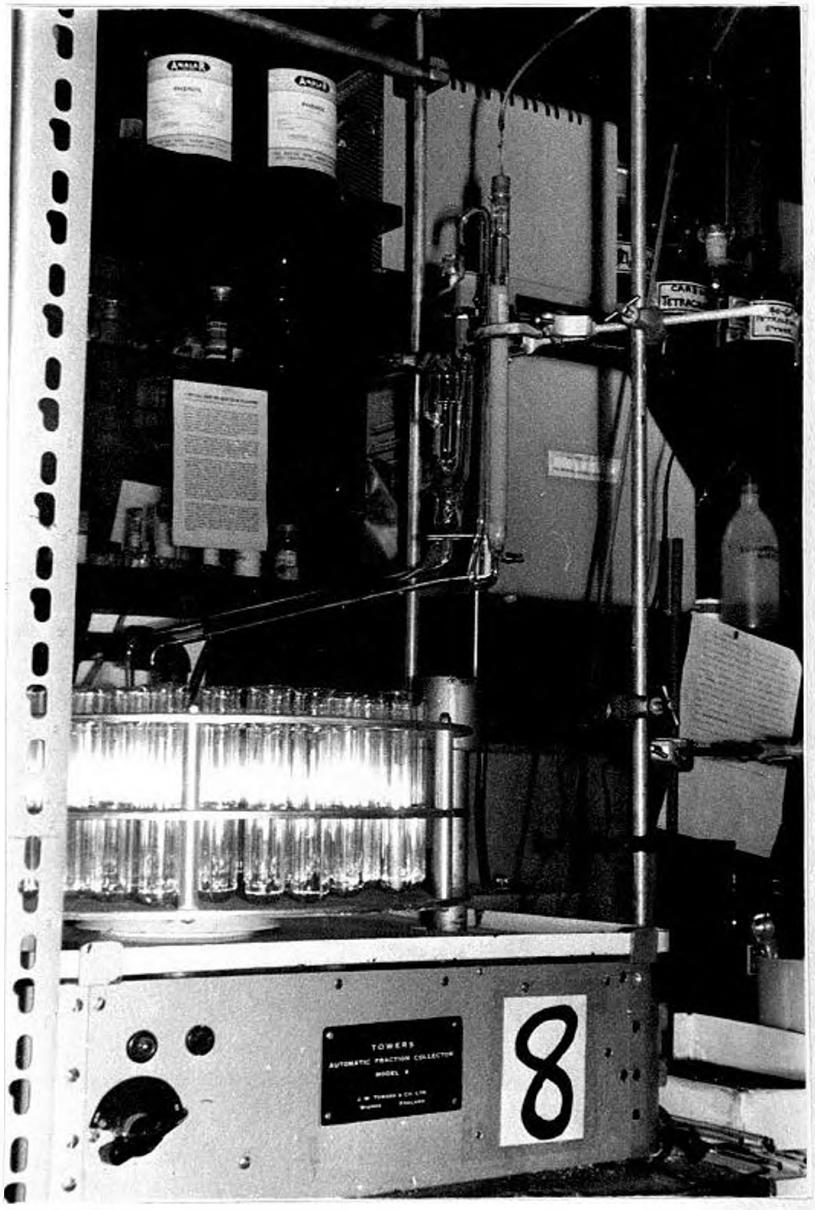


Plate I.

Column Chromatography

EXPERIMENTS AND RESULTS

Feeding and Injections

In a typical experiment, rats*, which had previously been fed on standard laboratory diet, were starved overnight and then fed by stomach tube; they were not anaesthetised. Two animals were used for each experiment; the control animal was fed with 0.75 ml of 25% glucose and the other with the same volume of olive oil. During the feeding of the animal, the mouth was kept open by means of a metal gag. After feeding, two hours were allowed for absorption to proceed; each animal was then lightly anaesthetised with ether and injected with a solution of carrier-free (^{32}P)-orthophosphate. The isotope was administered intravenously (see Part II section (i) for isotope details) and quantities varied from experiment to experiment between 0.5 mC and 1.0 mC, although control and fat-fed animals in the same experiment were given equal doses. The animals were killed by concussion thirty minutes after injection of the isotope. The small intestine, stomach and caecum were immediately removed and the contents washed out with 0.9% sodium chloride. The mucosal cells

were scraped off** with a wooden spatula and immediately homogenised for 1 minute in 10 ml ice-cold 10% TCA. Acid-soluble compounds and phospholipids were then extracted as described in part I.

Measurement of fat absorbed

The washings from intestine, stomach, and caecum of the fat-fed animal were extracted with 2 x 25 ml portions of chloroform-methanol, 2:1, v/v, and 4 x 20 ml of ether and after taking to dryness, the fat residue was weighed. In experiments 3 and 4, as much fat was recovered as was administered; this could have been due to insufficient starvation, so that the animals had fat in the stomach or intestine before the experiment began. In the other experiments, however, similar proportions of administered fat were recovered, and these figures are recorded in the tables.

The results of a series of experiments are recorded in Tables I - VII. In experiments 1 - 4, analyses were done only with the aid of paper chromatography; experiment 5 made use of column chromatography to check the identity of the highly labelled glycerophosphate.

The results of experiment 1, table I, are not typical of the rest of the series. This is regarded very much as a preliminary experiment, in which the correct conditions

were being sought; chromatograms were very poor and only a few results from the fat-fed animals are included.

The figures in column 3 of each table represent the number of counts in 100 seconds given by the stated quantity of phosphorus. This figure has been corrected for decay, background, and dead-time of the counter as I have explained in Part II. Specific activity (S.A.) and Relative specific activity (R.S.A.) have also been defined in part II.

Experiment 5, using column chromatography

In this case, control and fat-feeding experiments were carried out separately owing to the difficulties involved in dealing with large numbers of animals. Even so, considerable difficulty was experienced in scraping the intestinal mucosa quickly and efficiently within the time limit set by the frequency of injections. Consequently, while the mean time of isotope incorporation was kept down to 37 minutes in the control experiment, (compare 30 minutes in previous experiments), due to unforseen difficulties, the mean time in the fat-feeding experiment was as much as 63 minutes. Hence these experiments are not so valuable from the point of view of comparison of relative specific activities, but are nevertheless still

useful as a proof that the highly labelled compound is really glycerophosphate.

The experimental procedure was exactly the same as described for experiments 1 - 4; chromatographic details have been described in the "Methods" section.

Column separations on silicic acid (total lipid mixture), Nalcite SAR (GP and GPGPG) and Dowex 1 x 10, (hydrolysis products of lecithin and inositides) are reproduced in figures 2 - 4. Figures 2 and 3 are taken from control results; (the recovery of phosphatidic acid was low in the fat-feeding experiment due to slowness in scraping mucosa from the intestine). Figure 4 is taken from fat-feeding results as a much better separation on Dowex 1 x 10 was obtained in this case; the separation in the control experiment was too poor to compare results, (see table VII).

* All rats were male albinos of the department strain, weighing approx. 250 g.

** The assistance of Dr. B.Clark is acknowledged.

TABLE I

Experiment 1

Feeding data: fat-fed animal: 0.75 ml olive oil, 0.665 g.
control: 1.0 ml 25% glucose

Oil extracted: 0.037 g.
Oil absorbed: 0.628 g.

Time between feeding and ^{32}P injection: 2.13 h.
Time between ^{32}P injection and death: 0.67 h.

SAMPLE		Phosphorus extracted µg	Phosphorus counted µg	Counts per 100 sec.	S.A.	R.S.A.
Acid soluble	control	-	713	1138×10^3	1662	-
	fat	-	232	1185×10^3	368	-
Lipid mixture	control	1226	613	16305	26.6	16
	fat	240	118	1514	12.8	35
GPC	control	-	47.5	984	20.7	13
	fat	-	-	-	-	-
	control	-	50.4	938	18.6	11
	fat	-	-	-	-	-
GPE	control	-	11.5	137	11.9	7
	fat	-	-	-	-	-
	control	-	14.7	169	11.5	7
	fat	-	-	-	-	-
GPS	control	-	-	-	-	-
	fat	-	2.0	31	15.5	42
	control	-	-	-	-	-
	fat	-	-	-	-	-
GPI	control	-	-	-	-	-
	fat	-	-	-	-	-
	control	-	-	-	-	-
	fat	-	-	-	-	-
GP	control	-	3.65	611	168	101
	fat	-	-	-	-	-
	control	-	2.0	710	355	214
	fat	-	3.2	336	168	456
GPGPG	control	-	2.25	55	24.5	15
	fat	-	-	-	-	-
	control	-	1.2	19	15.8	10
	fat	-	0.5	6	12.0	33
cyclic GP	control	-	8.4	385	45.9	28
	fat	-	-	-	-	-
	control	-	7.5	411	54.9	33
	fat	-	-	-	-	-

TABLE II

Experiment 2

Feeding data: fat-fed animal: 0.75 ml olive oil, 0.665 g.
control: 0.75 ml 25% glucose

Oil extracted: 0.170 g.
Oil absorbed: 0.495 g.

Time between feeding and ^{32}P injection: 1.75 h.
Time between ^{32}P injection and death: 0.50 h.

SAMPLE		Phosphorus extracted µg	Phosphorus counted µg	Counts per 100 sec.	S.A.	R.S.A.
Acid soluble	control	-	489	2122×10^3	4339	-
	fat	-	527	1172×10^3	2224	-
Lipid mixture	control	724	362	13381	37.0	9
	fat	1160	585	27780	47.5	21
GPC	control	-	52.2	924	17.7	4
	fat	-	53.3	2720	51.0	23
	control	-	46.0	915	19.9	5
	fat	-	47.6	2610	54.8	24
GPE	control	-	24.9	368	14.8	3
	fat	-	25.5	414	16.2	7
	control	-	9.8	205	20.9	5
	fat	-	14.5	359	24.8	11
GPS	control	-	6.3	12	1.9	0.4
	fat	-	6.5	58	8.9	4
	control	-	4.6	29	6.3	1.4
	fat	-	5.4	9	1.7	7
GPI	control	-	2.5	441	176	41
	fat	-	2.8	442	158	70
	control	-	-	-	-	-
	fat	-	-	-	-	-
GP	control	-	3.4	1530	450	104
	fat	-	3.4	1192	349	156
	control	-	3.2	1520	475	110
	fat	-	3.4	1200	353	158
GPGPG	control	-	1.8	41	22.7	5
	fat	-	2.6	36	13.8	6
	control	-	-	-	-	-
	fat	-	-	-	-	-
cyclic GP	control	-	4.2	205	48.8	11
	fat	-	5.4	511	94.6	42
	control	-	2.1	327	156	36
	fat	-	2.7	438	162	72

TABLE III

Experiment 3

Feeding data: fat-fed animal: 0.70 ml olive oil, 0.621 g.
control: 0.70 ml 25% glucose

Oil extracted: 0.624 g.
Oil absorbed: -

Time between feeding and ^{32}P injection: 2.16 h.
Time between ^{32}P injection and death: 0.5 h.

SAMPLE		Phosphorus extracted μg	Phosphorus counted μg	Counts per 100 sec.	S.A.	R.S.A.
Acid soluble	control	-	583	182×10^3	312	-
	fat	-	330	143×10^3	433	-
Lipid mixture	control	1400	720	4270	5.9	19
	fat	906	453	7653	16.9	39
GPC	control	-	44.3	116	2.6	8
	fat	-	51.7	1071	20.6	48
	control	-	-	-	-	-
	fat	-	-	-	-	-
GPE	control	-	25.0	40	1.6	5
	fat	-	28.0	257	9.2	21
	control	-	-	-	-	-
	fat	-	-	-	-	-
GPS	control	-	5.2	19	3.7	12
	fat	-	3.2	34	10.6	25
	control	-	-	-	-	-
	fat	-	-	-	-	-
GPI	control	-	3.5	76	21.7	70
	fat	-	3.3	78	23.6	55
	control	-	-	-	-	-
	fat	-	-	-	-	-
GP	control	-	3.3	147	44.1	142
	fat	-	3.1	296	95.7	221
	control	-	-	-	-	-
	fat	-	-	-	-	-
GPGPG	control	-	3.0	16	5.3	17
	fat	-	3.9	25	6.4	15
	control	-	-	-	-	-
	fat	-	-	-	-	-
cyclic GP	control	-	3.5	9	2.6	8
	fat	-	5.7	118	20.6	48
	control	-	-	-	-	-
	fat	-	-	-	-	-

TABLE IV

Experiment 4

Feeding data: fat-fed animal: 0.75 ml olive oil; 0.665 g.
control: 0.75 ml 25% glucose

Oil extracted: 0.716 g.
Oil absorbed: -

Time between feeding and ^{32}P injection: 2.18 h.
Time between ^{32}P injection and death: 0.50 h.

SAMPLE		Phosphorus extracted µg	Phosphorus counted µg	Counts per 100 sec.	S.A.	R.S.A.
Acid soluble	control	1064	190	61×10^3	322	-
	fat	1004	193	66×10^3	344	-
Lipid mixture	control	430	215	1500	7.0	22
	fat	460	230	2740	11.9	35
GPC	control	-	47.0	181	3.9	12
	fat	-	50.0	953	19.1	56
	control	-	45.0	167	3.7	12
	fat	-	50.4	787	15.6	45
GPE	control	-	23.0	85	3.7	12
	fat	-	25.0	135	5.4	16
	control	-	25.0	129	5.2	16
	fat	-	24.0	114	4.8	14
GPS	control	-	-	-	-	-
	fat	-	-	-	-	-
	control	-	-	-	-	-
	fat	-	-	-	-	-
GPI	control	-	2.7	86	31.8	99
	fat	-	2.9	76	26.2	76
	control	-	2.0	52	26.2	81
	fat	-	2.6	69	26.5	77
GP	control	-	3.0	185	61.6	192
	fat	-	3.2	194	60.5	176
	control	-	2.3	123	53.4	166
	fat	-	2.9	141	48.5	141
GPGPG	control	-	-	-	-	-
	fat	-	-	-	-	-
	control	-	-	-	-	-
	fat	-	-	-	-	-
cyclic GP	control	-	-	-	-	-
	fat	-	-	-	-	-
	control	-	-	-	-	-
	fat	-	-	-	-	-

TABLE V

Composition of the alkali-labile phospholipid fraction from rat intestinal mucosa.

Compound	Phosphorus recovered from paper as % of alkali-labile phosphorus.	Number of Determinations	Variation
GPC	50.5 (53.5)*	5	44.3-54.4
GPE	25.4	5	23.1-29.5
GPS	5.2	5	3.4-6.3
GPI	2.9 (4.1)*	3	2.5-3.5
GP	3.2	4	2.8-3.4
GPGPG	2.8	4	1.8-4.1
Cyclic-GP	5.5	5	3.5-8.6

* Corrected for breakdown.

Fig 1 INCORPORATION OF ^{32}P INTO RAT MUCOSAL PHOSPHOLIPIDS DURING FAT ABSORPTION

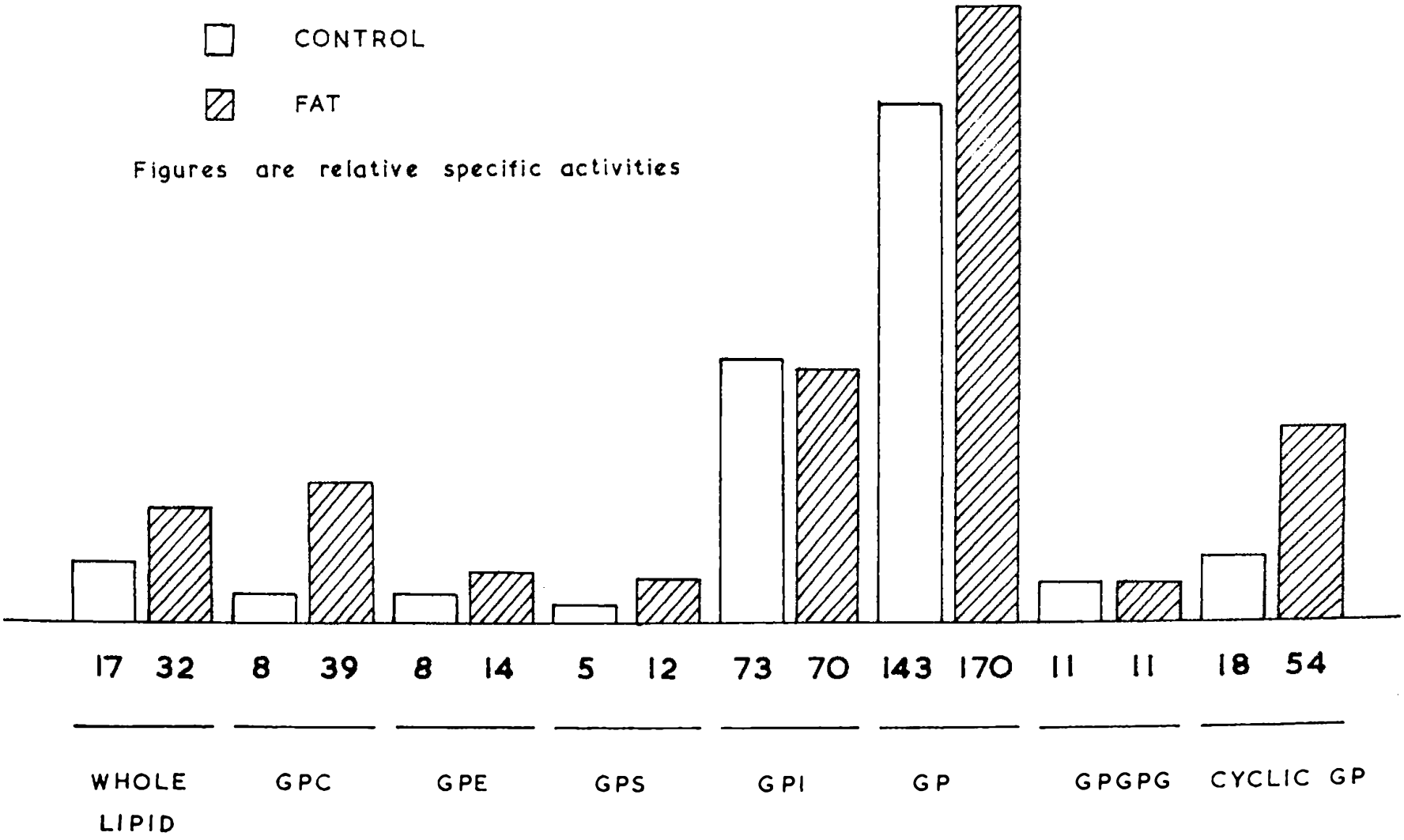


TABLE VI

Experiment 5. Data on feeding and injections.

Feeding Data:

fat-fed animal: 0.75 ml olive oil to each of 6 rats

control: 0.75 ml 25% glucose to each of 6 rats

amount of oil fed: 3.9900 g.

amount of oil recovered: 1.6966 g.

amount of oil absorbed: 2.2930 g.

amount absorbed per rat: 0.36 g.

Mean time between feeding and ^{32}P injection:

fat : 2.20 hours

control : 2.17 hours

Mean time between ^{32}P injection and death:

fat : 1.05 hours

control : 0.62 hours

Amount of radioactivity injected:

1.0 mC per animal.

TABLE VII

Results from Column chromatography

SAMPLE		Phosphorus extracted µg	Phosphorus counted µg	Counts per 100 s. x 10^{-4}	S.A.	R.S.A.
Acid Soluble	control	10,297	1,392	1061	7622	-
	fat	-	534	191	3583	-
Total Lipid Mixture	control	5,946	2,417	21.8	90.4	12
	fat	4,463	1,750	25.1	143	40

SILICIC ACID COLUMN

Peak		Phosphorus recovered µg	Counts per 100 s. x 10^{-2}	S.A.	R.S.A.
A	control	660	1,259	191	25
	fat	144	369	256	72
B	control	1,388	794	57.2	8
	fat	1,120	962	86.0	24
C	control	3,902	2,351	60.2	8
	fat	2,725	3,340	123	34

TABLE VII (Continued)

Peak		Phosphorus recovered μg	Counts per 100 s.	S.A.	R.S.A.
NALCITE SAR COLUMN					
A (1)	control	91	1229	13.5	2
	fat	8	412	53.6	15
A (2)	control	123	69054	561	74
	fat	66	16126	244	68
DOWEX 1 x 10 COLUMN					
GPC	control	813	45900	56.4	7
	fat	1360	334027	246	69
C (1)	(fat)	48	20328	425	119
C (2)	(fat)	269	40805	152	42
C (3)	(fat)	290	42902	148	41

FIGURE 2

Chromatography of ^{32}P phospholipids from rat intestinal mucosa on silicic acid/hyflo-super-cel. 10 ml fractions were collected and the graph represents the counts per 100s. per fraction x 10^{-3}. Peak A contains phosphatidic acids and diphosphatidylglycerol, (11% of the phosphorus applied to column), peak B mainly phosphatidylethanolamine and phosphatidylserine, (22.5% of the total phosphorus), and peak C, mainly phosphatidylcholine and phosphatidylinositol (67% of the total phosphorus).

Fig 2 SILICIC ACID CHROMATOGRAPHY OF MUCOSAL PHOSPHOLIPIDS

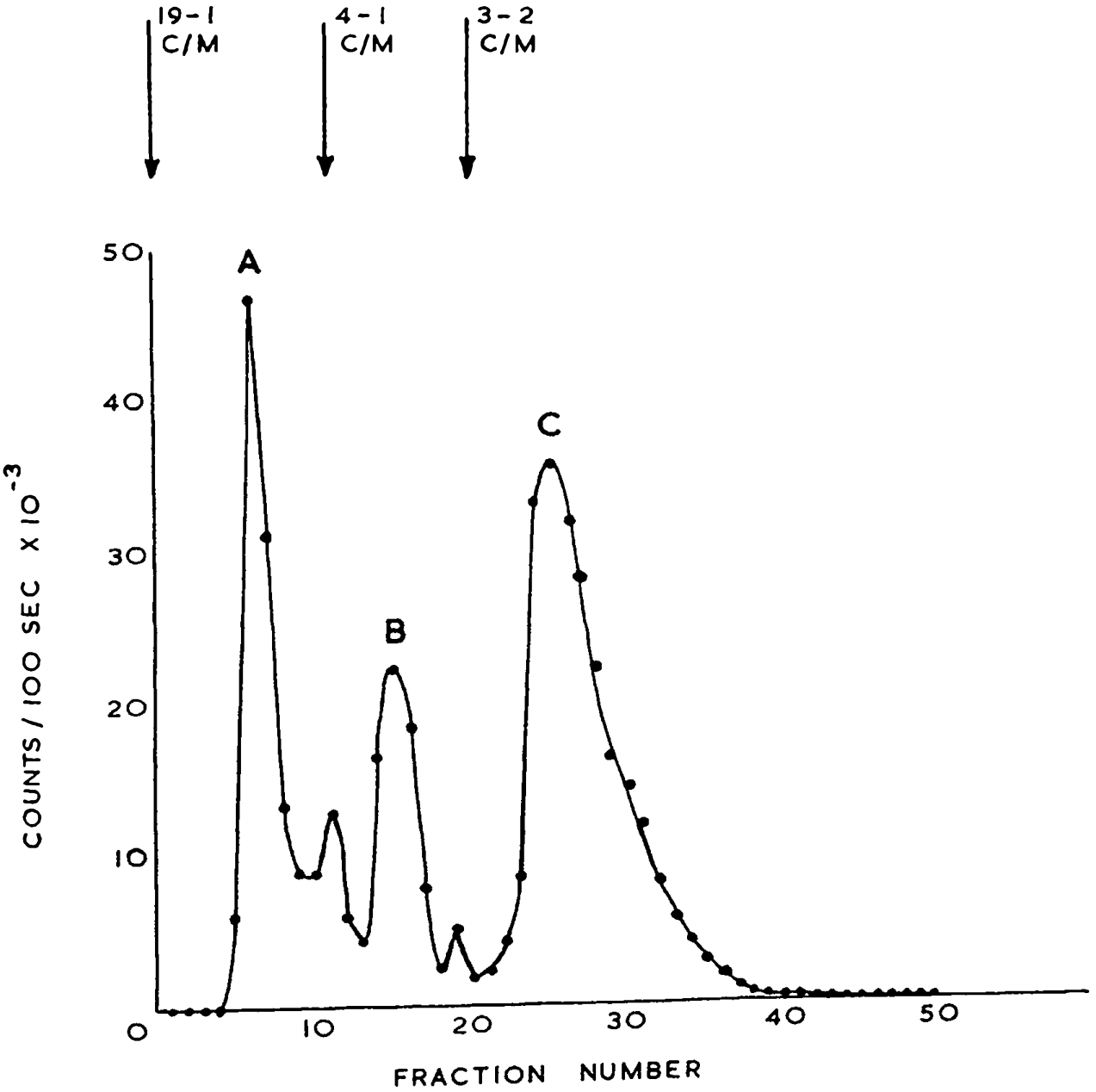


FIGURE 3

Ion-exchange chromatography on Nalcite SAR, of the water-soluble hydrolysis products, GPGPG and GP from diphosphatidylglycerol, (A.1) and phosphatidic acid (A.2). The solid line represents the radioactivity in counts per 100s x 10^{-3} and the shaded portion indicates the amount of phosphate material in each peak. A.1 contained 91 μg or 1.6% of the total lipid phosphorus applied to the silica column, and peak A.2, 123 μg or 2.1% of the total lipid phosphorus applied to the silica column.

Fig 3 ION-EXCHANGE CHROMATOGRAPHY OF GP AND GPGPG ON NALCITE-SAR

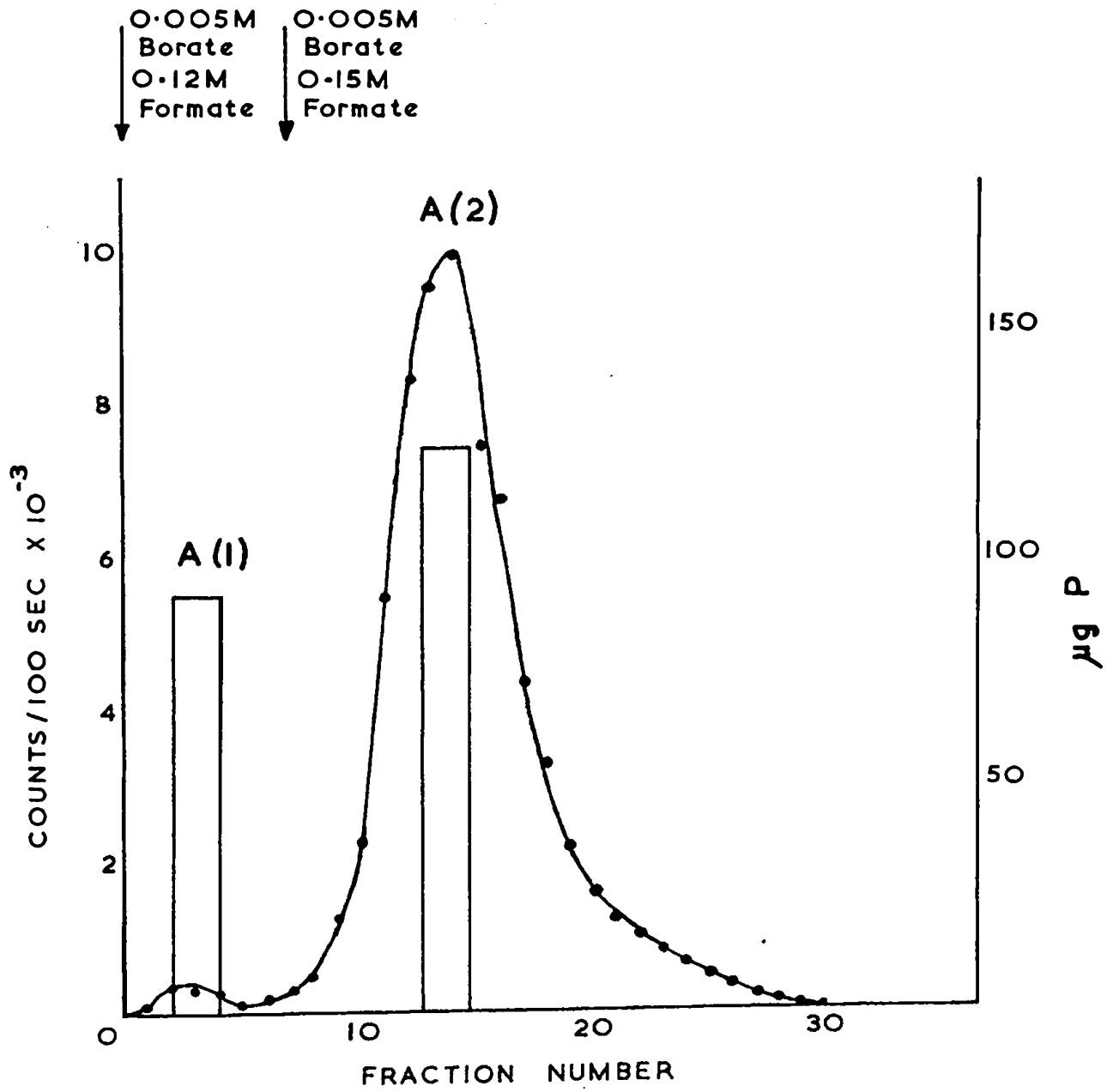


FIGURE 4

Ion-exchange chromatography on Dowex 1 x 10 of the water-soluble hydrolysis products obtained by mild alkaline hydrolysis of the phospholipids recovered in peak C of the silicic acid column. The total amount of phosphorus loaded on the column was 2222 μg, and the total recovered from the four peaks was 1967 μg. The first peak represents the material coming straight through the column during loading and is mainly GPC. Recovered phosphorus was 1360 μg, recovered radioactivity, 334027 c/100s., and relative specific activity was 69.

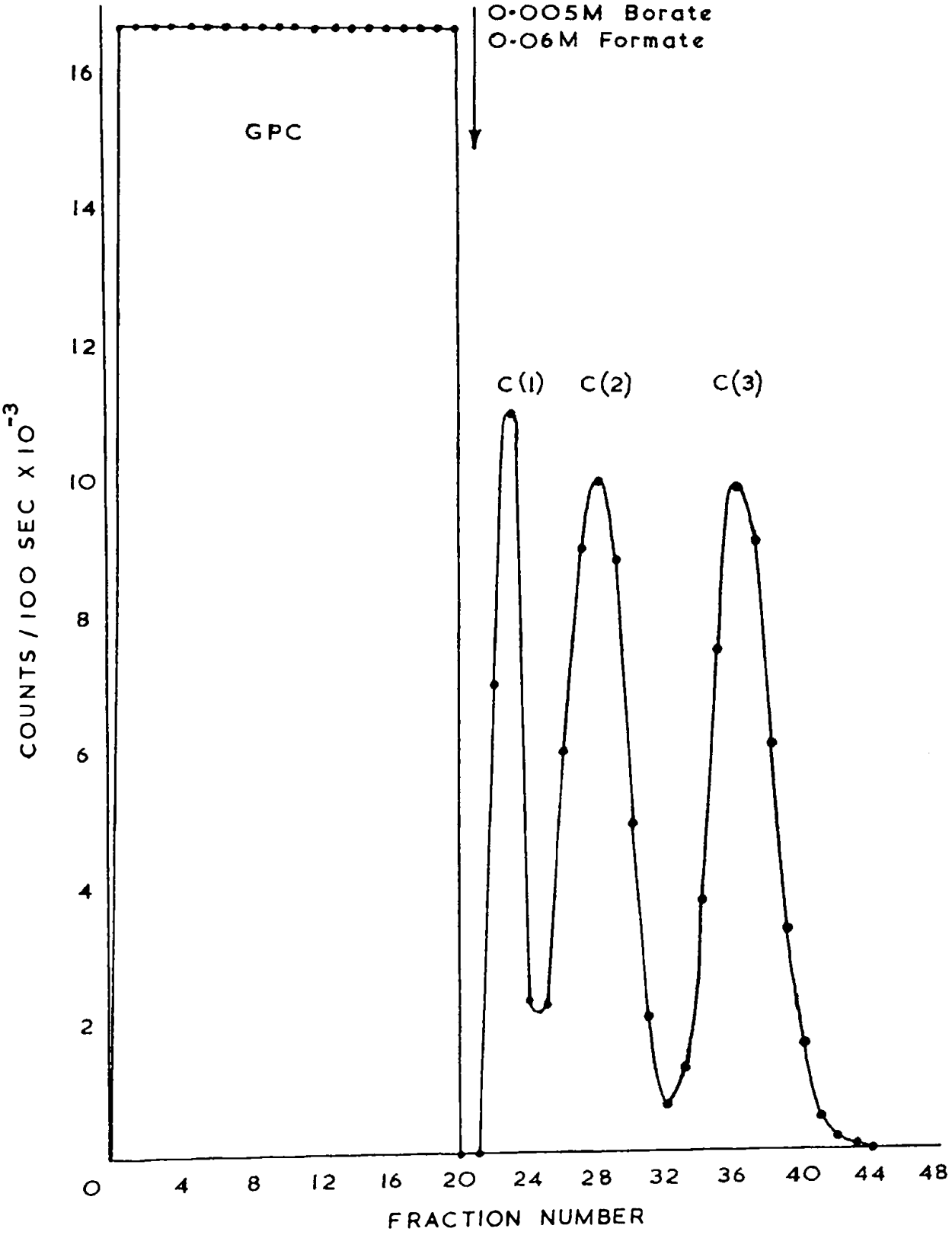
Peak C (i) contained 48 μg P, 20,328 c/100s.,RSA=119. This peak contained a large quantity of inositol.

Peak C (ii) contained 269 μg P, 40,805 c/100s.,RSA=42. No inositol was found in this peak.

Peak C (iii) contained 290 μg P, 42,902 c/100s., RSA=41.

Only a very slight trace of inositol was found.

Fig 4 ION EXCHANGE CHROMATOGRAPHY OF HYDROLYSIS PRODUCTS OF 'PEAK C' PHOSPHOLIPIDS ON DOWEX 1 X 10



DISCUSSION OF RESULTS

The results of this series of experiments on the incorporation of ^{32}P into phospholipids of intestinal mucosa of the rat during the absorption of olive oil may be summarised as follows.

The pattern of phospholipids is similar to that of liver. The chief component is phosphatidylcholine, which accounts for roughly half of the lipid phosphorus. It is impossible, from the methods used, to tell how much, if any, of this was originally lyso-phosphatidylcholine. In the control animals, the turnover of phosphatidylcholine is relatively low (RSA 8) when compared with that of phosphatidic acid (RSA 143), or phosphatidylinositol (RSA 73), but increases up to 6-fold in the fat-fed animal.

Phosphatidic acid is present in amounts representing roughly 3% of the phosphorus of the alkali-labile phospholipids, and has the highest turnover in both control and fat-fed animals. The turnover does not change significantly during fat absorption. The identity of this compound has been confirmed by silicic acid chromatography. The phosphatidic acid-containing peak has been hydrolysed and

the water-soluble hydrolysis products separated on Nalcite SAR ion-exchange resin; when this was done, the low-activity GPGPG from cardiolipin was well separated from the high-activity GP from phosphatidic acid.

Phosphatidylinositol has been found in small quantity, but with a high turnover, second only to phosphatidic acid. The turnover did not change significantly during fat absorption.

Phosphatidylethanolamine accounts for about 25% of the alkali-labile lipid phosphorus but has a low turnover; its turnover in this tissue is therefore in marked contrast to the turnover in liver. A two-fold increase in the turnover from control to fat fed animal was observed in experiment 2 (Table II), four-fold in experiment 3 (Table III), and no increase in experiment 4 (Table IV). Diphosphatidyl-glycerol contributed very little to either phosphorus or turnover and although the turnover of phosphatidylserine showed a rise in each case, it was so poorly labelled and present in such small quantity that errors in specific activity are likely to be quite large. The high specific activity of cyclic glycerophosphate rising consistently in fat-fed rats is additional evidence that this compound originates from lecithin and monophosphoinositide.

The results from column chromatography have been useful in that they permitted a check on the identity of phosphatidic acid, but owing to the fact that the time of incorporation of the isotope was much longer in the fat-feeding experiment than the control, all specific activities in that experiment are necessarily higher and comparison of turnovers in each case is impossible. Also much of the phosphatidic acid was probably lost due to delay in the scraping of intestine after death (Table VII).

Phospholipid composition of mucosa

No attempt has been made to compare the amount of phospholipid in control and fat-fed rats, but previous studies (93,106,222) indicate that there is little change in the total amount of phospholipid in the mucosa during fat absorption. Only the alkali-labile phospholipids have been investigated in the present study. The pattern of these compounds appears to be the same in mucosa from control and fat-fed rats.

There are relatively few figures in the literature for the phospholipid composition of intestinal mucosa. For dog intestine McKibbin (223) recorded the following figures (expressed as % total lipid nitrogen):

phosphatidylserine, 14.4; phosphatidylethanolamine, 13.8; phosphatidylcholine, 41.9; sphingomyelin, 17.3; undetermined, 12.6. In the same tissue (224) the molar ratio: inositol/total lipid-P was found to be 0.0713. Assuming that phosphatidylinositol is the only inositide present, it would therefore account for 7.13% of the total lipid-P (including sphingomyelin). Calculating from figures quoted by Wittcoff (222), sphingomyelin accounted for 22.7 and 18.8% of the total lipid-P in beef intestine.

The present results on rat intestinal mucosa are not strictly comparable with these figures. Again phosphatidylcholine is the major component. We have found less phosphatidylserine but more phosphatidylethanolamine than found by McKibbin in dog intestine.

^{32}P incorporation during fat absorption

The finding that the relative specific activity of the whole lipid fraction of rat intestinal mucosa rises during fat absorption agrees with the results of Schmidt-Nielsen (106), and, qualitatively, with those of Zilversmit (107), although quantitatively the increase observed in my experiments is much greater than Zilversmit's.

TABLE VIII

Author	Control		Fat (Peanut Oil)		Comment
	Wt. of Animal	R.S.A.	Wt. of Animal	R.S.A.	
Schmidt-Nielsen (106)	190*	0.35	205	1.02	Amount of oil = 2.5g
	180*	0.29	210	0.78	feeding-injection = 5h
	160**	6.7	260	32.6	injection-death = 15 min*
	250**	8.6	225	23.3	= 60 min**
Zilversmit et al (107)	150*	12	150	24	Amount of oil = 2.1g
	136*	15	140	18	feeding-injection = 5h
	192**	89	218	100	injection-death = 15 min*
	185**	55	167	140	= 60 min**

The present work is the first investigating the effect produced on individual phospholipids, and the results suggest that this increased turnover is due almost entirely to phosphatidylcholine, (with perhaps slight contribution from phosphatidylethanolamine and phosphatidylserine). The biochemical mechanism responsible for this increase is obscure, but it seems likely that increased synthesis of phosphatidylcholine will be required during fat absorption, since it is the chief phospholipid stabilising the chylomicrons (225). In early experiments from this department (112), olive oil was fed to rats with and without choline. When 916 mg olive oil was given, 10 mg choline produced a 52% increase in fat absorption over a three hour period. This may also be a reflection of the need for phosphatidylcholine. Artom and Cornatzer's (110) work would also tend to support this hypothesis.

The role of phosphatidic acid

From the evidence presented, there seems to be little doubt that the compound of highest specific activity is phosphatidic acid. The finding that the specific activity of phosphatidic acid does not increase during fat absorption appears to conflict with the results of Johnston and Bearden (113). The apparent discrepancy would be explained

by the following differences in experimental conditions: Johnston and Bearden used an *in vitro* system, incubating hamster intestine with fatty acid, whereas this work was carried out *in vivo* after feeding olive oil.

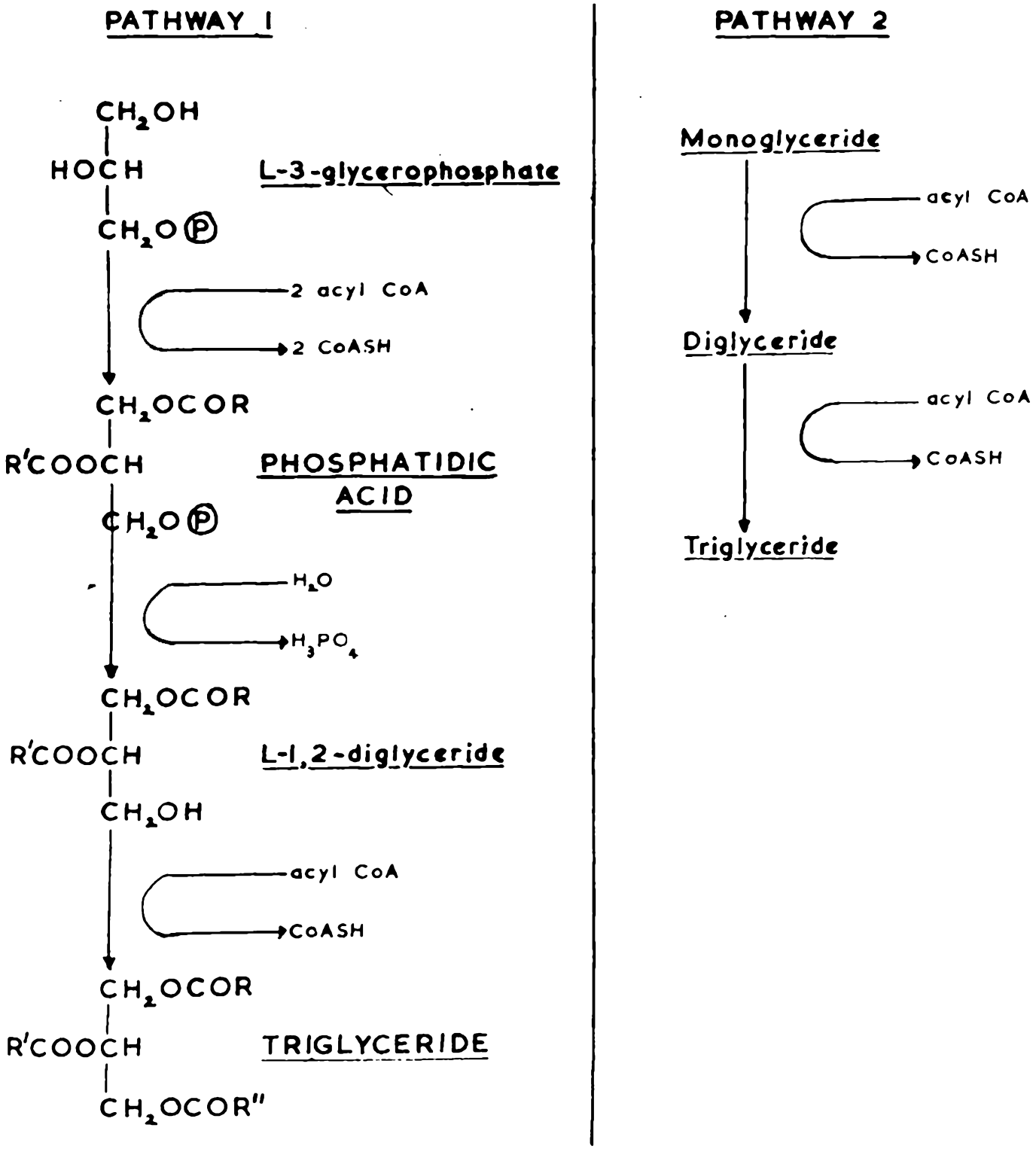
Phosphatidic acid has been shown by Clark and Hübscher (226) to be an intermediate in triglyceride biosynthesis when L-3-glycerophosphate is the precursor of the glycerol moiety of the triglyceride (see figure 5, scheme I). This was demonstrated with rabbit small intestinal mitochondria and the pathway is identical with that discovered by Weiss, Kennedy and Kiyasu (227) in chicken liver. The work of Johnston and Bearden (113) also points to this pathway.

The resynthesis of triglyceride can also take place by the monoglyceride pathway of Clark and Hübscher (226) (see figure 5, scheme 2), which does not involve phosphatidic acid. Evidence for this pathway has also been presented by Johnston and Bearden (228), who, using α-monopalmitin labelled with $^{3}H/^{14}C$ in the ratio 1:1.88, showed that the ratios of those labels in di- and tri-glycerides were 1:1.89, and 1:1.82 respectively.

One could postulate therefore, that when fatty acid is administered (viz. Johnston and Bearden) an increased

fig 5.

PATHWAYS FOR THE RESYNTHESIS OF TRIGLYCERIDE IN INTESTINAL MUCOSA

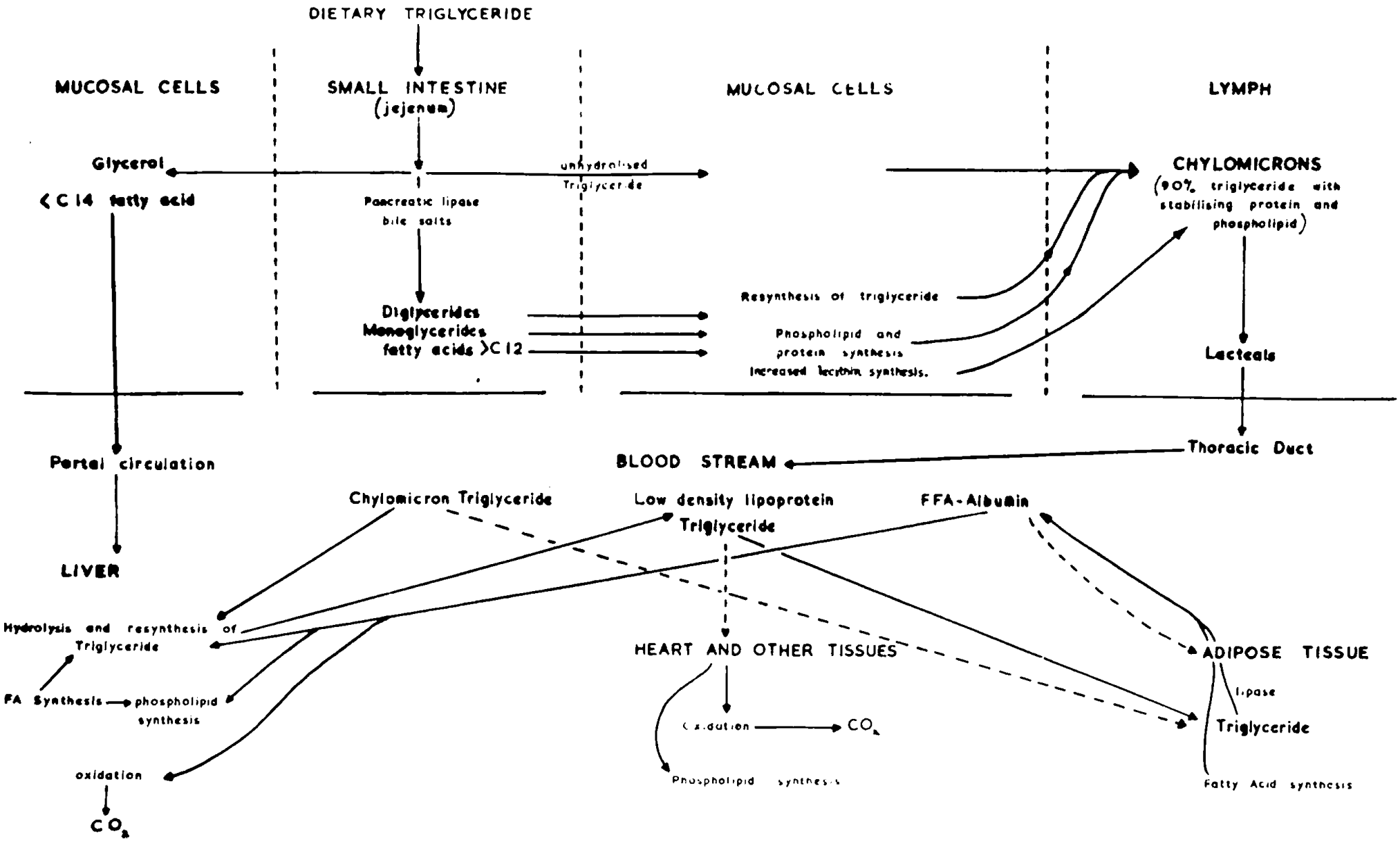


turnover of phosphatidic acid occurs, since this compound is on pathway 1. Hence Sinclair's predictions (93) concerning a specific phospholipid now seem to be correct, although the evidence is against all fatty acids passing through this pathway. When triglycerides, such as olive oil or peanut oil are fed, monoglyceride is produced by lipolysis in the lumen and this can act as precursor for the glycerol moiety of the triglyceride, and since pathway 2 does not involve phosphatidic acid, a higher turnover might not be expected in this case. Nevertheless, hydrolysis of olive oil would yield plenty of fatty acids (Borgström (229) has shown that on average 40% of triglyceride is fully hydrolysed, 10% absorbed as triglyceride, and 50% as mono- and di-glycerides), which have the possibility of using pathway 1. The present results would suggest that the monoglyceride pathway is the major one in the resynthesis of triglyceride in intestinal mucosa. This is not proved, however, and many variations on this type of experiment are required in order to establishthe relative importance of these two pathways. To modify the present experiments by feeding various fatty acids instead of oil would probably yield some useful information.

The high turnover of phosphatidic acid even in non-fat-

fig 6

GLYCERIDE METABOLISM



absorbing intestine is noteworthy; this is not altogether surprising since it is an intermediate in the synthesis of other phospholipids and it may be connected with the fact that intestine is a rapidly regenerating tissue. Zilversmit (230) has suggested that the reason why there is no increase in turnover is because the specific activity is already so close to that of its immediate precursor that no change can be observed. The highest specific activity of the phosphatidic acid in these experiments was 456 relative to the <u>total</u> acid-soluble specific activity (given a value of 1000). However, this acid-soluble fraction contains a large number of compounds of which the direct precursor of phosphatidic acid would make up only a very small part. The precursor is glycerophosphate and could originate from one of three possible sources: (1) triose phosphate from the glycolytic pathway, (2) triose phosphate from the pentose phosphate pathway, or (3) from phosphorylation of glycerol. It might be that such precursors have a specific activity well above the average specific activity of the pool, in which case the specific activity of the phosphatidic acid need not be near the "saturation point" as suggested by Zilversmit. On the other hand, if such precursors had specific activities approaching that of the

pool, the specific activity of 456 recorded for phosphatidic acid might well be too close to that of the precursor for any increase in turnover to be observable under our conditions. The data available here are insufficient for a decision between these two possibilities. The construction of precursor-product time-curves would be necessary if further work on this subject were to be carried out.

There remains the possibility that the glycerophosphate measured on chromatograms, originates not from phosphatidic acid, but lyso-phosphatidic acid. Pieringer and Hokin (21) have recently shown that monoglyceride can be phosphorylated directly to lysophosphatidic acid by a brain enzyme:

ATP + monoglyceride ⟶ lyso-phosphatidic acid + ADP

Only minute amounts are found because most is converted to phosphatidic acid by the reaction:

lyso-phosphatidic acid + fatty acyl-Co-A ⟶ phosphatidic acid + CoASH.

The reactions: 2 lyso-phosphatidic acid ⟶ phosphatidic acid + GP

lyso-phosphatidic acid ⟶ GP + Pi,

also occur. If, however, phosphatidic acid and lyso-phosphatidic acid were derived from this source, the turnover of phosphatidic acid would be expected to increase

during fat-feeding, since monoglyceride precursors would be supplied in greatly increased amount.

It is interesting to discover that problems of intestinal absorption have recently been investigated by methods quite different from the general tracer techniques. Przelcka et al (231) have used staining methods to show considerable lipid synthesis in intestinal mucosa accompanied by a marked rise in alkaline phosphatase activity, chiefly in the Golgi zone. The possibility that this enzyme complex contains phosphatidic acid phosphatase activity is being investigated. A high fat diet fed to rats has been shown to produce morphological and functional adaptations of the organs of the digestive tract, and these are induced by the volume and by the composition of the diet. The influence of saturated fatty acids is more marked than that of unsaturated fatty acids. It seems that although our insight into the pathways of fat metabolism is becoming clearer, there still remains to be unravelled a large number of complex changes in this tissue. Experimental feeding has tended to be unphysiological!

The high phosphoinositide turnover is of interest in view of the observed parallelism in many tissues between high phosphoinositide turnover and high phosphatidic acid

turnover. This effect has already been described in the introductory section on lipids and ion transport and has been discussed in its broader aspects by Hawthorne (79). The so-called "phosphatidic acid effect" seems to be a feature of secretory tissues or those involved in large scale transport of ions and other materials. Intestine is such a tissue but if one considers the suggestion of the Hokin's that such an effect is concerned with the transport of materials into or out of the cell, then it is difficult to understand why the turnover of these substances should remain constant in spite of a strong stimulation such as the application of a large quantity of fat.

CONCLUSION

The structure and physiology of animal cells are strongly dependent on membrane systems. Our knowledge is coming to the stage where distinction between structural and metabolic function is meaningless; few biochemical compounds are merely "bricks and mortar"; they exert their function by close structural co-operation with other substances in a vast molecular complex, and disruption of the unique form of this complex invariably leads to impotence of the individual molecular species.

The gross structure of these membranes, too, has been elucidated in the last two decades. Thus the importance of phospholipids in such membranes has been firmly established and a great deal of work published which demonstrates the way in which phospholipid composition and arrangement influences the properties of the membrane.

Van Deenen and his group (232) demonstrated a specific phospholipid pattern in the erythrocyte membrane of each member of a group of different species. Furthermore, there occurred a gradation in the phosphatide composition throughout the series which corresponded well with a

similar gradation in permeability properties and haemolysis times of the respective membranes. For example, in the sequence: sheep, ox, pig, man, rabbit, rat, there was a steady increase in the percentage of lecithin from 0 to 58, and a decrease in sphingomyelin content from 63% to 23%. The "cephalin" content was rather more constant, but nevertheless decreased regularly from 37% to 19%; there was a similar gradation in fatty acid composition.

A similar finding was made by Dawson and co-workers (233) and Turner et al (234). Dawson measured the composition of the phospholipid of erythrocyte "ghosts" and plasma from six species (man, pig, horse, cow, sheep and goat). The percentage of lecithin in the non-ruminant "ghosts" was over three times as high as in ruminant "ghosts", whereas the concentration of sphingomyelin and choline plasmalogen were higher in ruminant "ghosts", so that effectively there were no differences between concentrations of total choline-containing phospholipid in various species. Phosphatidylethanolamine was present in markedly higher concentration in "ghosts" of omnivores than in those of herbivores and phosphatidylinositol varied from 1.6% in man to 5.8% in the goat. Phosphatidylserine was present in relatively high concentration in all species.

In marked contrast to the pattern in the membranes, the plasma contained over 94% of choline-containing phospholipids in all species.

Groups at the Enzyme Institute, Wisconsin, (67) have studied the phospholipid composition of different sites in the mitochondrion, showing that the phospholipid mixture associated with the enzyme systems of the electron transport chain has a different composition from that of the external membrane system.

My own work emphasises the great similarity between the phospholipid pattern of the particulate liver cell fractions: nucleus, mitochondrion, and the microsomal membrane system. This is surely a reflection of the fact, established largely by electron microscopy, that all intracellular membranes are built on a similar pattern. Such differences as there are may well reflect the different processes occurring at the different membrane surfaces.

The composition of what I might loosely term "the standard membrane phospholipid mixture" could possibly have significance from the point of view of charge distribution. Calculating from figures in Table XXI, Part I. the "standard membrane phospholipid mixture" contains the following distribution of charged groups:

Phospholipid	Moles P per 100 moles P in mixture	$\overset{+}{N}(CH_3)_3$ groups	$\overset{+}{N}H_3$ groups	PO_4^- groups	CO_2^- groups
PC	58	58	--	58	--
PE	24	--	24	24	--
PS	4	--	4	4	4
PI	7	--	--	7	--
SPH	5.5	5.5	--	5.5	--
(others	1.5)				
total	100.0	63.5	28	98.5	4

Total positive charge = 91.5

Total negative charge = 102.5

Net negative charge = 11.0

This necessarily assumes that at neutral pH, the amino groups are completely ionised, which is not the case. Nevertheless this simple calculation shows that there is a net negative charge associated with the membrane phospholipid mixture and this may be important for the optimum bonding conditions with protein in the membrane or in determining the permeability properties of the membrane.

The mitochondrion alone, (as far as is known) has a

unique phospholipid component. "Cardiolipin", according to the data of Fleischer et al (67) and Marinetti et al (235) is concentrated primarily in the cytochrome oxidase enzyme complex. This component could well be absent from the external membrane, as from other cytoplasmic membranes.

As was originally predicted, the phospholipids of the microsomal membrane fraction have been proved similar, both in composition and metabolic activity, to those of the nucleus. Some differences are to be expected since the membranes of the endoplasmic reticulum are the most heterogeneous and most complex in the cell (236). In contrast to the mitochondrial membranes, the appearance and disposition of the membranes of the endoplasmic reticulum vary with the tissue and the organism; even within a single cell it is often possible to distinguish half a dozen local differentiations (236). In view of the present results, it would be interesting to find out whether there is any difference, in respect to phosphatides, between the "fixed" and "labile" portions of the nuclear membrane; this seems unlikely. Barer (64) has described the formation of the nuclear membrane (in insect spermatozoa) as follows: "The nuclear membrane appears to be formed by the fusion of vesicular elements which are probably part

of the endoplasmic reticulum. The process may require the co-operation of the mitochondria, but their exact role is not clear. The close connection between the nuclear membrane and the endoplasmic reticulum has frequently been demonstrated (237), and it appears that the two are composed of essentially the same material which can be laid down in different places according to the requirements of the cell at any given time".

It could be said that the absence of any striking differences between the metabolic activities of the different phospholipids throughout the cell is to be expected in "resting cells". Any speciality of function of any particular phospholipid might be brought to light by inflicting upon the cell some kind of 'stimulus'.

Such a stimulus was partial hepatectomy, causing the liver cells to divide at many times their normal rate. The original idea behind this work was based on the findings of Murray et al (238) that myo-inositol was capable of blocking the colchicine-induced arrest of mitosis of certain cells at metaphase. Since the phospholipid fraction which differed from all others in the liver regeneration studies of Johnson, Albert et al was the nuclear "cephalin", there was the possibility that the

inositol-containing phosphatide was exerting a special function during cell division. The present results strongly suggest that no specific phospholipid is involved in cell division.

I have discussed the similarity in phospholipid pattern within the different intracellular membranes of one tissue - liver. The similarity of this pattern to that of the intestinal mucosa is striking. Intestine is quite a different tissue, with a different function, and a rather more complex structure. Intracellular distribution studies would be of interest here, especially with regard to the brush border, which is at the site of active absorption. Before more can be learned about the difference in ^{32}P incorporation of liver and intestine, a time curve will need to be constructed for intestinal phosphatides since no comparative figures are available for intermediate times (3 - 12 hours). Indications are that they may not be so different as at first supposed.

The 'stimulus', analogous to the partial hepatectomy imposed upon the liver cell, is in this case the presentation of a large volume of neutral fat to the intestinal cell. While the present findings suggest a chiefly physical role for phospholipids (especially lecithin) during fat

absorption, further investigation of the role of phosphatidic acid and the relative importance of the phosphatidic acid and monoglyceride pathways for glyceride resynthesis is of interest. More light could be possibly thrown on the subject by the feeding of labelled fatty acids instead of triglycerides.

The cell membrane is in a dynamic state, and is therefore to be considered as a four-dimensional system, in which different chemical species function only by full co-operation with other molecules. Since this is the case, the type of chemical bonding must be important in determining the precise mode of action of each of these substances. The nature of the chemical bonding of phospholipids to proteins at the cell surface is as yet, purely conjectural. The bonds are undoubtedly strong since separation of the lipid and protein moieties often causes denaturation of the protein. That the major proportion of the bonds are ionic, can be deduced from the fact that phospholipids are extractable from lipoproteins by polar organic solvents. Such polar bonding might be expected between the negative carboxyl groups of the protein and the basic nitrogen of phosphatidylcholine, phosphatidylethanolamine, or phosphatidylserine. A small amount of bonding could be

attributable to a covalent linkage between the phospholipid base and hydroxyamino acids of the protein. Thirdly, a certain proportion of the bonding might come from weak hydrogen bonds. The quaternary ammonium ions of the phospholipid polar regions are likely to be surrounded by a zone of water molecules; -OH, -COOH, -CO, and $-\overset{+}{N}H_3$ groups of proteins would become linked to water molecules by means of hydrogen bonding; hence water molecules could act as a "glue" for binding phospholipid to protein (239). Little or nothing is known about the strength or relative importance of these different bonds.

The question of surface charge and surface pH is also likely to be of importance in membrane function. According to Danielli (240) the negative ends of phospholipid dipoles are the more likely to be oriented towards the aqueous phase. This would lead to an accumulation of cations at the membrane surface, among which would be hydrogen ions leading to a significantly lower pH at the lipid interface than in the bulk phase. This may be expected to have considerable influence on surface enzyme reactions. The finding by Bangham and Dawson (241,242), that the electrical charge on a lipid substrate can affect the substrate - enzyme reaction may be of great importance here, in that

changes at the cell surface could be brought about by cellular enzymes in response to alterations in surface charge.

This work has been an attempt to make a careful analysis of the lipid constituents of biological membranes, then to enquire into the similarities or difference between membranes of different types or from different sites. From these data, it has been possible to make deductions about the nature of the phospholipid part of membranes, especially that the nucleus yields sufficient phospholipid to supply a double membrane structure, the outer of which is less firmly bound. Following this, it was a logical step to determine the dynamic state of these various lipids, first in resting cells, and then in cells upon which some 'stimulus' had been imposed.

I think that only closer attention to, firstly, the chemical bonding between lipids and proteins, and secondly, a study of the enzyme systems bringing about phospholipid reactions and the way in which the functioning of the enzyme system depends on its lipoprotein structure, will yield more information about phospholipid metabolism and function. The logical steps would seem to lead to the construction of synthetic membrane systems using "tailor-made" phospholipid

molecules. The way is already being paved by the synthetic phospholipid studies of de Haas and van Deenen (243). The use of the *in vivo* isotope experiment can lead us to ask many questions; a more physico-chemical approach may be the logical one for the future.

APPENDIX I

The incorporation of ^{32}P into the phospholipids of rabbit liver homogenate and nuclei at different times *in vivo*.

APPENDIX I. Experiment 1. (3h. exchange)

HOMOGENATE						NUCLEI					
sample	P recovered µg	P counted µg	corrected count per 100s.	S.A.	RSA.	sample	P recovered µg	P counted µg	corrected count per 100s.	S.A.	RSA.
Acid Soluble	-	587	709,408	1210	1000						
Whole lipid	-	562	56,457	100.4	83	Whole lipid	447	297	30,088	103.0	84
PC	-	48.2	4,150	86.2	71	PC	-	47.8	6,840	143.0	118
PE	-	25.3	3,615	143.0	118	PE	-	23.8	3,320	139.5	115
PS	-	6.5	162	24.9	21	PS	-	8.0	111	13.9	12
PI	-	4.7	615	131.0	108	PI	-	4.5	995	222.0	183
PA	-	<1.0	423	>423.0	>349	PA	-	<1.0	394	>394.0	>325

APPENDIX I. Experiment 2. (6h. exchange)

HOMOGENATE						NUCLEI					
sample	P recovered µg	P counted µg	corrected count per 100s.	S.A.	RSA.	sample	P recovered µg	P counted µg	corrected count per 100s.	S.A.	RSA.
Acid Soluble	-	513	705,059	1370	1000						
Whole lipid	-	428	73,400	172	126	Whole lipid	228	145	18,750	129.0	95
PC	-	41.3	5,580	135.0	99	PC	-	46.3	9,900	213.0	156
PE	-	24.5	7,670	313.0	228	PE	-	20.2	5,200	257.0	188
PS	-	4.7	294	62.5	46	PS	-	4.1	318	77.5	57
PI	-	3.4	652	191.5	140	PI	-	3.0	850	284.0	206

APPENDIX I. Experiment 3. (6h. exchange)

HOMOGENATE						NUCLEI					
sample	P recovered µg	P counted µg	corrected count per 100s.	S.A.	RSA.	sample	P recovered µg	P counted µg	corrected count per 100s.	S.A.	RSA.
Acid Soluble	-	780	865,000	1110	1000						
Whole lipid	3250	650	86,400	134.0	120	Whole lipid	403	255	40,900	160.0	144
PC	-	41.2	6,200	150.5	136	PC	-	54.6	11,000	202.0	182
PE	-	28.0	3,910	139.5	125	PE	-	34.3	5,810	169.0	153
PS	-	4.0	.110	27.5	25	PS	-	4.3	247	58.1	52
PI	-	3.3	443	134.0	121	PI	-	3.8	893	235.0	212
PA	-	<1.0	207	>207.0	>186	PA	-	<1.0	194	>194.0	>175

APPENDIX I. Experiment 4. (9h. exchange)

HOMOGENATE						NUCLEI					
sample	P recovered µg	P counted µg	corrected count per 100s.	S.A.	RSA.	sample	P recovered µg	P counted µg	corrected count per 100s.	S.A.	RSA.
Acid Soluble	-	320	266,000	832.0	1000						
Whole lipid	-	688	117,500	171.0	206	Whole lipid	461	242	59,700	246.0	296
PC	-	37.0	7,670	207.0	249	PC	-	43.7	16,200	371.0	446
PE	-	21.6	9,000	416.0	500	PE	-	25.8	11,800	459.0	550
PS	-	3.0	273	91.0	110	PS	-	4.2	204	48.7	59
PI	-	3.3	545	165.0	198	PI	-	2.5	877	351.0	422
						PA	-	<1.0	282	>282.0	>327

APPENDIX II

The incorporation of ^{32}P into the phospholipids of the mitochondrial and microsomal fractions of rat liver at different times *in vivo*.

APPENDIX II. Experiment 1. (3h. exchange)

MITOCHONDRIAL FRACTION						MICROSOMAL FRACTION					
sample	P recovered µg	P counted µg	corrected count per 100s.	S.A.	RSA.	sample	P recovered µg	P counted µg	corrected count per 100s.	S.A.	RSA.
Acid Soluble	-	92	29,551	321	1000	Acid Soluble	-	76	18,651	245	1000
Whole lipid	1000	500	16,378	32.8	101	Whole lipid	1334	667	23,045	34.6	141
PC	-	58.5	2,310	39.4	123	PC	-	67.5	2,940	43.5	177
PC	-	54.3	2,120	38.9	121	PC	-	72.5	3,260	45.0	183
PE	-	40.2	3,720	92.5	288	PE	-	33.3	4,000	120.5	492
PE	-	39.0	3,420	87.6	273	PE	-	36.6	4,310	117.5	479
PS	-	8.6	41	4.8	15	PS	-	6.1	36	5.9	24
PI	-	6.5	89	13.7	43	PI	-	7.8	161	20.6	84
PA	-	<1.0	90	>90.0	>280	PA	-	<1.0	50	>50	>204
CL	-	4.8	51	10.7	33						

APPENDIX II. Experiment 2. (6h. exchange)

MITOCHONDRIAL FRACTION						MICROSOMAL FRACTION					
sample	P recovered µg	P counted µg	corrected count per 100s.	S.A.	RSA.	sample	P recovered µg	P counted µg	corrected count per 100s.	S.A.	RSA.
Acid Soluble	-	85	56,632	667	1000	Acid Soluble	-	58	20,585	355	1000
Whole lipid	984	492	44,080	89.8	134	Whole lipid	1,892	947	92,690	98.0	276
PC	-	54.6	3,525	64.3	97	PC	-	62.0	4,575	73.9	208
PC	-	60.0	3,930	65.7	99	PC	-	67.6	5,100	75.3	212
PE	-	37.7	3,910	103.5	155	PE	-	19.6	2,975	152.0	427
PE	-	35.8	3,925	109.5	164	PE	-	23.8	3,350	140.0	394
PS	-	3.7	19	5.1	8	PS	-	4.2	50	11.8	32
PI	-	4.2	-	-	-	PI	-	5.7	169	29.7	84
PA	-	-	-	-	-	PA	-	<1.0	75	>75.0	>211

APPENDIX II. Experiment 3. (6h. exchange)

MITOCHONDRIAL FRACTION						MICROSOMAL FRACTION					
sample	P recovered µg	P counted µg	corrected count per 100s.	S.A.	RSA.	sample	P recovered µg	P counted µg	corrected count per 100s.	S.A.	RSA.
Acid Soluble	400	100	4,650	46.5	1000	Acid Soluble	244	61	1,770	29.1	1000
Whole lipid	934	540	7,030	13.1	281	Whole lipid	1302	823	11,700	14.2	489
PC	-	44.3	595	13.4	288	PC	-	36.5	561	15.4	528
PC	-	43.8	542	12.4	266	PC	-	50.3	707	14.0	480
PE	-	29.8	705	23.6	506	PE	-	17.4	590	33.8	1161
PE	-	34.2	830	23.3	500	PE	-	25.4	738	29.0	1000
PS	-	4.0	17	4.3	92	PS	-	7.5	36	4.8	164
PI	-	4.0	10	2.6	55	PI	-	5.0	39	7.9	270
PA	-	-	-	-	-	PA	-	-	-	-	-
CL	-	-	-	-	-						

APPENDIX II. Experiment 4. (9h. exchange)

MITOCHONDRIAL FRACTION						MICROSOMAL FRACTION					
sample	P recovered µg	P counted µg	corrected count per 100s.	S.A.	RSA.	sample	P recovered µg	P counted µg	corrected count per 100s.	S.A.	RSA.
Acid Soluble	-	201	10,132	50.4	1000	Acid Soluble	-	407	14,043	34.5	1000
Whole lipid	707	350	12,900	36.9	733	Whole lipid	1506	625	27,110	43.3	1250
PC	-	55.6	1,858	33.4	663	PC	-	59.5	2,050	34.4	1000
PC	-	64.7	2,170	33.5	665	PC	-	77.5	2,750	35.4	1025
PE	-	33.7	1,770	52.6	1022	PE	-	21.7	1,330	61.4	1780
PE	-	43.8	2,080	47.6	945	PE	-	28.0	1,780	63.7	1840
PS	-	-	-	-	-	PS	-	6.3	51	8.0	233
PI	-	3.0	70	23.3	462	PI	-	-	-	-	-
PA	-	<1.0	15	>15.0	>296	PA	-	<1.0	34	>34.0	>990
PA	-	<1.0	13	>13.0	>257	PA	-	<1.0	60	>60.0	>1740
CL	-	4.6	24	5.3	105						

APPENDIX II. Experiment 5. (9h. exchange)

MITOCHONDRIAL FRACTION						MICROSOMAL FRACTION					
sample	P recovered µg	P counted µg	corrected count per 100s.	S.A.	RSA.	sample	P recovered µg	P counted µg	corrected count per 100s.	S.A.	RSA.
Acid Soluble	480	120	9,780	81.3	1000	Acid Soluble	352	88	5,670	64.4	1000
Whole lipid	844	442	31,110	70.3	865	Whole lipid	739	308	23,190	75.0	1160
PC	-	44.6	3,000	67.3	828	PC	-	48.0	3,460	72.0	1120
PC	-	38.5	2,750	71.5	880	PC	-	49.3	3,540	71.9	1115
PE	-	21.5	1,930	89.5	1100	PE	-	17.0	1,560	92.0	1430
PE	-	23.1	1,860	80.5	990	PE	-	17.3	1,575	91.0	1410
PS	-	5.3	82	15.6	194	PS	-	-	-	-	-
PI	-	-	-	-	-	PI	-	2.1	41	19.5	303
PA	-	<1.0	232	>232	>2850	PA	-	<1.0	50	>50	>780
CL	-	2.2	79	35.6	429						

APPENDIX II. Experiment 6. (12h. exchange)

MITOCHONDRIAL FRACTION						MICROSOMAL FRACTION					
sample	P recovered µg	P counted µg	corrected count per 100s.	S.A.	RSA.	sample	P recovered µg	P counted µg	corrected count per 100s.	S.A.	RSA.
Acid Soluble	-	75	74,387	995	1000	Acid Soluble	-	52.5	42,635	813	1000
Whole lipid	835	345	254,505	738.0	744	Whole lipid	1586	755	782,756	1038.0	1275
PC	-	34.3	23,400	683.0	687	PC	-	52.5	40,200	765.0	940
PC	-	42.1	27,500	655.0	658	PC	-	49.6	37,100	749.0	920
PE	-	32.0	22,750	714.0	718	PE	-	14.3	10,800	755.0	930
PE	-	24.3	19,400	797.0	800	PE	-	15.7	12,250	780.0	960
PS	-	6.2	1,082	175.0	176	PS	-	3.3	1,030	313.0	384
PI	-	5.3	1,310	247.0	248	PI	-	4.7	1,880	400.0	491
PA	-	<1.0	1,230	>1230	>1240	PA	-	< 1.0	1,180	>1180	>1450
CL	-	5.0	580	116.0	116						

APPENDIX II. Experiment 7. (19h. exchange)

MITOCHONDRIAL FRACTION						MICROSOMAL FRACTION					
sample	P recovered µg	P counted µg	corrected count per 100s.	S.A.	RSA.	sample	P recovered µg	P counted µg	corrected count per 100s.	S.A.	RSA.
Acid Soluble	-	156	17,600	112.5	1000	Acid Soluble	-	51	3,900	76.5	1000
Whole lipid	919	515	38,900	75.6	672	Whole lipid	1132	488	40,600	83.5	1090
PC	-	37.8	3,475	92.0	818	PC	-	46.8	4,780	102.0	1335
PE	-	29.6	3,340	112.5	1000	PE	-	22.8	2,680	117.5	1540
PS	-	3.8	164	40.5	360	PS	-	2.5	173	69.2	905
PI	-	3.6	95	26.2	233	PI	-	3.6	128	35.7	467
CL	-	7.5	143	19.1	170	PA	-	<1.0	92	>92.0	>1200

APPENDIX II. Experiment 8. (19h. exchange)

MITOCHONDRIAL FRACTION						MICROSOMAL FRACTION					
sample	P recovered μg	P counted μg	corrected count per 100s.	S.A.	RSA.	sample	P recovered μg	P counted μg	corrected count per 100s.	S.A.	RSA.
Acid Soluble	496	124	15,750	138.0	1000	Acid Soluble	114	38	3,600	95.0	1000
Whole lipid	772	385	36,100	93.8	680	Whole lipid	968	430	39,907	92.9	980
PC	-	38.3	4,750	124.0	900	PC	-	48.3	6,200	128.0	1345
PE	-	32.3	4,650	144.0	1042	PE	-	22.5	3,530	156.5	1650
PS	-	-	-	-	-	PS	-	2.2	210	95.6	1050
PI	-	2.8	375	133.5	970	PI	-	3.8	233	61.3	645
						PA	-	<1.0	127	>127.0	>1330

APPENDIX III

The incorporation of ^{32}P into the phospholipids of cell fractions of regenerating liver.

NUCLEI: UNOPERATED CONTROLS AND REGENERATING LIVER

APPENDIX III. Experiment 1.

NUCLEI: UNOPERATED CONTROLS*						NUCLEI: REGENERATING LIVER					
sample	P recovered µg	P counted µg	corrected count per 100s.	S.A.	RSA.	sample	P recovered µg	P counted µg	corrected count per 100s.	S.A.	RSA.
Acid Soluble	-	10	505	50.5	1000	Acid Soluble* Group I	-	1.6	668	417.0	1000
						Acid Soluble** Group II	-	3.2	3,100	966.0	1000
Whole lipid	203	2.9	113	38.9	770	Whole lipid Group I	-	9.3	3,580	385.0	925
						Whole lipid Group II	-	12.3	5,680	462.0	478
PC	-	27.2	1,158	42.5	845	PC	-	34.8	14,750	424.0	613
PC	-	27.0	1,135	42.0	833	PC	-	37.9	16,600	438.0	632
PE	-	10.8	483	44.9	885	PE	-	13.8	6,330	459.0	662
PE	-	11.8	535	45.4	895	PE	-	13.2	5,900	446.0	644
PS	-	3.7	10	2.7	54	PS	-	5.9	590	100.0	144
PI	-	3.3	91	27.6	546	PI	-	3.7	1,195	323.0	466
PA	-	<1.0	36	>36.0	>712	PA	-	<1.0	424	>424.0	>613
*	Mean results from 10 animals.					*	Mean result from 10 animals.				
						**	Mean result from 9 animals.				

APPENDIX IV

The incorporation of ^{32}P into the phospholipids of cell fractions of regenerating liver.

MITOCHONDRIAL AND MICROSOMAL FRACTIONS: UNOPERATED CONTROLS.

APPENDIX IV. Experiment 1.

MITOCHONDRIAL FRACTION						MICROSOMAL FRACTION					
sample	P recovered μg	P counted μg	corrected count per 100s.	S.A.	RSA.	sample	P recovered μg	P counted μg	corrected count per 100s.	S.A.	RSA.
Acid Soluble	-	22.8	25,750	1130	1000	Acid Soluble	-	22.5	11,950	532.0	1000
Whole lipid	-	16.1	11,050	686.0	608	Whole lipid	-	23.3	21,300	915.0	1820
PC	-	38.0	28,700	755.0	666	PC	-	54.3	43,600	805.0	1510
PC	-	34.0	26,400	775.0	684	PC	-	54.0	44,400	820.0	1520
PE	-	18.8	23,200	1240.0	1095	PE	-	14.8	19,700	1335	2500
PE	-	19.7	25,400	1285.0	1140	PE	-	19.1	25,900	1355	2550
PS	-	3.4	538	158.0	140	PS	-	4.2	773	185.0	347
PI	-	3.7	1,160	314.0	279	PI	-	4.3	990	230.0	432
PA	-	<1.0	1,830	>1830	>1620	PA	-	<1.0	2,210	>2210	>4150
CL	-	5.1	438	86.0	76						

APPENDIX V

The incorporation of ^{32}P into the phospholipids of cell fractions of regenerating liver.

MITOCHONDRIAL AND MICROSOMAL FRACTIONS: SHAM-OPERATED CONTROLS.

APPENDIX V. Experiment 1.

MITOCHONDRIAL FRACTION						MICROSOMAL FRACTION					
sample	P recovered µ g	P counted µ g	corrected count per 100s.	S.A.	RSA.	sample	P recovered µ g	P counted µ g	corrected count per 100s.	S.A.	RSA.
Acid Soluble	-	15.4	10,220	665.0	1000	Acid Soluble	-	15.2	4,640	305.0	1000
Whole lipid	-	12.2	6,290	515.0	775	Whole lipid	-	13.1	7,140	544.0	1785
PC	-	38.4	16,400	428.0	642	PC	-	52.1	26,700	512.0	1675
PC	-	37.8	15,700	416.0	625	PC	-	48.9	24,600	503.0	1670
PE	-	21.8	13,400	616.0	926	PE	-	20.4	16,100	790.0	2590
PE	-	21.4	13,000	610.0	928	PE	-	19.1	15,100	790.0	2590
PS	-	5.2	337	64.8	97	PS	-	4.8	425	88.3	290
PI	-	2.3	382	165.5	249	PI	-	4.5	663	147.0	482
PA	-	-	-	-	-	PA	-	-	-	-	-
CL	-	6.1	475	78.1	118						

APPENDIX V. Experiment 2.

MITOCHONDRIAL FRACTION						MICROSOMAL FRACTION					
sample	P recovered µg	P counted µg	corrected count per 100s.	S.A.	RSA.	sample	P recovered µg	P counted µg	corrected count per 100s.	S.A.	RSA.
Acid Soluble	-	308	64,500	209.0	1000	Acid Soluble	-	144	18,350	127.5	1000
Whole lipid	968	432	45,300	105.0	501	Whole lipid	1208	485	54,100	111.5	875
PC	-	40.7	4,850	119.0	569	PC	-	42.0	4,425	105.0	823
PC	-	28.8	3,130	108.5	520	PC	-	34.6	4,200	121.5	950
PE	-	31.8	5,710	180.0	860	PE	-	21.6	4,140	191.0	1500
PE	-	29.7	5,400	181.5	870	PE	-	9.4	1,819	193.5	1515
PS	-	4.7	131	27.0	133	PS	-	6.2	59	9.7	76
PI	-	5.3	312	59.0	282	PI	-	2.6	87	33.4	262
PA	-	1.8	-	-	-	PA	-	-	-	-	-
CL	-	6.1	-	-	-						

APPENDIX V. Experiment 3.

MITOCHONDRIAL FRACTION						MICROSOMAL FRACTION					
sample	P recovered µg	P counted µg	corrected count per 100s.	S.A.	RSA.	sample	P recovered µg	P counted µg	corrected count per 100s.	S.A.	RSA.
Acid Soluble	-	318	110,500	347.5	1000	Acid Soluble	-	115	25,500	221.0	1000
Whole lipid	1086	517	87,300	169.0	486	Whole lipid	880	398	74,000	186.0	842
PC	-	38.6	5,620	145.0	419	PC	-	54.5	8,850	162.0	735
PC	-	42.5	6,980	164.0	472	PC	-	54.2	8,720	160.5	729
PE	-	29.5	8,000	271.5	781	PE	-	21.5	7,030	326.0	1475
PE	-	26.3	7,820	297.0	854	PE	-	19.3	6,150	318.0	1440
PS	-	3.6	147	40.8	117	PS	-	5.7	176	30.8	139
PI	-	6.2	-	-	-	PI	-	3.8	152	40.1	181
PA	-	-	-	-	-	PA	-	-	-	-	-
CL	-	6.6	-	-	-						

APPENDIX VI

The incorporation of ^{32}P into the phospholipids of cell fractions of regenerating liver.

MITOCHONDRIAL AND MICROSOMAL FRACTIONS: REGENERATING LIVER.

APPENDIX VI. Experiment 1.

MITOCHONDRIAL FRACTION						MICROSOMAL FRACTION					
sample	P recovered µg	P counted µg	corrected count per 100s.	S.A.	RSA.	sample	P recovered µg	P counted µg	corrected count per 100s.	S.A.	RSA.
Acid Soluble	-	140	21,000	150.0	1000	Acid Soluble	-	133	10,400	78.2	1000
Whole lipid	813	387	52,000	134.0	895	Whole lipid	816	320	46,200	144.4	1845
PC	-	46.2	7,750	168.0	1120	PC	-	39.9	5,760	144.2	1840
PC	-	45.7	7,500	164.0	1090	PC	-	47.9	6,880	143.5	1835
PE	-	27.4	7,960	290.0	1930	PE	-	20.8	4,870	233.5	2990
PE	-	26.2	7,080	270.0	1800	PE	-	17.5	4,200	240.0	3060
PS	-	3.3	169	51.2	341	PS	-	4.3	141	32.8	418
PI	-	5.6	350	62.2	416	PI	-	3.9	299	76.8	980
PA	-	<1.0	107	>107	>711	PA	-	<1.0	87	>87	>1110
CL	-	4.2	178	42.3	282						

APPENDIX VI. Experiment 2.

MITOCHONDRIAL FRACTION						MICROSOMAL FRACTION					
sample	P recovered µg	P counted µg	corrected count per 100s.	S.A.	RSA.	sample	P recovered µg	P counted µg	corrected count per 100s.	S.A.	RSA.
Acid Soluble	-	145	39,000	268.5	1000	Acid Soluble	-	92	13,800	150.0	1000
Whole lipid	879	433	81,500	188.0	701	Whole lipid	498	258	49,700	192.0	1280
PC	-	38.7	5,080	131.0	489	PC	-	42.7	7,450	175.0	1165
PC	-	34.3	4,540	132.0	494	PC	-	44.3	7,500	169.0	1125
PE	-	25.2	5,140	204.0	760	PE	-	17.8	5,830	327.0	2180
PE	-	18.8	3,790	201.5	750	PE	-	13.8	4,975	360.0	2390
PS	-	3.8	130	34.3	128	PS	-	2.3	98	42.6	284
PI	-	3.9	241	61.8	230	PI	-	5.6	356	63.6	423
PA	-	<1.0	56	>56	>208	PA	-	<1.0	34	>34	>226
CL	-	3.8	103	27.1	101						

APPENDIX VI. Experiment 3.

MITOCHONDRIAL FRACTION						MICROSOMAL FRACTION					
sample	P recovered µg	P counted µg	corrected count per 100s.	S.A.	RSA.	sample	P recovered µg	P counted µg	corrected count per 100s.	S.A.	RSA.
Acid Soluble	-	52	50,300	969.0	1000	Acid Soluble	-	29	19,700	679.0	1000
Whole lipid	558	223	102,400	458.0	474	Whole lipid	705	282	156,000	552.0	814
PC	-	49.3	21,300	432.5	446	PC	-	51.3	24,800	483.0	710
PC	-	45.1	19,675	437.0	450	PC	-	49.0	24,600	503.0	741
PE	-	33.3	17,950	538.5	555	PE	-	25.2	15,200	602.0	886
PE	-	30.8	17,000	552.0	570	PE	-	24.3	16,800	690.0	1020
PS	-	4.0	262	65.6	68	PS	-	4.7	362	77.0	114
PI	-	3.3	700	212.0	219	PS	-	2.0	343	171.0	252
PI	-	2.2	495	224.0	232	PI	-	2.8	579	206.0	305
PA	-	< 1.0	231	> 231	> 238	PI	-	1.7	399	234.0	346
CL	-	4.0	278	69.8	72	PA	-	< 1.0	163	> 163	> 241
CL	-	3.3	297	90.2	93						

APPENDIX VI. Experiment 4.

MITOCHONDRIAL FRACTION						MICROSOMAL FRACTION					
sample	P recovered µg	P counted µg	corrected count per 100s.	S.A.	RSA.	sample	P recovered µg	P counted µg	corrected count per 100s.	S.A.	RSA.
Acid Soluble	-	7.7	2,330	303.0	1000	Acid Soluble	-	3.3	889	269.0	1000
Whole lipid	-	13.7	2,240	163.5	539	Whole lipid	-	14.1	2,890	205.0	762
PC	-	47.1	8,950	189.7	625	PC	-	60.3	11,650	193.5	720
PC	-	47.5	9,170	192.5	634	PC	-	56.9	10,850	191.0	710
PE	-	24.3	6,370	262.0	864	PE	-	27.8	8,640	310.0	1150
PE	-	25.8	6,780	261.0	863	PE	-	27.1	8,430	310.0	1150
PS	-	3.0	70	23.4	77	PS	-	4.0	123	30.8	114
PI	-	3.6	256	71.3	235	PI	-	4.3	344	80.0	294
PA	-	<1.0	89	>89	>294	PA	-	<1.0	193	>193	>718
CL	-	3.8	102	27.0	89						

MITOCHONDRIAL FRACTION

sample	P recovered µg	P counted µg	corrected count per 100s.	S.A.	RSA.
Acid Soluble	-	5.3	1,180	223.0	1000
Whole lipid	-	10.4	1,213	117.0	525
PC	-	50.8	6,710	132.2	595
PC	-	48.2	6,190	128.2	575
PE	-	29.0	5,490	189.0	846
PE	-	26.3	4,730	179.5	807
PS	-	1.7	27	15.9	71
PI	-	3.4	221	65.0	291
PA	-	<1.0	34	>34	>153
CL	-	4.3	51	11.9	53

Experiment 5.

MICROSOMAL FRACTION

sample	P recovered µg	P counted µg	corrected count per 100s.	S.A.	RSA.
Acid Soluble	-	3.0	552	184.5	1000
Whole lipid	-	11.7	1,390	118.7	644
PC	-	61.8	8,520	138.0	747
PC	-	56.9	7,920	139.0	755
PE	-	25.1	5,600	222.5	1210
PE	-	26.8	5,630	210.0	1140
PS	-	5.5	128	23.4	127
PI	-	5.9	410	69.5	376
PA	-	<1.0	39	>39	>211

APPENDIX VII

Published work.

APPENDIX VIII

ABBREVIATIONS USED IN THIS THESIS

1. Lipids

PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PS	Phosphatidylserine
PI	Phosphatidylinositol
PA	Phosphatidic acid
CL	Cardiolipin (Diphosphatidylglycerol)
SPH	Sphingomyelin
DPI	Diphosphoinositide (phosphatidylinositol phosphate)
TPI	Triphosphoinositide (phosphatidylinositol diphosphate)
TG	Triglyceride
DG	Diglyceride
MG	Monoglyceride
L	Total lipid mixture

2. Phospholipid hydrolysis products

GPC	Glycerylphosphorylcholine
GPE	Glycerylphosphorylethanolamine
GPS	Glycerylphosphorylserine

Phospholipid hydrolysis products (continued)

GPS	Glycerylphosphorylserine
GPI	Glycerylphosphorylinositol
GP	Glycerophosphate
GPGPG	Diglycerylphosphorylglycerol
GPIP	Glycerylphosphorylinositol phosphate
GPIPP	Glycerylphosphorylinositol diphosphate

3. Non-lipids

TCA	Trichloroacetic acid
AS.	Acid-soluble fraction
DNA	Deoxyribonucleic acid
Pi.	Inorganic phosphate
ATP	Adenosine triphosphate
C/M	Chloroform-methanol

4. Other abbreviations

S.A.	Specific activity
R.S.A.	Relative specific activity

All other abbreviations are found in Biochimica et Biophysica Acta, Suggestions and instructions to authors, Elsevier Publishing Company, Amsterdam. The arrangement of references is also that of Biochimica et Biophysica Acta,

and details of journals quoted can be found in "List of periodicals abstracted by chemical abstracts", Chemical Abstracts Service, Columbus, U.S.A.

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