A THESIS

SUBMITTED

by

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A Candidate for the Degree of

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

Publications, pp. 200-201

BIOSYNTHESIS OF DIPHOSPHOINOSITIDE

To

MY PARENTS

" ..... The formation of lecithin thus demands relatively much ATP; at times the organism seems to waste its energy-rich phosphate."

Karlson (1963).

SYNOPSIS.

1. A "mitochondrial" fraction from brain, incubated with $^{32}P_i$, synthesised di- and tri-phosphoinositides.(DPI and TPI).

2. A mitochondrial fraction from liver, incubated under similar conditions, synthesised DPI.

3. The synthesis was associated with the presence of mitochondria.

4. Oligomycin prevented the labelling of both ATP and DPI. Thus, DPI is not involved in mitochondrial divalent cation accumulation.

5. Phosphatidylinositol (PI) can be phosphorylated to DPI by subcellular fractions from liver, with γ-^{32}P-ATP as the phosphate donor.

6. The biosynthesis of DPI requires Mg^{++} (or Mn^{++}) ions, PI and ATP.

7. The synthesis occurs mainly in the nuclear and microsomal fractions from rat liver. The mitochondrial fraction is almost inactive.

8. Marker enzymes studies have shown that the distributions of DPI synthesising activity and 5'-nucleotidase are very similar, indicating that DPI synthesis may be localised in the plasma membrane of the liver cell.

9.The possibility that DPI and TPI may be characteristic constituents of plasma membrane systems is discus-ed in relation to high turnover rates and possible functions.

ACKNOWLEDGEMENTS

This is an account of original work in the Department of Medical Biochemistry and Pharmacology. I am grateful to Professor A.C.Frazer for his interest and support and to the Medical Research Council for a Research Studentship.

Any credit for the work herein must belong mainly to my supervisor, Dr. J.N.Hawthorne, who combined great patience with just the right degree of encouragement and discipline. Many of my colleagues gave invaluable help and advice, particularly Drs. R.Coleman, T.Galliard, G.Hübscher, M.Kai and P.Kemp and Mr. D.N.Brindley. Mrs. B.Evans and Mrs. Johnson saved must valuable time by the alacrity with which they washed up behind me.

The effort of preparing this Thesis was considerably reduced by the timely publication of reviews by Ansell and Hawthorne, Hawthorne and Kemp, and Hawthorne. Miss Elizabeth Evans helped in the preparation of an initial typescript. The more beautiful of the figures were drawn by Miss Pauline Taylor and all were copied by Mrs. D.Clarke. I thank them all, and also I thank all, whether named above or not, who helped to make these three years so enjoyable.

CONTENTS.

DISCUSSION (continued). PAGE

ABBREVIATIONS

ABBREVIATIONS

Generally accepted abbreviations (ATP, etc.) are not listed here.

Others used herein are:

Phospholipids (and their deacylation products).

PS	phosphatidylserine	(GPS)
PC	phosphatidylcholine	(GPC)
PE	phosphatidylethanolamine	(GPE)
PI	phosphatidylinositol	(GPI)
DPI	diphosphoinositide	(GPIP)
TPI	triphosphoinositide	($GPIP_2$)
PA	phosphatidic acid	(3-GP)*
	cardiolipin	(GPGPG)

Other phosphate esters.

IP, IP_2, IP_3	inositol mono-, di- and triphosphates respectively.
I-4,5-P_2, I-1,5,P_2, I-1,4-P_2	the appropriate inositol-n,n'-diphosphates.
I-1,4,5-P_3, I-2,4,5,-P_3	the appropriate inositol-n,n',n''-triphosphates.
3-GP* and 2-GP*	3- and 2- glycerophosphates, respectively.
G-6-P	glucose-6-phosphate.

* Footnote: the convention of Hirschmann (1960) is used, in which L-3-GP becomes 3-GP.

Miscellaneous

P_i	inorganic orthophosphate.
TCA	trichloroacetic acid.
AF	ammonium formate.
FA	formic acid.
TLC	thin-layer chromatography.
INT	2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl-tetrazolium chloride.

INTRODUCTION

INTRODUCTION:

Occurrence of Inositol.

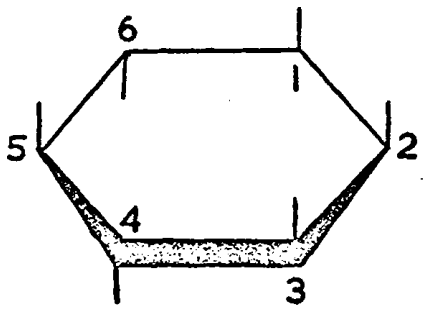
Scherer first isolated inositol from beef muscle in 1850 and after this initial appearance its presence was reported in a variety of organisms. The first indication that inositol might have any metabolic significance was its identification as a constituent of "Bios", a growth factor for micro-organisms (Eastcott, 1928). This observation first stimulated interest in inositol and paved the way for the development of very valuable methods for microbiological assay.

Bound inositol was first reported by Rosenburger (1908), but the nature of the compound involved remained obscure until inositol was recognised as a component of the lipids of bacteria (Anderson and Roberts, 1930) and of plants (Klenk and Sakai, 1939) and finally animal tissues (Folch and Woolley, 1942).

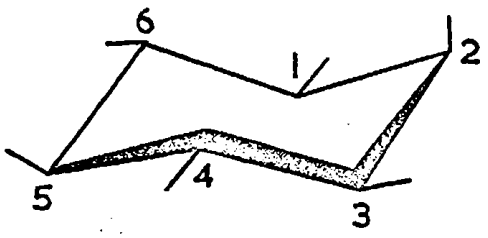
Inositol (hexahydroxycyclohexane) has nine possible isomers, all of which have been chemically synthesised (see review by Angyal and Anderson, 1959). Only two of these isomers, myo- and scyllo-, have been shown to occur in animal tissue. It is however the myo- isomer which is the most effective growth factor and it is only this which has been detected in phospholipids: even in tissues rich in scyllo-inositol it does not occur as a lipid constituent (Hawthorne, 1961). Future reference to inositol in this thesis will refer to myo-

inositol specifically.

Inositol is shown below in the usual planar representation (Ia) and in the preferred "chair" form, with a single axial hydroxyl group at the 2 position (Ib).



Ia



Ib

Biosynthesis of inositol.

Although some micro-organisms and some cells in tissue culture (Eagle et al. 1957) show an absolute requirement for inositol, studies with intact animals do not show such clear-cut results. This field has been reviewed by Cunha (1954). Whatever the needs for exogenous inositol may be, though, it has recently become clear that inositol biosynthesis occurs in fungi, higher plants and in animals. There was doubt for some time about the conclusion that inositol synthesis takes place in the rat, but this was recently dispelled by the demonstration that germ-free rats do convert glucose to inositol

(Freinkel and Dawson, 1961). Slices of rat kidney and brain have since been shown to synthesise myo- inositol (Hauser, 1963).

Little reference will be made here to early work on inositol biosynthesis, as much new light has recently been shed on this problem, mainly by the use of sophisticated chemical techniques to analyse isotope distribution in inositol synthesised from specifically labelled glucose.

In 1945 Fischer suggested that inositol could be produced by direct cyclisation of the glucose molecule, or possibly of glucose-6-phosphate. The situation remained relatively static from that time until Charalampous (1957) found evidence that this pathway might not be operative in the yeast Torulopsis utilis. However, the situation has since then changed completely. Several groups of workers, including that of Charalampous, have shown conversion of ^{14}C-glucose specifically labelled in the 1, 2 or 6 position, to ^{14}C-inositol, specifically labelled in the 6, 5 or 1 positions, respectively. Evidence for the cyclisation of glucose and its conversion to inositol as an intact unit has been found in yeast (Chen and Charalampous, 1964), Sinapsis alba (Kindl and Hoffman-Ostenhof, 1964), parsley leaves (Loewus et al., 1962; Loewus and Kelly, 1962), the intact rat (Imai, 1963) and in slices and homogenates of rat seminal vesicles and testes (Imai, 1964; Eisenberg et al., 1964). In general the degree of specificity with which the cyclisation has

occurred has been such that more than 60% of the ^{14}C has been localised in the expected carbon atom of the inositol. In some cases isotope localisations of better than 90% of the correct carbon atom have been found.

So far the best-characterised system is that of Chen and Charalampous (1964). They have purified a yeast enzyme system 100-fold, till it contained no glycolytic or pentose phosphate pathway enzymes except for traces of phosphoglucomutase and/or phosphohexose-isomerase. This preparation converted 1-^{14}C-glucose to 6-^{14}C-inositol with 99% localisation of the isotope. In the conversion of 6-^{14}C-glucose to 1-^{14}C-inositol no ^{14}C was found in other than the 1-position of the synthesised inositol. The cyclisation required NAD and Mg^{++} ions and was stimulated by NH_4^+ ions, (K^+ was less effective and Na^+ ineffective). Other metals inhibiting the system (with decreasing efficiency) were: Ag^+; Hg^{++}; Cu^{++}; Zn^{++}; and Co^{++}. A feature of this enzyme was that it showed a preference for glucose-6-phosphate over glucose as its substrate. Eisenberg and Bolden (1965) have shown that a heat-treated homogenate of rat testis (Eisenberg and Bolden, 1963) also shows a specific requirement for glucose-6-phosphate.

The next major question, whether phosphate remained attached during the reaction sequence, was recently answered by Chen and Charalampous (1965 a, b), who showed that if they

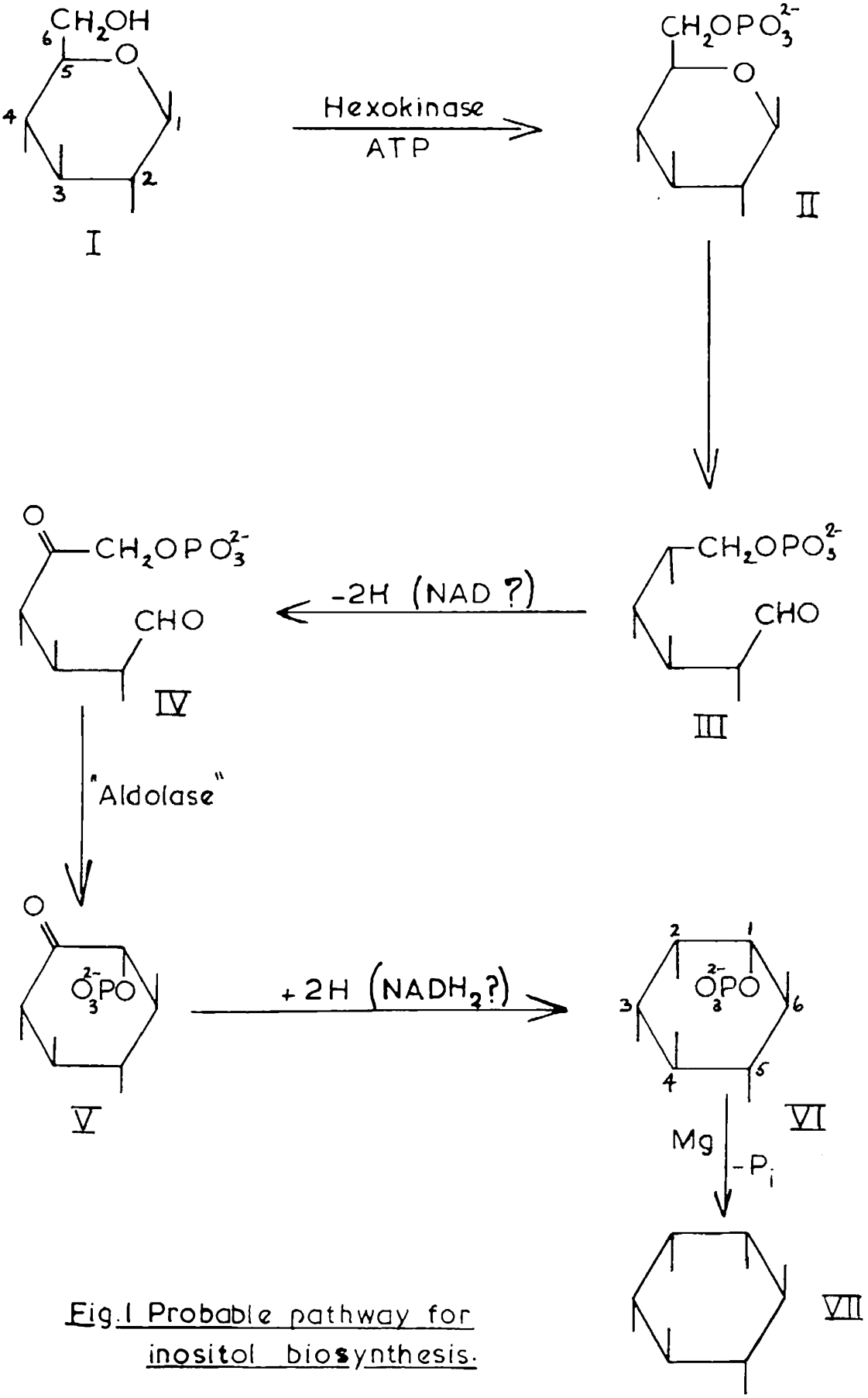


Fig.1 Probable pathway for inositol biosynthesis.

omitted Mg^{++} ions from their incubations they got conversion of glucose-6-phosphate, not to inositol, but to inositol-1-phosphate. The stereochemistry of this product has not yet been established, but starting from D-glucose-6-phosphate it is almost certainly D-inositol-1-phosphate, which is identical with L-inositol-3-phosphate. NAD and NH_4^+ were required for the glucose-6-phosphate to inositol-1-phosphate conversion, which was inhibited by -SH inhibitors, heavy metals and a variety of phosphorylated compounds. The final step in the conversion to free inositol, dephosphorylation of the inositol-1-phosphate, was Mg^{++}-dependent and was twenty times faster than the synthesis of the phosphate. This left the problem of the mechanism of the glucose-6-phosphate to inositol-1-phosphate conversion open, but the requirement for NAD supported the idea that D-glucose-6-phosphate (III, Figure 1) might be dehydrogenated to a keto derivative, possibly D-5-keto-glucose-6-phosphate (IV, Figure 1). Loewus and Kelly (1962) have proposed the existence of a 5-keto intermediate in the parsley leaf system. Cyclisation of the phosphorylated 5-keto-glucose by an aldolase type of reaction (Fischer, 1945; Loewus and Kelly, 1962) could give D-myo-2-inosose-1-phosphate (V, Figure 1). Reduction of this would yield D-inositol-1-phosphate. During the conversion of glucose-6-phosphate to inositol, a low steady-state level of $NADH_2$ is attained. The net reduction observed is small and

it must be concluded that the $NADH_2$ is re-oxidised by a subsequent reductive step. The reduction of NAD probably occurs in the conversion of III to IV and the reoxidation in the step from V to VI (Chen and Charalampous, 1965a). Dephosphorylation of the inositol-1-phosphate (VI) would give inositol (VII). The probable full pathway from glucose to inositol is shown in Figure 1.

It is interesting to note that the interconversion of myo- inosose-2-, scyllo-inositol and myo-inositol has been shown by Scholda et al. (1965) in Sinapsis alba. The conversion of myo- inosose-2 to myo-inositol would be analogous to that of the proposed phosphorylated inosose to inositol phosphate in Figure 1 (V, VI). That to scyllo-inositol suggests that Sinapsis alba contains two reductases capable of attacking the keto-group from opposite sides, one yielding myo-inositol, the other scyllo-inositol (See figure 2).

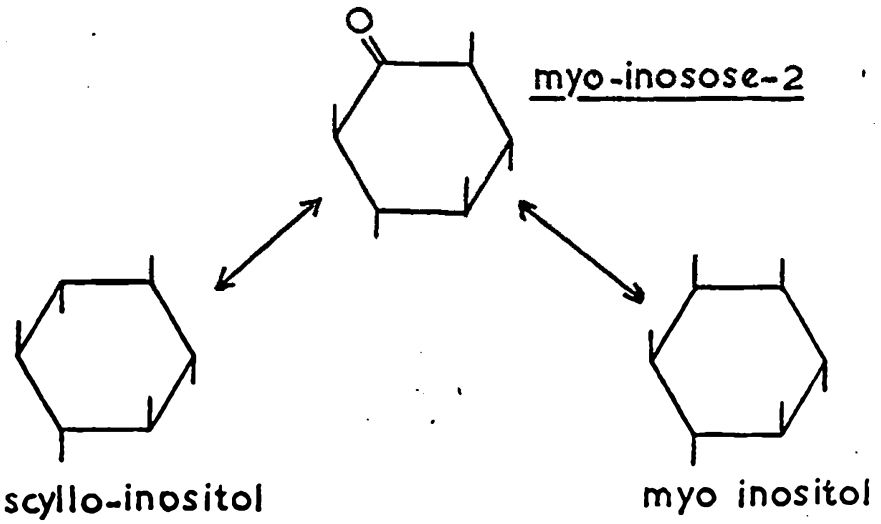


Fig 2 Interconversion of myo-inosose-2, myo-inositol and scyllo-inositol

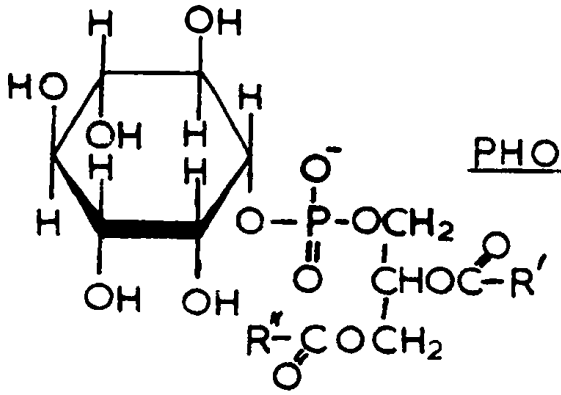
Phospholipid structure.

This field has been recently reviewed by Ansell and Hawthorne (1964) and by Hanahan and Brockerhoff (1965). Only the structures of the inositol phospholipids will be discussed here. As these have been reviewed in some detail by Hawthorne (1964b) and by Hawthorne and Kemp (1964), this will be limited to a fairly brief summary.

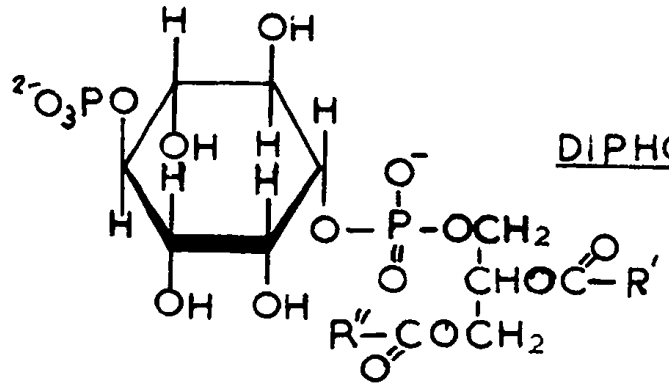
Three inositol lipids have so far been characterised in extracts from animal tissues. They are 1-phosphatidyl-L-myo-inositol, 1-phosphatidyl-L-myo-inositol-4-phosphate and 1-phosphatidyl-L-myo-inositol-4,5-diphosphate. These are generally known, respectively, as phosphatidylinositol (or monophosphoinositide), diphosphoinositide, and triphosphoinositide. Their structures are given in Figure 3 and for the purposes of this thesis their names will be abbreviated to PI, DPI and TPI.

PI has been identified from several sources (see Hawthorne and Kemp, 1964), but DPI and TPI have only been fully characterised as components of the mixed inositide fraction from ox-brain described by Folch (1949) as diphosphoinositide. Herein this mixture will be referred to as the "Folch inositide fraction", to avoid the ambiguity inherent in describing a mixture of at least four phospholipids as "diphosphoinositide".

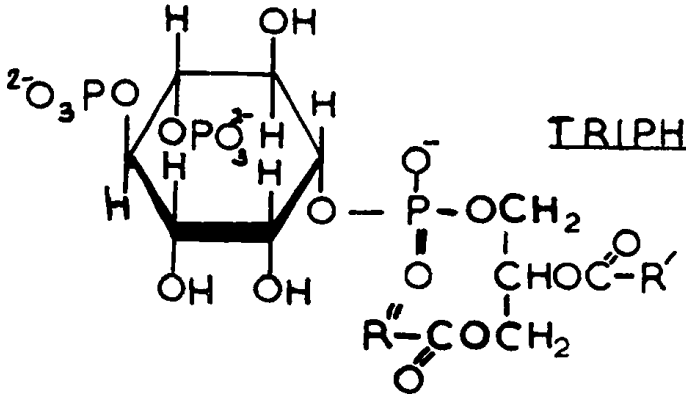
These three inositides were originally identified by a full characterisation of the phosphate esters produced by their



PHOSPHATIDYLINOSITOL.



DIPHOSPHOINOSITIDE.



TRIPHOSPHOINOSITIDE.

FIG. 3 Structures of Inositol phospholipids (animal).

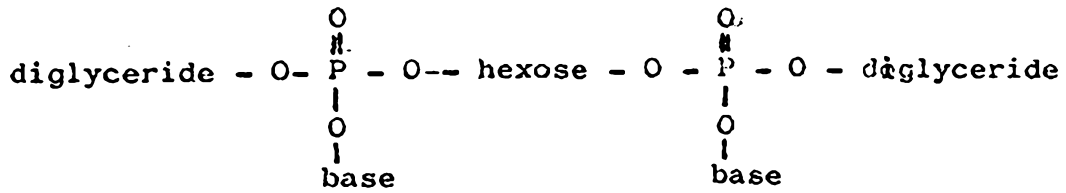
deacylation. The assumption that the intact lipids are, in fact, the diacyl derivatives of these phosphate esters was confirmed by Dittmer and Dawson (1961) who isolated TPI, and by Hendrickson and Ballou (1964), who isolated DPI and TPI as intact, pure lipids. That DPI can be separated from TPI in a pure form in two different systems (chromatography on DEAE-cellulose and on formaldehyde-impregnated paper) seems, together with the data from degradation studies, to refute firmly the recent suggestion (Rouser, 1965) that there is insufficient evidence for the existence of DPI as a separate entity.

The Folch inositide fraction probably also contains at least one other inositide, which yields inositol triphosphate on mild alkaline hydrolysis (Ellis et al., 1963). This material contains no glycerol and Hawthorne and Kemp (1964) have speculated on the possibility of its being an inositol polyphosphate with fatty acids esterified directly to the inositol. A report suggesting the existence of a tetraphosphoinositide in brain (Santiago-Calvo et al., 1963) was withdrawn (Santiago-Calvo et al. 1964) after further analyses.

Another, but ill-characterised and more complex, inositide was isolated from brain by Klenk and Hendricks (1961). This contained inositol and phosphorus in a molar ratio of 1:4, and also glycerol, glucosamine, mannose and batyl alcohol. A tentative structure was proposed, although the substance may be a

mixture of lipids. Unfortunately no further studies have been published.

The only other inositide so far reported from animal tissue was described by Morgan et al. (1963). This lipid, found in kidney, was stable to mild alkali, and acid hydrolysis released inositol, inositol monophosphate and a hexose (probably glucose). Whether this hexose bears any relation to the occurrence of a glucose derivative in a liver phosphatidyl-inositol preparation (Kemp, 1960) is uncertain. These observations together do, however, make more interesting the recently advanced evidence for the existence of tissue lipids in a novel triester form (Galanos and Kapoulas, 1965 a, b, c). One of the suggested structures is:



These authors suggest that TPI is a degradation product, probably of the material described by Klenk and Hendricks (1961), but there is at present little evidence to support this hypothesis.

Inositol lipids can be extracted from tissues tightly bound to protein (Folch, 1952; Huggins and Cohn, 1959). As prepared from brain (LeBaron et al., 1962) and kidney (Andrade and Huggins, 1964) these complexes contain both DPI and TPI

bound to protein. In a study of the esters released by mild alkaline hydrolysis (deacylation) from the kidney phosphatido-peptide, Andrade and Huggins also found a component which was less polar than GPI, yet yielded inositol triphosphate on strong alkaline hydrolysis. This compound contained glycerol and α-amino nitrogen in a 1:1 molar ratio and may be a derivative of a hitherto undescribed complex inositol lipid. Huggins and Cohn have found that these phosphatido-peptides can be extracted from a variety of tissues and that the preparations from different sites have similar compositions. These observations may be related to the identification of inositol di- and tri- phosphates as components of several rat tissues by Seiffert and Agranoff (1965).

It is clear that our present knowledge of the chemical form and of the physical state of inositol lipids in tissues still leaves much to be desired.

As well as the compounds discussed above, several other inositides have been reported as components of plant and bacterial lipids. This work was reviewed by Hawthorne (1960). Since then the most marked progress has come in the elucidation of the structures of the phosphatidylinositol mannosides of Mycobacteria. Although these compounds will not be discussed in detail here it should be noted that all of the compounds so far characterised (Vilkas, 1960; Ballou et al., 1963; Lee and Ballou, 1964) contain the same basic 1-phosphatidyl-L-myo-inositol

structure common to the three mammalian inositides.

Distribution of inositol phospholipids.

PI is widely distributed in a variety of plant and animal tissues. Hawthorne and Kemp (1964) have listed some of the many organisms in which this lipid is known to occur. In mammalian tissues PI usually comprises between 2 and 12% of the total phospholipid.

In contrast DPI and TPI were for several years thought to be exclusively components of the nervous system. Recently it was found that dog adrenal glands contain DPI and TPI in concentrations comparable to those present in the brain (Lo Chang and Sweeley, 1963). As the adrenal medulla may be considered an extension of the sympathetic portion of the autonomic nervous system, this is not entirely unexpected and it may be hoped that studies of the metabolism of DPI and TPI in this highly specialised offshoot of the nervous system will also help in elucidating their possible functional significance in the brain.

The view that DPI and TPI are only components of nervous tissues has been convincingly exploded in recent years by work from several laboratories, primarily those of Hörhammer, Kerr, Hokin, Hawthorne and Huggins. No other tissues, though, have been shown to contain concentrations of the polyphosphoinositides comparable to those in brain and adrenals. In fact, only in liver (Galliard et al., 1965; Kfoury and Kerr, 1964), kidney

(Morgan, 1964; Andrade and Huggins, 1964) and pancreas (Kfoury and Kerr, 1964) have they been detected in sufficient quantities to permit any reasonable degree of chemical characterisation. It is encouraging, however, that there is agreement between several laboratories, although a variety of techniques have been used in attacking the problems.

The first detection of DPI and TPI outside the nervous system came from Hörhammer's group, who have now shown the presence of these lipids in rat heart, lung, kidney, liver and spleen (Hörhammer et al., 1961; Wagner et al., 1963; Hölzl and Wagner, 1964). In this work the intact lipids were separated on formaldehyde-impregnated papers (Hörhammer et al., 1959).

Dawson et al. (1962), using chromatography and electrophoresis of the phosphate esters produced by deacylation of the lipids as their detection method, found that DPI was confined to the nervous system, but that TPI occurred as "traces in other tissues".

Kfoury and Kerr (1964) partially purified DPI from pork liver lipids and identified IP_2 and GPIP in, respectively, acid and alkaline hydrolysates of the material. They reported minimum concentrations of DPI of 11 mμmoles per gram in pork liver and 4 mμmoles per gram in beef liver.

The same authors found that pork pancreas contains at least 120 mμmoles DPI per gram of tissue. No evidence was found

for the presence of TPI in either liver or pancreas.

Santiago-Calvo et al. (1964) developed a method for separating PI, DPI and TPI on silicic-acid-impregnated papers, using phenol:ammonia for development. By this method they detected labelled DPI and TPI in the lipids of brain, kidney, liver, heart, pancreas and gull salt gland. The lipids were extracted from tissue slices which had been incubated with ^{32}P-orthophosphate.

Hokin and Hokin (1964c), also using this separation procedure, showed that erythrocyte ghosts would synthesise labelled DPI and TPI if incubated with γ-^{32}P-ATP.

Huggins and Cohn (1959) have shown that a phosphatido-peptide complex somewhat similar to that found by Folch and LeBaron (1951) in brain extracts can be isolated from a variety of tissues (brain, kidney cortex, heart, liver, lung, spleen, pancreas, intestine, and Ehrlich ascites tumour). Analysis of the kidney material showed that it contained inositol, phosphate, glycerol and a variety of other components. Andrade and Huggins (1964 a, b) have since shown that the kidney complex contains DPI and TPI, the identification being mainly based on the chemical compositions, electrophoretic mobilities and ion-exchange chromatographic behaviour of the deacylated materials.

In 1963 Garbus et al. found that incubation of liver or kidney mitochondria with ^{32}P-orthophosphate under conditions suitable for limited oxidative phosphorylation produced a labelled lipid, possibly DPI or TPI. It was identified as DPI independently by Galliard and Hawthorne (1963) and by Morgan (1964). This present thesis describes further studies of the biosynthesis of DPI in rat liver. Some of the work has been reported in the journals (Galliard, Michell and Hawthorne, 1964; 1965; Michell, Galliard and Hawthorne, 1964; Hawthorne and Michell, 1965).

Biosynthesis of phosphatidylinositol.

Although some studies have suggested that tissue free inositol may not be the only source of inositol for the biosynthesis of PI, almost all of the studies so far conducted on this problem have been designed to find a pathway for the incorporation of free inositol.

Kennedy's work showed the importance of cytidine nucleotides in the biosynthesis of phosphatidylcholine and phosphatidylethanolamine, via the phosphorylated bases and their cytidylyl derivatives. Reactions 1 - 3 show the pathways, X representing either choline or ethanolamine.

$$X + ATP \xrightarrow{\text{kinase}} \text{phosphoryl-}X + ADP \quad (1)$$

$$\text{phosphoryl-X} + \text{CTP} \longrightarrow \text{CMP-P-X} + PP_i \quad (2)$$

$$\text{CMP-P-X} + \text{1,2-diglyceride} \longrightarrow \text{phosphatidyl-X} + \text{CMP} \quad (3)$$

Knowing this reaction sequence, it was a very short mental step to substitute inositol for X. Several tissues are known to contain inositol phosphate (Hübscher and Hawthorne, 1957) and possibly nucleotide-bound inositol (Hübscher, 1958; Galliard, 1963; Galliard and Hawthorne, 1963; Redman and Hokin, 1961). Until recently, though, no tissue had been shown to contain an inositol kinase, despite searches by Paulus and Kennedy (1957, 1960), Hübscher (1958) and Galliard (1963). This enzyme has now been reported to exist in mung beans, sycamore cells, Escherischia coli, and ox liver by Dietz and Albersheim (1965), but little is yet known of its properties. The apparent absence of this enzyme was sufficient discouragement for many years to prevent anybody from attempting to show any synthetic activity with CDP-inositol. This was recently found by Redman and Hokin (1964) to be a well-founded pessimism. They showed that the cytidylyl derivative of L-myo-inositol-1-phosphate (the naturally occurring lipid component) was completely inactive as an inositol phosphate donor for lipid synthesis in brain, liver and pancreas.

Although CDP-inositol is not utilised in PI synthesis, it is now certain that cytidine nucleotides have a key role in

inositide biosynthesis in at least three tissues (Agranoff et al., 1958; Paulus and Kennedy, 1960; Thompson et al., 1963; Redman and Hokin, 1964).

The first observations implicating cytidine derivatives were those of Agranoff et al. (1958), who found that CDP-choline, CMP, CDP and CTP all stimulated ^{3}H-inositol incorporation into PI in a frozen-dried kidney preparation. If inositol was omitted from the medium it was found that a chloroform-soluble cytidine derivative accumulated, and it was suggested that this might be CDP-diglyceride, formed by the transfer of a cytidylyl group from CDP-choline to phosphatidic acid, with the elimination of phosphorylcholine (Reaction 1, Figure 4). This novel liponucleotide (it showed properties both of a nucleotide and of a phospholipid) was then postulated to react with inositol, giving PI, with the elimination of CMP (Reaction 5, Figure 4).

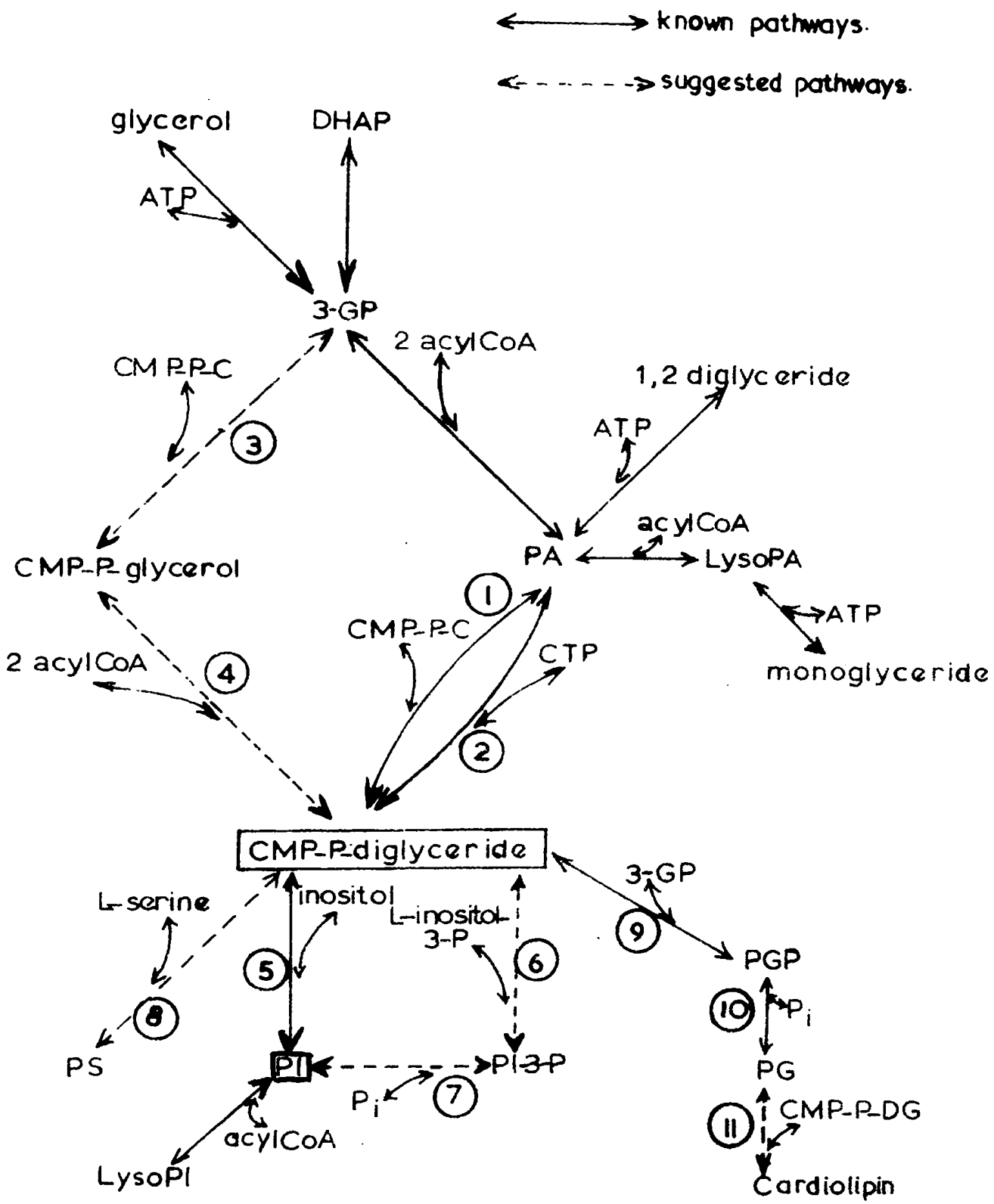
Subsequent work from the laboratories of Kennedy, Rossiter, Agranoff and Hokin has cast no doubts on the validity of reaction 5, but the biosynthetic pathway to CDP-diglyceride is still not altogether clear. The variety of tissues and conditions which have been used for these studies have so far only served to confuse the issue further. This is regrettable because of the key position that CDP-diglyceride is known to occupy in the biosynthesis of acidic phospholipids (Reactions 5, 8, 9, 11, Figure 4; Kiyasu et al., 1960, 1963; Kanfer and Kennedy, 1962).

Fig. 4.

Abbreviations other than those given on pp. 1 and 2;

DHAP	dihydroxyacetone phosphate
CMP-P-C	cytidine diphosphate choline
CMP-P-glycerol	cytidine diphosphate glycerol
CMP-P-DG	cytidine diphosphate diglyceride
PGP	phosphatidylglycerophosphate
PG	phosphatidylglycerol
PI-3-P	phosphatidylinositol 3-phosphate
lysoPA	lysophosphatidic acid
lysoPI	lysophosphatidylinositol

Fig. 4. Metabolic interrelationships involved in PI biosynthesis.



In Agranoff's original paper the suggestion that CDP-choline was the cytidylyl donor for CDP-diglyceride production was based solely on the observation that in dilute Tris buffer ^{3}H-inositol incorporation into lipid was stimulated by the cytidine nucleotides in the following order of effectiveness: CDP-choline > CMP > CDP > CTP. The seeds of doubt were sown later in the same paper, when a comparison of the efficiencies of different cytidylyl derivatives in different buffers was made. If dilute phosphate was used the situation was virtually unchanged, but with a raised phosphate concentration CDP and CTP rose in efficiency to levels similar to, or higher than, those with CDP-choline.

Other features of the system were that it was stimulated by Mg^{++} and inhibited by Tween 20 or Ca^{++}. Phosphatidic acids, both dioleoyl and dimyristoyl, stimulated far more effectively than the corresponding diglycerides.

At the same time Paulus and Kennedy (1958, 1959, 1960) were studying liver systems. Their experiments used rat, guinea-pig or chicken liver homogenates or microsomes, and in most cases it is not clear how they chose the system for each experiment. In the rat and guinea-pig tissues they found that two systems for inositol incorporation into PI existed, one of which was simply a Mn^{++}-dependent exchange reaction, inhibited by high concentrations of phosphate. They claimed that when this

reaction was eliminated the incorporation of inositol into lipid became specifically CTP-dependent: Agranoff's CDP-choline activation was ascribed to the exchange reaction. This was supported by the statement that CDP-choline breakdown did not occur during their assay, so it could not be the cytidylyl donor. However, there was no statement of which assay system was used for this measurement. The observation is rendered less than convincing by two features. One is that, having eliminated CDP-choline breakdown, the authors then suggest that CMP (formed by breakdown of the other nucleotides!) is the active species in the stimulation of the exchange reaction. Secondly, their 1960 paper includes a table of comparative activities of different cytidine nucleotides in phosphate buffer, when exchange activity should have been minimal. The activities are CDP-choline > CTP > CMP, all of the incorporations being of the same order (83 - 126 mμmoles inositol incorporated).

Paulus and Kennedy, though, showed that Reactions 2 and 5 (Figure 4) do occur in liver microsomal fractions. They confirmed that the intermediate was CDP-diglyceride, by utilising the synthetic liponucleotide as substrate for Reaction 5. It was also shown that ^{32}P-phosphatidic acid was incorporated intact into PI and that in Reaction 5 α-^{32}P-CTP yielded ^{32}P-CMP.

In most of their studies these workers did not use

^{32}P-phosphatidic acid as their phosphate source, but ^{32}P-3-glycerophosphate. In an incubation containing this labelled compound, together with ATP, CoASH, free fatty acid, Mg^{++} ions and microsomes, it was assumed that ^{32}P-phosphatidic acid was endogenously produced. In this system CDP-choline was completely ineffective as a cofactor.

The third study of reactions leading to PI was that of Thompson et al. (1963) which followed up the observation (McMurray et al., 1957) that CTP stimulated PI labelling in brain dispersions. They found that in these dispersions synthesis of PI from ^{32}P-phosphatidic acid was specifically CTP-dependent, as Kennedy has found in liver. However, in a pair of earlier notes (Thompson et al., 1959; Strickland et al. 1960) they reported that if ^{32}P-3-glycerophosphate and a "phosphatidic acid generating system" were used, CDP-choline was a far better cofactor than CTP. This completely opposed the Kennedy view. Moreover, at the end of their incubations the specific activity of PI was higher than that of phosphatidic acid, apparently eliminating phosphatidic acid as an intermediate on the pathway to PI. CDP-choline also decreased the phosphatidic acid concentration of the medium. This simultaneous fall in both activity and concentration relative to the CTP-dependent synthesis clearly pointed to an alternative synthetic route for the production of the phosphatidyl unit.

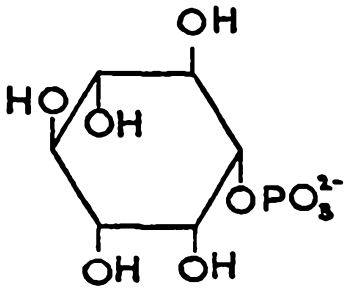
These results could be explained by proposing that CDP-diglyceride can be synthesised, not only *via* phosphatidic acid, but also *via* CDP-glycerol, a compound which has been detected in *Lactobacillus arabinosus* (Baddiley *et al.*, 1956). This compound was first mentioned as a possible intermediate by Paulus and Kennedy (1957), but was discarded when no evidence could be found for its synthesis in liver. The results from Rossiter's group suggest that it may be active in brain, participating in Reactions 3 and 4 (Figure 4). Kiyasu and Mobley (1962) have shown that CDP-diglyceride synthesis in kidney may not be phosphatidic acid-dependent. CDP-choline, rather than CTP, was the nucleotide required by the kidney system of Agranoff *et al.* (1958) and this might reflect the presence of the alternative pathway.

Recently the situation has been further confused by the work of Redman and Hokin (1964). They found, in complete contradiction of Rossiter's group, that in brain homogenates the conversion of ^{32}P-3-glycerophosphate to PI in the presence of an acylating system was stimulated by CTP ten times better than by CDP-choline.

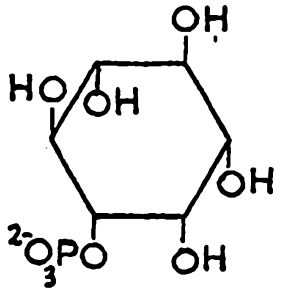
In summary, it seems that in several tissues inositol will accept phosphatidic acid from CDP-diglyceride, giving PI, and that the liponucleotide can be formed from phosphatidic acid and CTP. CDP-diglyceride can probably also be formed by an

alternative pathway, not involving phosphatidic acid. As noted above, resolution of this confusion would be significant, not only for the clarification of PI biosynthesis, but also that of diphosphatidylglycerol, phosphatidylglycerol and possibly also phosphatidylserine.

Studies of incorporation of inositol or its precursors into lipid inositol in the rat brain in vivo have suggested that endogenously synthesised inositol may be a better precursor of lipid inositol than injected free inositol (Hauser, 1963; Margolis and Heller, 1965). Explanation of this requires either the postulate of two pools of inositol available for lipid synthesis or of a biosynthetic route to PI not requiring free tissue inositol as an intermediate. A suggestion for a pathway of this type arises from the recent work of Chen and Charalampous (1965) and of Eisenberg and Bolden (1965). If the cyclisation of D-glucose-6-phosphate to inositol-1-phosphate follows the route suggested earlier in both plant and animal tissues, then D-glucose-6-phosphate is likely to be converted to D-inositol-1-phosphate (L-inositol-3-phosphate). The inositol of PI, however, is in the form of the L-1-phosphate (see Figure 5).



L-myo-inositol-1-phosphate
(D-myo-inositol-3-phosphate)



D-myo-inositol-1-phosphate
(L-myo-inositol-3-phosphate)

Fig. 5. Configurations of myo inositol 1- and 2- phosphates.

A simple way of achieving this configuration would involve the L-inositol-3-phosphate in a series of reactions analogous to those which incorporate 3-glycerophosphate into phosphatidylglycerol, (Reactions 9 and 10, Figure 4). CDP-diglyceride would react with the inositol phosphate, yielding 1-phosphatidyl-L-myo-inositol-3-phosphate (Reaction 6). Dephosphorylation of this in Reaction 7 would complete a reaction sequence in which glucose was converted to the lipid inositol of PI without the intermediate formation of free inositol at any stage; also the pathway is closely analogous to a known reaction sequence. In Figure 6, the suggested pathway is drawn out in more detail.

The synthetic pathways discussed above have only been shown to synthesise a phosphatidylinositol. Nothing is known of how the lipid acquires its specific fatty acid pattern.

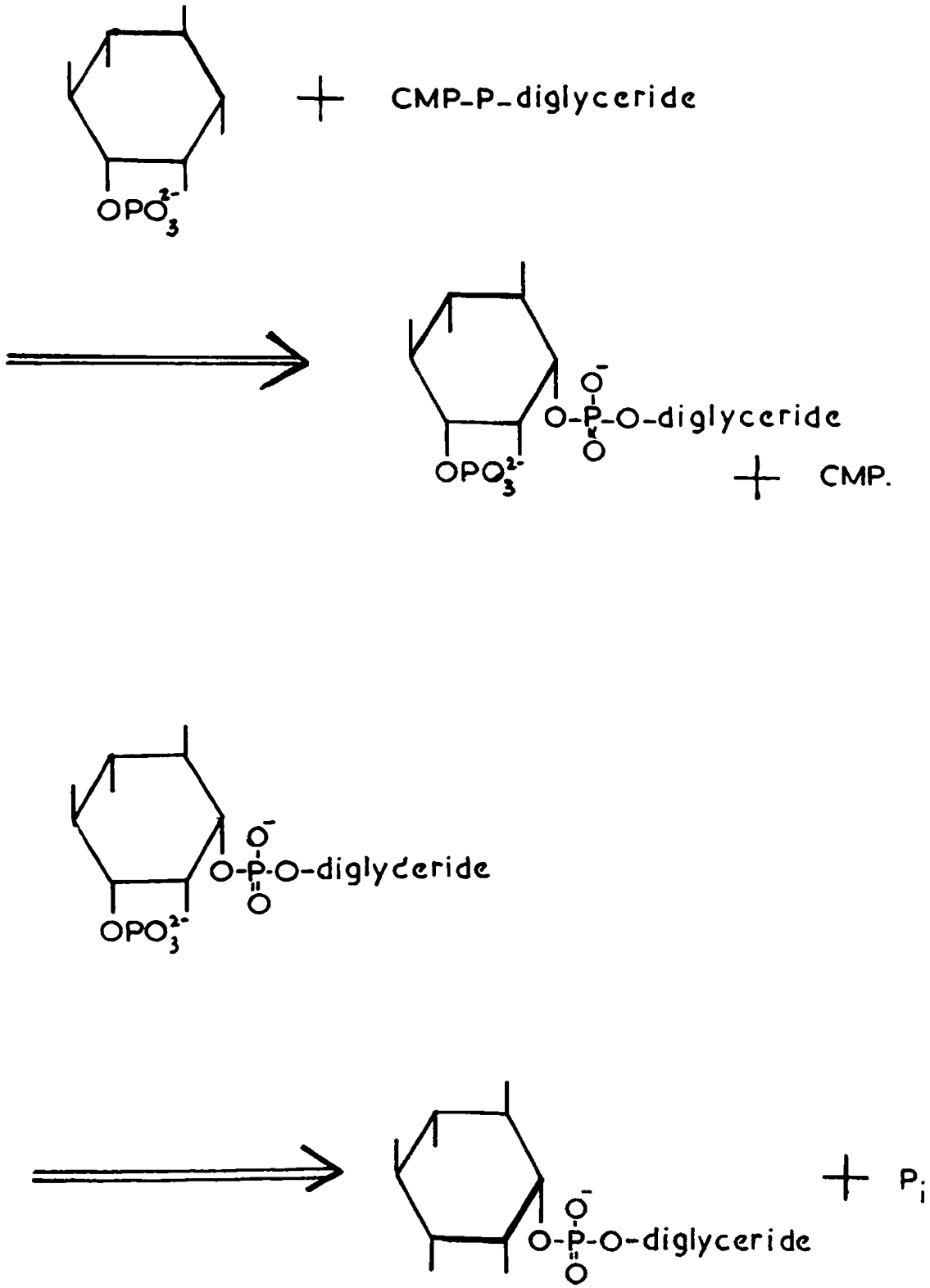


Fig. 6. Possible pathway for PI biosynthesis.

Reference to published data (Ansell and Hawthorne, 1964; Appendix 5; Hendrickson and Ballou, 1964; Keenan and Hokin, 1964) clearly shows the great variations in fatty acid pattern which exist, even between samples of metabolically related lipids from one tissue. For example, rat liver PI contains 39% of its acyl content as stearic acid and 28% as arachidonic. 34% of its fatty acids are either C_{20} or C_{22}. Phosphatidic acid from rat liver, from which the PI is supposed to be derived, contains only 2.7% stearic acid and a total of 3.2% of C_{20} and C_{22} acids. It contains 72% linoleic acid, but PI contains only 5.8%. Assuming their metabolic inter-relationship to be genuine there are two ways in which this specificity of composition may come about. First, the lipid may be synthesised by an enzyme system which is specific for those molecules of the precursor pool which already have the correct composition, ignoring the bulk of the available "substrate". The second alternative is that the lipid as initially synthesised may have the fatty acid composition of the precursor pool, but that this is modified after synthesis. This modification could be catalysed either by a transacylase of the type described by Lands (1960), or by a cycle of enzymes catalysing a deacylation (Phospholipase A), followed by a reacylation of the resulting lyso-compound (Keenan and Hokin, 1962).

Biosynthesis and metabolism of DPI and TPI.

The close structural relationship between PI, DPI and TPI suggested that there might be a stepwise phosphorylation pathway from PI to DPI and then to TPI. Incorporation of $^{32}P_i$ into the inositides of brain *in vivo* (Ellis *et al.*, 1962; Wagner *et al.*, 1962; Rossiter *et al.*, 1964) and *in vitro* (Brockerhoff and Ballou, 1962 a) showed that the labelling of the three inositides, expressed as molar radioactivities, fell in the order: TPI > DPI > PI. Brockerhoff and Ballou (1962 b) also showed that if rabbit brain slices were incubated with ^{3}H-inositol or ^{14}C-glycerol the specific activities of the lipids fell in the reverse order: i.e. PI > DPI > TPI. Studies of the incorporation of ^{3}H-inositol into the brain of the mature cat or rat *in vivo* gave similar results (Rossiter and Palmer, 1965). These observations supported the stepwise phosphorylation hypothesis. Further support came from the finding that the fatty acid patterns of the three inositides from ox brain were very similar (Hendrickson and Ballou, 1964).

However, the situation seems to be considerably more complex than was at first thought. In cat brain slices, incubated with ^{3}H-inositol or ^{14}C-glycerol as precursor, the molar radioactivities of the lipids were: DPI > PI > TPI (Rossiter *et al.*, 1964). In young rats (7 or 18 days old) incorporation of ^{3}H-inositol *in vivo* gave the sequence PI > TPI > DPI, but by the

35th day the adult pattern (PI > DPI > TPI) was established (Rossiter and Gardiner, 1965). Agranoff (1960) studied the labelling of lipid and phosphatidopeptide inositol after the injection of free inositol into rats of various ages. In the young rat brain radioactivity was primarily incorporated into the phosphatidopeptide (presumably DPI and TPI) but by 30 days labelling was mainly in the lipid (presumably PI), the activities being similar at about the 9th day. As these results were expressed in terms of incorporation per mg. of brain, rather than as specific activities, they are difficult to compare with those of Rossiter and his co-workers. However, it seems that before and during myelination it is the phosphatidopeptides and TPI that are most actively synthesised.

There are two possible explanations for the above results. There might be more than one biosynthetic pathway to DPI and TPI. If this applies, and the two pathways are operative at different stages of development, the resulting variations with age might be similar to those described above. The alternative is that the stepwise phosphorylation pathway is the only one and that there is intracellular compartmentation. On this hypothesis the differences observed at different stages of development would be a reflection of changes in the relative rates of DPI and TPI turnover in different compartments. The recent results of Kai and Hawthorne (1965) suggest that in the

brain of young rats such compartmentation may occur. Using the fractionation procedure of Nyman and Whittaker (1963) they studied the specific activities of the three inositides in brain cell fractions from animals killed four hours after the sub-arachnoid injection of $^{32}P_i$. Expressed as molar radioactivities, their results only showed the expected pattern of TPI > DPI > PI in the myelin fraction; in all of the others the relationship was DPI > TPI > PI. M.Kai (unpublished results) has also studied the ATP-dependent phosphorylations of PI to DPI and of DPI to TPI in the isolated cell fractions. The results indicated that the latter process was considerably more active in myelin than elsewhere. The former was less restricted in its distribution, being spread through the fractions in a similar pattern to that reported for the distribution of $(Mg^{++}/Na^{+}/K^{+})$-stimulated ATPase by Hosie (1965). These results add to the already considerable body of evidence that the poly-phosphoinositides, particularly TPI, are characteristic components of myelin (Hörhammer et al., 1960; Amaducci et al., 1962; LeBaron et al., 1962; Folch-Pi, 1963; Eichberg and Dawson, 1964; Soto et al., 1965; Rossiter and Gardiner, 1965; LeBaron, 1965; Dawson, 1965). They will be further discussed later in relation to the possible occurrence of DPI and TPI as characteristic components of plasma membranes in general.

Biosynthesis of phospholipids with monoester phosphate groups.

At the time when this work was started phosphatidic acid (PA) was the only phospholipid with a monoester phosphate group which had been studied from a metabolic point of view. There seem to be two biosynthetic pathways to it in animal tissues. These are the acylation of 3-glycerophosphate (Kornberg and Pricer, 1953) and the phosphorylation of 1,2-diglyceride (Hokin and Hokin, 1959 b). As described elsewhere, the other lipids with monoesterified phosphate groups (DPI and TPI) seem to have a biosynthetic pathway analogous to the second of these: the phosphorylation of PI to DPI and of DPI to TPI. No attempt has so far been made to demonstrate the direct acylation of GPIP and $GPIP_2$ to DPI and TPI respectively.

Metabolism and function of phospholipids.

Phospholipids and phagocytosis.

Karnovsky and Wallach (1961) studied the labelling of the phospholipids of guinea-pig polymorphonuclear leucocytes "at rest" and during phagocytosis. Labelling of PA, PS and an inositol lipid were greatly stimulated during phagocytosis but there was little change in the activity of the other lipids. These effects were also observed in leucocytes treated with bacterial endotoxins, which induce a "pseudo-phagocytosis", with the exception that phosphatidic acid labelling was unstimulated. Similar results were obtained with monocytes (Karnovsky, 1964a).

Karnovsky (1964 b) stated that labelling of PI, and not of DPI or TPI, was stimulated. However, the published evidence for its identity (Karnovsky and Wallach, 1961) was based on its similarity to the Folch inositide fraction, so that some doubt must be felt about this identification. Karnovsky et al. (1964) have drawn parallels between phagocytosis and pinocytosis. They found that the changes in phospholipid metabolism during pinocytosis are similar to those of phagocytosis, with PS the most consistently active lipid during pinocytosis.

Phospholipids, ion-transport and secretory functions.

Hokin and Hokin (1959) suggested that phosphatidic acid could act as a carrier molecule in sodium transport. This theory has been widely discussed and criticised (see reviews by Ansell and Hawthorne, 1964; Hawthorne and Kemp, 1964). It was based on the stimulation of phosphatidic acid labelling observed when slices of salt-gland (gull, duck, albatross or goose) or brain were stimulated with acetylcholine. Ansell and Hawthorne (1964) considered that the theory was only tenable in the salt-gland, and not as a general mechanism. This was the only tissue in which the acetylcholine-stimulated phosphatidic acid turnover was inhibited by ouabain, an inhibitor of monovalent cation transport (Nicholls et al. 1962). This type of inhibition is one of the requirements to be fulfilled by any candidate for a role in this transport process. These requirements have been

discussed in a recent review by Skou (1965), and they seem to be well met by the Na^+/K^+-activated ATPase first described in crab nerve by Skou (1957).

There seems to be no reason to believe that sodium transport in the salt-gland is mediated by a mechanism different from that in other tissues and recently an active Na^+/K^+-stimulated ATPase has been found in this tissue (Bonting et al., 1964). Furthermore, if gulls were maintained on fresh water for 7 weeks the size of the salt-gland fell by 65% and the specific activity of this ATPase in the remaining tissue was only 51% of that in the normal gulls. When the water in the diet of these gulls was replaced by 3% salt solution no regeneration of the gland occurred and the birds died within four days. As Hokin and Hokin (1964 a) have shown that phosphatidic acid does not fulfil the requirements of an intermediate of the ATPase reaction in the salt gland, it seems doubtful whether it is involved directly as a carrier of monovalent cations even in this tissue. A phosphorylated intermediate has been shown to be involved in the ATPase reaction by several workers (e.g. Post et al., 1965), and Skou (1965) speculated as to whether this might be phosphatidic acid. This now seems very unlikely, both from the work quoted above and from recent work (Hokin et al., 1965; Nagano et al., 1965) suggesting that the intermediate is an acyl phosphate.

Glynn et al. (1965) could find no evidence for the participation of any phospholipid in the Na^+/K^+-stimulated ATPase of Electrophorus or Torpedo electric organs. The Na^+/K^+-stimulated ATPase now falls into the growing category of enzymes which require lipid for activity (Skou, 1961; Swanson et al., 1964; Ohnishi and Kawamura, 1964) but do not operate via a lipid intermediate. The last authors found that after abolition of erythrocyte Na^+/K^+-ATPase activity by phospholipase A, the activity could be partially restored by the addition of PS, PE or PI, but that only PS restored the ouabain-sensitivity of the system.

In more recent work the Hokins (Hokin and Hokin, 1964 b) have somewhat changed their outlook. They have now produced evidence that in the salt gland a cycle of changes occurs in which PI and PA are interconverted, presumably via diglyceride. When salt gland is stimulated by acetylcholine a labelling of PA occurs (0.12 µmole per gram of tissue). On removal of the stimulus by atropine the tissue reverts to the "resting" state and the PA is converted to PI, the PI retaining the labelled phosphorus. If the tissue is re-stimulated this PI disappears and the PA is reformed, presumably via diglyceride since there is loss of the original labelled phosphate group. This cycle is shown in Figure 7. The Hokins' interpretation of this is that "the phosphatidylinositol-phosphatidic acid cycle is part of

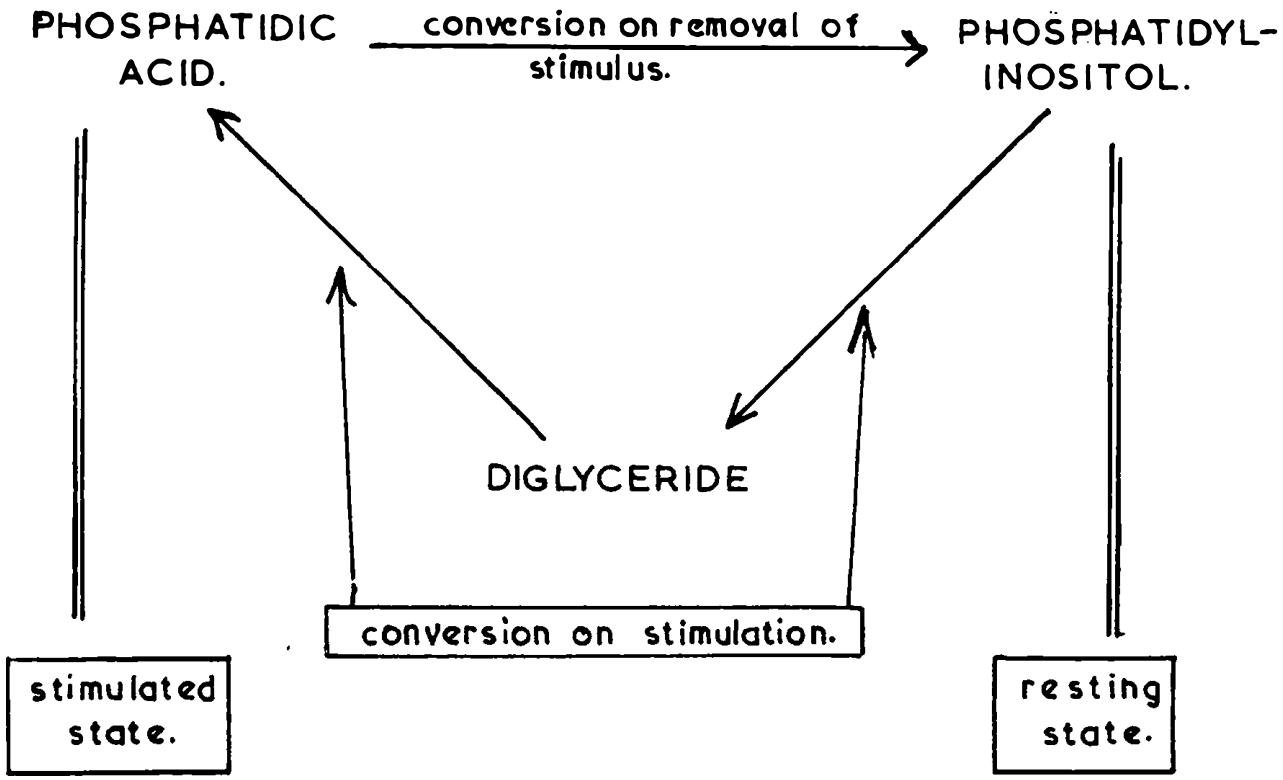


FIG. 7. A proposed cycle involved in the control of Na^+ transport in salt gland. (Hokin and Hokin, 1964b).

a mechanism which controls whether or not Na^+ transport sites are active in the gland. According to this interpretation, the transport sites would be inoperative when the lipoprotein at the site exists in the phosphatidylinositol form; the site would become operative when the phosphatidylinositol form is converted to the phosphatidic acid form, this change being brought about by the stimulation of secretory activity with acetylcholine ..... ; reversion of the phosphatidic acid form to the phosphatidylinositol form on removal of the stimulus would render the transport site inactive".

The observation that the acetylcholine-stimulated PA turnover in the salt-gland shows an absolute requirement for added monovalent cations, sodium being easily the most active (Hokin and Hokin, 1961), supports the view that it may be in some way involved in the Na^+ transport system, possibly by some mechanism like that described above. It seems reasonable that the controlling mechanism for a transport system through a phospholipid membrane could be based on a modification of the lipid pattern in the membrane.

In the discussions of PA turnover, which were stimulated by the work of the Hokins, the increased turnover of phosphoinositide which accompanies that of PA in most of the secretory tissues studied became somewhat overshadowed. This may have been because the labelled inositide studied was usually PI and

it was more difficult to implicate the turnover of the diester phosphate group of PI, than of a monoester, into a transport mechanism. Table 1 lists the effects of stimulation of secretory activity on phospholipid turnover in a variety of tissues. This table is based on that of Hawthorne (1960), with some additions, mainly those quoted by Ansell and Hawthorne (1964). Vilkki (1962) confirmed that PI is the most highly labelled lipid of thyroid, both in vivo and in slices, but he did not check Freinkel's observations on stimulation by thyrotrophic hormone. Other systems with a high turnover of PI and PA are intestinal mucosa, leucocytes (see also section on Phospholipids and Phagocytosis) and heart.

In summary, it seems certain that phosphatidylinositol and phosphatidic acid have some general function in secretory activity but at present the mechanism of this action is still well hidden. It may be that the recent work of the Hokins, suggesting a control, rather than a carrier function, may point the way to clarification of the phenomenon.

TABLE 1 Effects of stimulation of secretory activity on phospholipid turnover in various tissues.

Tissues with stimulated inositide and phosphatidic acid turnover during secretion.	Tissues not showing the stimulation.
Pigeon pancreas (during secretion of amylase).	Pigeon pancreas (during secretion of H_2O, HCO_3^-
Sheep thyroid.	Rat adrenal (during secretion of corticosteroids.)
Guinea-pig adrenal medulla.	Frog gastric mucosa.
Rat adenohypophysis.	
Duck, gull, albatross and goose salt glands.	
Pigeon oesophageal mucosa (secretion of pepsin).	
Denervated cat submaxillary gland.	

Phospholipids and nervous activity.

In brain slices incubated with acetylcholine and eserine a stimulation of PA and PI turnovers occurs (Hokin and Hokin, 1958), which is superficially similar to that observed with the secretory tissues. This was confirmed by Hayashi et al. (1962). Yoshida and Nukada (1961) found that the turnover of PA in brain slices was increased in the presence of high concentrations of K^+ or the absence of Ca^{++}. Both of these effects were dependent on the presence of Na^+ in the bathing medium. Brossard and Quastel (1963) extended this work, and

noted that these changes were found in the turnover of both PI and PA. Whether these phenomena are connected with the transport of K^+ associated with nervous activity (Whittam, 1962) or with the effects of Ca^{++} on membrane permeability (Edelman, 1961) is uncertain. In an extension of the studies with acetylcholine, Hokin and Hokin (1959 b) found that this compound stimulated the diglyceride kinase of brain microsomes, but that this effect was abolished by solubilisation with deoxycholate. They concluded that acetylcholine had a direct effect on diglyceride kinase.

Several attempts have been made to correlate changes in lipid turnover in nervous tissues with the conduction of nerve impulses. Most of the studies have been made with the stimulated superior cervical ganglion of the cat. Gardiner (1962) extracted from these ganglia two labelled lipids which had not been observed in the unstimulated tissue. Baranov (1960), on the other hand, found increased labelling in an acid chloroform:methanol extract, presumably containing DPI and TPI, but not of other lipids. This labelled fraction was broken down after prolonged stimulation, releasing P_i. Incorporation of $^{32}P_i$ into rat brain *in vivo* gives a very high labelling of protein-bound inositides (LeBaron *et al.*, 1962). These lipid-bound inositides are mainly concentrated in myelin (LeBaron, 1965; Eichberg and Dawson, 1964, 1965). Hayashi *et al.* (1962) found that during convulsions in the

rabbit the radioactivity of TPI fell, with a simultaneous rise in the level of PI or DPI. These lipids were not conclusively identified. Larrabee et al. (1963) found that during stimulation of the perfused rat cervical ganglion the specific activity of PI was approximately doubled, whilst there was no significant change in the activity of any of the other lipids examined. There was no change in the concentration of any of the lipids studied. Unfortunately, they did not make a simultaneous study of the changes in polyphosphoinositide labelling.

Recently Larrabee and Leicht (1965) have extended their studies on the labelling of PI in the stimulated cervical ganglion. ^{32}P incorporation was raised above the "resting" value only when the ganglion was in a conductive state. If it was blocked with tubocurarine, either partially or completely, the fall in the fraction of PI labelling induced by stimulation was correlated with the fall in the postganglionic action potential. As tubocurarine probably does not block the release of acetylcholine during preganglionic stimulation this was interpreted as evidence that the inositide effect was localised in a postsynaptic structure. Other nervous structures, not containing synapses, did not show the inositide effect. This showed the dependence of the inositide effect on the presence of synapses. An attempt to induce the labelling by antidromic

stimulation of the ganglion was unsuccessful.

Hawthorne and Kemp (1964) suggested that the results of electrical stimulation on lipid labelling in nervous tissue available when their review was written could all be explained by the breakdown of polyphosphoinositides to PI during stimulation. However, this was revised (Hawthorne, 1964 b) when the specific activity results of Larrabee et al. (1963) became available. As the diester phosphates of DPI and TPI have lower specific activities than that of PI this theory must now be treated with caution.

The role of lipids in mitochondrial shrinkage.

Vignais, P.V. et al. (1963, 1964) showed that "aged" mitochondria would not shrink when ATP was added to them. A 0.6M KCl-extract of mitochondria or submitochondrial particles restored the response to ATP in mitochondria which had swollen in the presence of phosphate, calcium, oleate or thyroxine, but not glutathione. Both the water-insoluble fraction (the "contractile protein" of Ohnishi and Ohnishi, 1962 a, b) and the soluble fraction showed "contraction factor" activity. Activity was highest in fractions which precipitated at less than 60% saturation of ammonium sulphate. This material contained all of the acyl ester and phosphate of the original extract. Boiling of the extract had no effect on its activity, whereas treatment with chloroform:methanol completely inactivated,

some of the activity being recovered in the extracted lipid. The phospholipid composition of this extract is given in Table 2, with figures for the composition of liver mitochondrial phospholipids taken from several sources.

TABLE 2. Phospholipid composition of a 0.6M KCl-extract of rat liver mitochondria and of the intact particles. (Figures in parentheses refer to the references given beneath the Table).

	Percentage of total lipid P						
	PC	PE	PI	PS	Cardiolipin	PG*	Other
Mitochondrial extract (1)	22.5	47.5	12		3.4	1.5	13
Mitochondria							
(2)	49	30	8		12	1	-
(3)	51.6	30.4	6.0	2.7	5.4	-	5.9
(4)	39	42	10	-	8	-	3
(5)	51	31	-	-	9	-	-
(6)	49.8	29.5	7.0	4.8	-	-	3.5
(7)	-	-	7+	-	-	-	93

Footnote: References.

(1) Vignais, P.V. et al. (1964)
(2) Strickland and Benson (1960)
(3) Gurr et al. (1965)
(4) Getz et al. (1962)
(5) Macfarlane et al. (1960)
(6) Youngs and Cornatzer (1963)
(7) Spiro and McKibbin (1956)

* PG - phosphatidylglycerol.
\+ - calculated from ref: 7. It was assumed that the inositol:P molar ratio of mitochondrial phosphoinositide was 1.0.

Thus the extract was depleted of PS, PC and cardiolipin, but enriched in PI and PE.

In an extension of the above studies, Vignais, P.M. et al. (1963, 1964) showed that when a wide range of lipids was tested, only PI, palmitoyl-CoA and palmitoylcarnitine showed significant contraction factor activity. Inositol-1-phosphate, inositol-2-phosphate, GPI and the fatty acids from PI all completely lacked activity. During the PI-dependent contraction they detected no incorporation of label from ^{32}P-ATP into "mono- and diphosphatidylinositol".

In two studies of the related turnover of phospholipids rather contrasting results have been obtained. Wojtczak and Lehninger (1961) and Wojtczak et al. (1963) studied the incorporation of oleate and 3-glycerophosphate into mitochondrial lipids during contraction. Oleate was incorporated into phosphatidic acid (or cardiolipin) and "cephalin" (presumably a synonym for PE, as the reference was to a spot separated on silicic acid-impregnated paper). If 3-glycerophosphate was the labelled precursor only phosphatidic acid was appreciably labelled. The PA labelling was stimulated by CoA and oleate, inhibited by inorganic pyrophosphate; presumably an acylation of 3-GP to PA was occurring. It was inhibited by all of the inhibitors of mitochondrial contraction that were tested, with the exception of azide, and thus seems to be in some way connected

to the process of contraction. A parallel is drawn by Wojtczak et al. (1963) between this process and the phosphatidic acid cycle of the Hokin's. This seems somewhat invalid, as Wojtczak studied the acylation of glycerophosphate, whereas the Hokin cycle involved production of phosphatidic acid by phosphorylation of diglyceride. The diglyceride kinase pathway does, however, probably exist in mitochondria from peanut (Bradbeer and Stumpf, 1960) and rat liver (results herein).

In contrast to the above results, Hilt et al. (1964) found that the incorporation of labelled fatty acids into mitochondrial phospholipids during mitochondrial shrinkage was almost confined to PI. The reason for this difference is not clear and there were no data to indicate whether this PI labelling was directly related to the shrinkage process.

Mitochondrial ion-accumulation.

This is briefly discussed at the beginning of Part II of the Results and will not be further treated here.

The role of phospholipids in enzyme action.

No definite correlation has yet been made between the action of any enzyme system and the presence of poly-phosphoinositides. However, a brief general consideration of lipid requirements by enzymes should indicate the possibilities open in this direction.

Phospholipids may be involved in enzyme activity in two basic ways. Physical interaction of the enzyme or of its substrate with a phospholipid may be required, either to keep the enzyme in its active form, or to convert the substrate into a physical form in which it is acceptable to the enzyme. In this case the phospholipid involved in activating the enzyme would undergo no chemical change. On the other hand a lipid might act as an intermediate in an enzymic reaction, the lipid undergoing a cycle of chemical changes, in a manner analogous to a coenzyme.

In the group of reactions requiring physical interaction of an enzyme with a phospholipid before the enzyme becomes active there is a broad spectrum of different specificities in both the phospholipid requirement and the enzymic activity evoked by the lipid. A few examples should clarify this point, each showing increased specificity in one or other direction over those preceding it.

A general requirement for phospholipid has now been reported for many enzyme systems, the best characterised of these being the respiratory chain of acetone-extracted mitochondria, each segment of which shows an absolute requirement for phospholipid. This can be satisfied by many phospholipid preparations of widely varying composition. If acetone-extraction is continued until the mitochondria have lost more than about 92% of their original

lipid they begin to lose their acidic lipids. At this stage they show a requirement for acidic phospholipid. This requirement can only be partially met by anionic detergents. This seems to differ from the activation of phospholipase B by either acidic phospholipids or other anions (Dawson, 1964). At the next level of specificity an enzyme may show an absolute requirement for a single phospholipid. D-β-hydroxybutyric dehydrogenase shows an absolute requirement for lecithin, with the activity of the individual lecithin sample depending on the degree of unsaturation of its fatty acids. Saturated lecithins are almost inactive. (The above examples of phospholipid requirement by mitochondrial systems are discussed by Green and Fleischer, 1963). The PI required for the ATP-dependent shrinkage of aged mitochondria, discussed elsewhere, cannot be replaced by any other phospholipid.

A different specificity, that involving a change in the properties of the enzyme, occurs when a mitochondrial soluble ATPase (Racker's coupling factor 1 (F_1)) which is insensitive to oligomycin is treated with T-U particles (submitochondrial particles which have been treated with trypsin and urea). It then regains the oligomycin-sensitivity which it is assumed to have had in the intact mitochondrion. The activity appeared to be present in an insoluble lipoprotein prepared from T-U particles. Oligomycin-sensitivity could be

partly restored by the addition of the phospholipids of this lipoprotein (Kagawa and Racker, 1965). As mentioned above, Na^+/K^+-stimulated ATPase, after treatment with phospholipase A, requires phospholipid for Na^+-stimulated ATPase activity and phosphatidylserine specifically before this shows its characteristic ouabain-sensitivity. The last, and so far unique, example is that of an enzyme which changes its specificity if its phospholipid is destroyed or removed. NAD and NADP transhydrogenase activities of heart mitochondria are thought to be functions of a single enzyme. This enzyme, when intact, will catalyse the transhydrogenation from either reduced nucleotide to 3-acetylpyridine-adenine dinucleotide with equal facility. After treatment with phospholipase A or extraction with acetone the activity towards $NADH_2$ is either unchanged or slightly enhanced, whereas that towards $NADPH_2$ is abolished (Pesch and Peterson, 1965). Reactivation of this system has not yet been achieved.

These physical functions are normally catalysed either by the mixed lipids or by the lipids present in relatively high concentrations. The lipids which are tentatively supposed to be involved in enzyme systems as intermediates (PA, DPI and TPI) all have monoester phosphate groups, are rapidly metabolised and comprise only a very small proportion of the tissue lipids.

Catabolism of phosphoinositides.

Work in this field has been reviewed by Ansell and Hawthorne (1964) and by Hawthorne and Kemp (1964).

Both Sloane-Stanley (1951, 1952, 1953) and Rodnight (1956) found that in nervous tissues the Folch inositide fraction was broken down to a mixture of products, including both organic and inorganic phosphates. Kemp et al. (1961 a) described a liver enzyme which broke down PI by two pathways:

$$PI + H_2O \longrightarrow \text{1,2-diglyceride} + \text{L-myo-inositol-1-phosphate.}$$

$$PI + H_2O \longrightarrow \text{2 fatty acids} + GPI.$$

This enzyme system would also attack the Folch fraction, with the release of inositol di- and tri-phosphates, but not of P_i. The observations of Rodnight and of Sloane-Stanley were confirmed and extended by Thompson and Dawson (1962 a, b). They found that extracts of brain acetone powders would catalyse the reactions:

$$TPI + H_2O \longrightarrow DPI + P_i$$

$$DPI + H_2O \longrightarrow PI + P_i$$

$$TPI + H_2O \longrightarrow \text{1,2-diglyceride} + \text{inositol triphosphate}$$

$$DPI + H_2O \longrightarrow \text{1,2-diglyceride} + \text{inositol diphosphate.}$$

These workers have recently studied this system further and they have purified from it both a TPI phosphomonoesterase and

a TPI phosphodiesterase (Thompson and Dawson, 1964; Dawson and Thompson, 1964). Both enzymes were activated by Mg^{++}, which was essential for monoesterase activity. Both were also activated by cationic amphipathic substances (e.g. cetyltrimethylammonium bromide) which decreased the negative charge of the substrate. Na^{+} ions stimulated both enzymes, and K^{+} ions the monoesterase. The physicochemical aspects of these and other phospholipases have been discussed by Dawson (1964). Dawson and Eichberg (1965) consider that the inositidases described by Thompson and Dawson are responsible for the very rapid post-mortem breakdown of DPI and TPI in brain.

OBJECTIVE OF THE WORK

OBJECTIVE OF THE WORK.

In 1963 very little was known of the biosynthesis of polyphosphoinositides. The work which follows is the result of an attempt to characterise a biosynthetic pathway to diphosphoinositide and to elucidate the function of this lipid.

MATERIALS AND METHODS

MATERIALS AND METHODS.

Materials

Analytical grade reagents, supplied by British Drug Houses, Hopkin and Williams or Judex, were used for most purposes.

In the case of 72% perchloric acid, the Judex product was preferred for phosphate assays, since it gave appreciably lower blanks than the other brands.

The sources of materials for special purposes are listed below.

Radiochemical Centre, Amersham: carrier-free ^{32}P-orthophosphate.

Boehringer und Soehne (obtained through Courtin and Warner, Lewes, Sussex): ATP, ADP, AMP, 3-phosphoglycerate kinase, glyceraldehyde-3-phosphate dehydrogenase, hexokinase, sodium succinate, sodium pyruvate.

Sigma (London) Chemical Co. Ltd. : ATP, ADP, AMP, CTP, GTP, UTP, NAD, NADP, 3-phosphoglycerate kinase, glyceraldehyde-3-phosphate dehydrogenase, hexokinase, glucose-6-phosphate, 6-phosphogluconate, crystalline bovine plasma albumin, cysteine (free base), tris (hydroxymethyl)amino-methane (Tris, type 7-9).

L.Light and Co., Colnbrook, Bucks. : phenolphthalein glucuronide, crystalline bovine plasma albumin, calf intestinal alkaline phosphatase.

British Drug Houses: 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium chloride (INT), aluminium oxide (for chromatographic

adsorption analysis).

Fluka, Switzerland. : sodium 3-glycerophosphate.

V.A.Howe: Ion-exchange resins (Dowex 1, Dowex 50W), Norit A charcoal, Potter-Elvehjem homogeniser (Tri-R).

Henry Simon Ltd., Cheadle Hume, Cheshire: nylon St. Martin's bolting cloth, 9 N.

Camlab Ltd. : silica gels G and H (Merck).

Savory and Moore Ltd. : silicic acid (Mallinkrodt), 100 mesh, powder, Analytical Reagent.

Shandon Ltd. : adjustable thin-layer spreader and other chromatography apparatus.

Birmingham Meat Market : fresh sheep and ox tissues.

American Lecithin Co. : Inosithin.

Oligomycin was a gift from Dr. S.Fleischer, Enzyme Institute, Madison, Wisconsin. Dr. P.Kemp and Mr. B.Sedgwick, both of this Department, kindly donated phosphatidylcholine (from egg) and phosphatidic acid (prepared from egg PC), respectively.

Spectrophotometry.

All spectrophotometric determinations were performed on either the Unicam SP 500 or SP 600, using 1 cm. glass or silica cells.

Chemical assay methods.

Protein was assayed by a biuret method (Weichselbaum,

1948) after precipitation of the protein with 8% or 10% TCA.

After addition of 1 ml. of water and 2 ml. of biuret reagent to the precipitate, stirring and incubating at 37°C for 15 min., many of the solutions showed considerable turbidity. This was usually most evident in the assays on homogenate and on the lysosomal and microsomal fractions. This led to artifacts in the apparent distribution of protein and also to low apparent recoveries of protein. These problems were overcome if, after incubation, each assay was extracted with an equal volume of water-saturated ether. Extinctions were measured at 555 mμ. Protein concentration was calculated in mg., based on bovine serum albumin as standard. This gave an optical density at 555 mμ of 0.09 per mg. protein.

Organic phosphate was measured by the method of King (1932) or by a more sensitive method (Galliard et al.,1965), based on that of Bartlett (1959). For the latter, samples containing less than 3 μg. phosphorus were boiled in a little water to remove any organic solvents. Each sample was then digested with 0.8 ml. of 72% perchloric acid until the digest was colourless and white fumes issued from the neck of the flask. Water was then added to the cooled digest to give a volume of 4.6 ml. Then 0.2 ml. 5% ammonium molybdate solution and 0.2 ml. 1-amino-2-naphthol-4-sulphonic acid reagent (Bartlett, 1959) were added and the tubes were heated in a

water bath at 90° for 15 min. Extinctions were measured at 830 mμ as described by Bartlett. This method of analysis is rapid and can be used for the analysis of phosphate-containing areas from paper chromatograms, provided that 0.01 ml. 5% ammonium molybdate is added prior to digestion. This appears to have a catalytic effect (Dawson, 1960). The colour yield of this method was only independent of perchloric acid concentration if more than 0.4 ml. of the 72% acid was used in a final volume of 5 ml. It was important, therefore, not to carry on the digestion for so long that the volume of perchloric acid was seriously reduced. Under the conditions given above approximately 0.2 ml. of acid is lost during the digestion. Often the volumes quoted had to be doubled due to the large amounts of paper in some of the samples analysed.

During the course of this work Judex Analytical Reagent 72% perchloric acid became available. This was found to be greatly superior to either the Hopkin and Williams or B.D.H. products, since it gave much lower blanks.

By the use of these two assay methods any quantity of phosphate in the range 0.5 - 60 μg. could be assayed quickly and easily.

Inorganic phosphate was measured by the method of King (1932).

RNA was assayed by the method of Schneider (1957) and DNA by that of Burton (1955, 1956).

Radiochemical assays.

Radioactivity (^{32}P) was measured using a type M6 liquid Geiger counter (20th Century Electronics Ltd.) and an Ekco scaler. All samples were counted in a volume of 10 ml.

Paper chromatographic methods.

Separation of phosphate esters

Samples were applied to Whatman No. 1 paper, and development was with either: a) n-propanol:ammonia (s.g. 0.88): water (5:4:1, v:v:v) for 12-40 hr., descending (Desjobert and Petek, 1956).

or : b) iso-propanol:ammonia (s.g. 0.88): water (7:1:2, v:v:v) for 2-5 days, descending (Markham and Smith, 1952).

Phosphate-containing spots were located with the perchloric acid:ammonium molybdate reagent of Hanes and Isherwood (1949) as modified by Dawson (1960). After the sprayed papers had dried they were irradiated with ultraviolet light (Hanovia Chromatolite with the filter glass removed), and the phosphate-containing compounds appeared as blue spots on a white background. Approximately 1 µg. of phosphorus was the minimum quantity detected by this method.

Separation of inositol.

Samples were applied to Whatman No.54 paper and development was with iso-propanol:acetic acid (glacial): water

(3:1:1, v:v:v) for 10-20 hr., (Hübscher and Hawthorne, 1957).

Polyols were detected with the silver nitrate dip of Trevelyan et al.(1950). The spots which appeared were fixed by dipping the chromatograms through 5% (w:v) sodium thiosulphate solution (Anet and Reynolds, 1954).

Separation of nucleotides.

The purity of ^{32}P-ATP was checked by chromatography on Whatman No. 1 paper, descending, for 4-6 hr. The solvent was a modification of the aqueous sodium hydrogen phosphate: iso-amyl alcohol mixture of Wyatt (1955), in which the phosphate was replaced by sodium hydrogen carbonate (sodium bicarbonate). Papers were suspended from the trough of a chromatography tank as usual. The trough was half-filled with 5% (w:v) aqueous sodium bicarbonate, saturated with iso-amyl alcohol. A layer of iso-amyl alcohol was then run on to the surface of this solvent and development allowed to proceed for the required time.

The nucleotide spots were detected by their ultra-violet absorption, using a Hanovia Chromatolite with the filter glass in place. Phosphates were detected as above and radioactive materials either by radioautography or with a Panax PM1 radiation monitor. ATP had an R_f of approximately 0.8, P_i of 0.3.

Preparation of formaldehyde-impregnated papers.

The method was essentially that of Letters (1964).

However, some variations were introduced.

No. 3MM or No. 1 Whatman papers were used for the preparations. Although those prepared from No. 1 gave a somewhat cleaner resolution of the inositides than the 3MM, less lipid could be loaded on to them and they were considerably more fragile when wet. For most of the assays described here, 3MM were used.

The papers were rolled inside paper towelling in bundles small enough to fit a 5-litre beaker. After standing overnight in 2:1 (v:v) formaldehyde:glacial acetic acid, the beaker containing the papers was placed (covered by a glass plate) in an oven which was heated to 110°, held at that temperature for 5-6 hours, and left to cool overnight. During the whole of this procedure the fumes from the vent at the top of the oven were removed by a water pump.

The following morning the papers were transferred to tap water, washed in this (either several changes or continuously flowing) for 24 hours and then in at least 3 changes of deionised water. After air-drying, they were pressed before use.

Chromatography of lipids on formaldehyde-impregnated papers.

The lipid samples to be separated were spotted on to the prepared papers and the chromatograms developed in n-butanol: acetic acid:water (4:1:5, v:v:v), top phase (for No.1 papers).

For 3MM papers it was found that equilibration of this solvent with 1/8 vol. diethyl ether for at least one day before use improved the chromatograms. The equilibration produced two layers, of which the upper was used. The chromatograms were run for 12-18 hours.

Detection of lipids on chromatograms.

When using labelled materials alone, the chromatograms were radioautographed with Kodirex film for 2-10 days.

When labelled materials were co-chromatographed with the Folch inositide fraction, or unlabelled samples were being studied, the lipids were detected with Nile Blue. The dried chromatograms were immersed in a 0.01% solution of the dye in 0.02N HCl for 30 min. and were washed in running tap water for 1-5 min. This did not elute any DPI or TPI from chromatograms having radioactive spots of these lipids on them, (M. Kai, unpublished). The background of the stained chromatograms could be partially cleared by brief exposure to ammonia.

Chromatograms on No. 1 papers could also be stained with the usual phosphate spray, but the results were not so good as those with Nile Blue.

The resolution of the three inositide spots from samples to which approximately 20µg. P of Folch inositide fraction had been added prior to chromatography was usually better than that of samples which were run without this addition.

Thin-layer chromatography.

Glass plates 20 cm. long were used, coated with a 0.3 mm. layer of silica gel G or H. The normal developing solvent was chloroform:methanol:water (95:35:5, v:v:v). The mobilities of all components could, if necessary, be increased by raising the water content of the solvent, and vice versa. The spots were visualised by three methods:

a) The plates were stood in a tank full of iodine vapour for about 15 min. This stained most organic compounds non-specifically.

b) Amino-containing compounds were detected by spraying the plates with 1% (w:v) ninhydrin (indane-trione hydrate) in acetone containing 1% (by volume) of pyridine. The sprayed chromatograms were then heated at 80° for 5 min.

c) The plates were sprayed with 25% (by volume) concentrated sulphuric acid and heated for 15 min. at 100° to char organic compounds.

Preparation of the Folch inositide fraction from ox brain.

A mixed inositide fraction (described by Folch as diphosphoinositide) was prepared from Folch fractions I and II (Folch, 1942) by 12 precipitations of the inositides from chloroform with methanol (Folch, 1949). The supernatants from these precipitations were combined and used as a source of PI (see below).

Isolation of phosphatidylinositol.

Three lipid mixtures have been used as sources of PI during the present work, these being:

a) The supernatants from the 12 precipitations of Folch fractions I and II (see above). These contained a mixture of PS, PI and PE.

b) A total lipid extract of beef liver. The liver was extracted twice with acetone and twice with chloroform: methanol (2:1, v:v). The chloroform:methanol extracts were washed by the method of Folch et al. (1957).

c) Inosithin, a mixture of phospholipids and neutral lipids derived from soya-beans.

These have been fractionated by column chromatography in several systems, as discussed below. During column fractionations it has usually been found more convenient and more informative to monitor the separations using TLC rather than phosphate analysis of the fractions. The separations discussed below were followed using the TLC method.

Silicic acid chromatography alone.

It has been found in this laboratory that in attempts to purify PI from animal lipid mixtures the greatest problem is the persistent way in which PI and PS stay together during fractionation procedures using silicic acid chromatography as the sole separation method. Even when a second silicic acid column (Hanahan, 1960, p. 29) was used in attempts to purify

further the inositide fraction from the first column (Hanahan et al., 1957), persistent contamination with PS occurred. This was particularly troublesome with the lipid derived from the brain inositide fraction, as the predominant component of the mixture applied to the column was PS. In one case the peak eluted from the second column as "PI" was found by chromatography on formaldehyde-impregnated paper to be composed of an approximately 7:3 mixture of PS and PI. Clearly this method was inadequate for the isolation of PI.

Chromatography on aluminium oxide.

In all experiments the alumina used was that supplied by British Drug Houses labelled as "Suitable for chromatographic adsorption analysis".

Two methods have been described by which PI and PS can be separated on columns of alumina. In that of Hanahan (1960, p. 115) PS was eluted before PI by a chloroform:ethanol mixture (5:2, v:v) to which increasing concentrations of water were added.

In the method of Long and Owens (1962) PI was eluted between PE and PS in a solvent system containing increasing amounts of water in 1:1 (v:v) chloroform:methanol. This was the better characterised of the systems available, (Long et al. 1960, 1962; Long and Staples, 1961). Also it had the advantage that PI, being eluted before PS, there was less risk of the PI

being contaminated with PS.

. The lipid mixture dissolved in chloroform, was applied to an alumina column and was eluted with 1:1 chloroform: methanol containing first 8% and then 14% water. With 8% water most of the PE was eluted from the column. The solvent containing 14% water eluted the remaining PE as a sharp peak, followed by a broad peak of PI. PS was not eluted unless the solvent was made basic by the addition of 1% by volume of concentrated ammonia (s.g. 0.88).

In some cases difficulties were still encountered in using this technique. These were usually avoided if the following restrictions were carefully observed.

1. The columns used were very short and wide (for dimensions see below). Long et al. (1962) also found this to be essential for the rapid and satisfactory separation of serine-containing lipids on alumina.
2. The columns were run as quickly as possible after the lipid mixtures had been loaded. This minimized the hydrolysis of the lipids to their lyso- derivatives by the alumina. In the purification of PI this was particularly relevant as a compound thought to be lyso-PE accumulated fairly rapidly on alumina columns and was eluted with PI both from alumina and silicic acid.

In summary, the best conditions for the partial purification of PI on an alumina column were as follows. A

column of approximately 9 cm. bed height was packed by pouring dry alumina into the column vessel containing sufficient chloroform to keep all of the alumina submerged. One g. of alumina was used per mg. of lipid phosphorus. Two hundred g. of alumina gave a column 9 cm. high with a diameter of 5.5 cm. The column was washed with approximately 1 bed volume of chloroform, after which the lipid sample, dissolved in chloroform, was run into the column. For a column of the dimensions quoted above, elution was with 2 l. chloroform:methanol:water (46:46:8, v:v:v), followed by 3 l. chloroform:methanol:water (43:43:14. v:v:v). Elution was always completed on the same day as the column was loaded, usually in 6-8 hours from loading. Normally under these conditions the PI emerged as a broad peak with its centre after about 1.5 l. of the final eluant. The fractions containing PI were detected using TLC and pooled. The combined fractions were evaporated in vacuo on a rotary evaporator until the chloroform and most of the methanol had been removed. At this stage the lipid was in the form of a turbid suspension. This was made approximately 0.01M with $MgCl_2$ and extracted with 2.2 volumes of 1:1 (v:v) chloroform: methanol. The lower phase was taken and the top phase extracted with 1.1 volume of chloroform. The extracts were combined, dried, and the lipid dissolved in chloroform prior to application to a silicic acid column.

Rechromatography of partially purified PI on silicic acid.

The technique used for rechromatography of the material isolated from the alumina column was as follows. A silicic acid:celite (2:1, w:w) column was packed in 1:1 (v:v) chloroform: methanol, using 2g. of silicic acid per mg. of lipid phosphorus to be applied. After the column had been washed with 1 bed volume of chloroform the lipid sample was applied in dry chloroform. The column was eluted with 1 volume of chloroform followed by 2 volumes of 11:2 (v:v) chloroform:methanol and 4 volumes of 5:2 (v:v) chloroform:methanol. The first chloroform:methanol mixture eluted the remaining PE, and the second eluted an almost pure peak of PI, the later fractions of which were contaminated with the compound thought to be lyso-PE. In all preparations from liver there was also a slight contamination with a fast-running (on TLC) lipid. This was ninhydrin-negative and may have been either phosphatidic acid, phosphatidylglycerol or cardiolipin.

Chromatographic behaviour of soya-bean lipids.

Before chromatography, "Inosithin" was subjected to two acetone and two ethanol precipitations, to remove most of the neutral lipids and PC. Chromatography on alumina as described above yielded a considerably purified PI preparation, with PE and lyso-PE (?) as the main contaminants. Further purification of this material by the above technique of silicic

acid chromatography gave the surprising result that the PI was almost quantitatively eluted before PE in the 11:2 chloroform:methanol, only a very small peak emerging in the 5:2 mixture. The major (11:2) peak from this column was contaminated with some six components which ran faster than PI on TLC. However, after standing the solution of lipid in chloroform at 0° for 24 hr., a heavy white precipitate was collected. This was insoluble in pure chloroform, but analysis by chromatography on formaldehyde-impregnated papers and by TLC showed it to be almost pure PI (estimated purity approximately 90%). The supernatant from this separation contained about 50% PI, the purity of which could be considerably improved by 2 ethanol precipitations from chloroform, followed by a series of precipitations at increasing ethanol concentrations at 0°, the precipitate being collected in 5 fractions as the ethanol concentration was increased to 80% (by vol.). This yielded a product of approximately 80-90% purity.

As PS was not a problem in the separation of the soya-bean lipids, an attempt was made to purify PI from the acetone- and ethanol-precipitated "Inosithin" using only a single silicic acid column. Again the PI eluted before the PE.

Great difficulty had been experienced in attempts to emulsify the previous soya-bean lipid prepared by the combined alumina/silicic acid technique (see above). However, if the lipid was exhaustively washed with disodium EDTA it became very

easy to emulsify.

Following the above observation, the PI peak from the single silicic acid column was dissolved in 2:1 chloroform: methanol and washed with 1/5 vol. of Na_2EDTA (0.05M). Rechromatography of the washed lipid on silicic acid gave two peaks of PI, one before PE and the other in the expected position in 5:2 chloroform:methanol. The material eluted with the 5:2 mixture was, as expected, very easily emulsified in water. This material was better than 90% pure.

In summary, it can be said that it is considerably easier to prepare PI from soya-bean lipids than from extracts of either brain or liver, mainly because of the absence of large quantities of PS. Whereas adequate purification of the animal PI requires the use of at least two columns the soya-bean lipid can be purified by chromatography of the sodium salts of the lipid mixture on a single column of silicic acid. The difference in the chromatographic properties of brain and soya-bean PI is unexplained.

Recently both Dr. M.L. Karnovsky (personal communication, 1965) and Dr. H.E. Carter (personal communication, 1965) have found that under certain conditions both animal and plant PI can be eluted from silicic acid columns with low concentrations of methanol in chloroform. Carter found that the Ca^{++} salt of PI was eluted in 7:1 chloroform:methanol, whereas the Na^{+} salt was

not eluted until a 3:1 mixture was applied. These results are in essential agreement with the observations on soya-bean described above.

Isolation of phosphatidylethanolamine.

In the isolation of PI from a brain lipid mixture containing PS, PE and PI (see above) the material eluted from an alumina column with chloroform:methanol:water (46:46:8, v:v:v) was approximately 80% pure PE, the main contaminant being lyso-PE(?). The fractions comprising the PE peak were pooled, most of the chloroform and methanol removed in vacuo, and the lipid extracted into 2.2 volumes of 1:1 chloroform:methanol, after the addition of $MgCl_2$ (final concentration 0.01M) to the aqueous suspension of lipid. This material was used as PE without further purification.

Preparation of ^{32}P-labelled ATP.

γ-^{32}P-ATP was synthesised from unlabelled ATP and ^{32}P-orthophosphate by the method of Glynn and Chappell (1964). The product was better than 95% radiochemically pure, the only contaminant being ^{32}P-orthophosphate.

Preparation of subcellular fractions.

All media for cell fractionation were neutralised to approximately pH 7.4 with sodium bicarbonate and all fractionation procedures were performed at 0-4^{0}.

The centrifuges used were the M.S.E. "13", M.S.E. "18", M.S.E. "40" and M.S.E. "50". The rotor normally used with the "13" and "18" was the 8 x 50 ml. angle rotor. Except where otherwise specified, quoted centrifuge speeds refer to this rotor. An 8 x 25 ml. angle rotor was used in the "40" and "50". All centrifuge data are quoted both as revs./min. for a certain rotor and as g values.

In a few experiments a 6 x 250 ml. and a continuous action rotor were used with the M.S.E. "18". The rotor speeds in these experiments were calculated to apply the same gravitational fields to the material being fractionated as those quoted for the same fractionation stages using the 8 x 50 ml. rotor.

All samples were centrifuged in polypropylene tubes, sealed with aluminium caps.

Fractionation of brain.

The "mitochondrial" fraction used in the studies on brain was an impure fraction prepared as follows (Aldridge, 1957).

Rats were killed by a blow on the back of the neck. Brains were rapidly removed. Each was homogenised in 7.5 ml. of 0.25M sucrose, using a teflon-tipped Potter-Elvehjem homogeniser with a diameter of 0.596 in. and a radial clearance of 0.002 - 0.003 in. The pestle was rotated at 1,100 revs./min. Twelve "up and down" strokes of the pestle were used, the whole operation taking $1\frac{1}{2}$ - 2 min. After homogenisation a further 12.5 ml.

sucrose per brain was added.

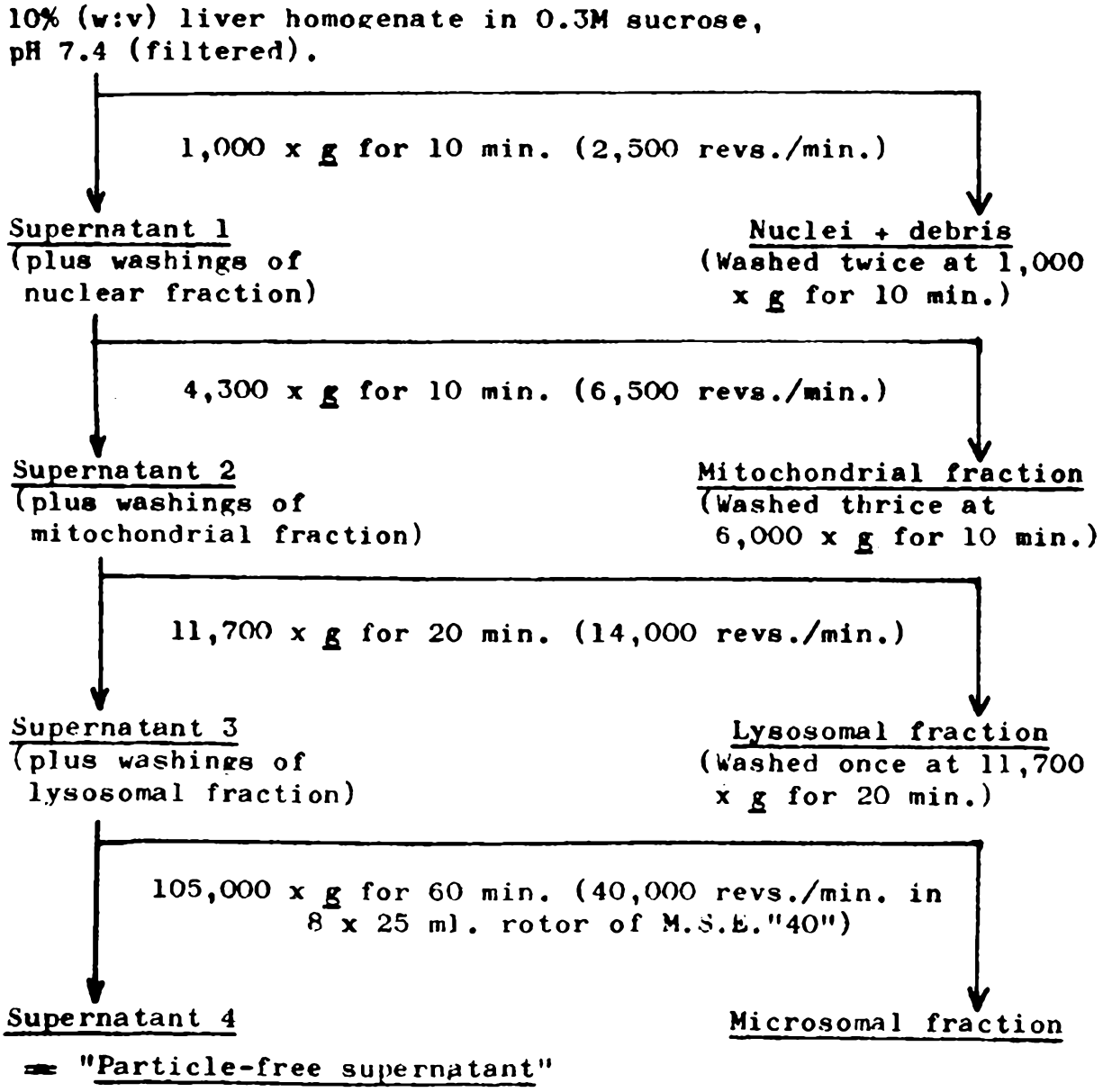
The homogenate was centrifuged at 1,050 x g (2,700 revs./min.) for 10 min. The sedimented "nuclear" fraction was discarded and the supernatant centrifuged at 10,000 x g(9,000 revs./min.) for 10 min. The pellet was rehomogenised in 0.3M sucrose and sedimented again at 10,000 x g for 10 min. The pellet ("mitochondrial fraction") was suspended in 0.3M sucrose and kept for enzyme assays.

Fractionation of liver.

During the work described, two different fractionation schemes were used. Initially mitochondrial preparations were made by the method of Schneider (1948).

The tissue was homogenised in a loosely-fitting Potter-Elvehjem homogeniser (diameter 0.966 in.; radial clearance 0.003 - 0.0045 in.) with a teflon pestle, which was rotated at 1,100 revs./min. Three ml. of sucrose were used for homogenisation of each gram of tissue. The homogenate was diluted with sucrose to approximately 10% (grams wet tissue/ml. sucrose) and centrifuged at 1,000 x g (2,600 revs./min.) for 10 min. The "nuclear" pellet was discarded. A mitochondrial pellet was sedimented from the supernatant at 12,000 x g (10,000 revs./min.) for 10 min. The pellet was homogenised in 0.25M sucrose and resedimented at the same speed. This mitochondrial pellet suspended in sucrose was kept for enzyme assays.

Fig. 8. **Fractionation of Liver Homogenate**
(Sedgwick and Hübscher, 1965)



During the course of the work described in this Thesis, Mr. B.Sedgwick and Dr. G.Hübscher of this Department were studying the subcellular fractionation of liver. This study produced an improved scheme for the fractionation of liver homogenates. This method (Sedgwick and Hübscher, 1965), based on that of de Duve et al. (1955), was adopted in the latter part of this work.

The preparation of a 10% (w:v) homogenate was as described above, except that the suspending medium used was 0.3M sucrose. Minor modifications were made of the published method for fractionating this homogenate during its routine usage. These were:

1. EDTA was omitted from the suspending medium, as it seemed neither to improve the separations obtained nor the stability of the resulting fractions. On the contrary, in studies of intestinal mucosa (Clark and Porteous, 1965; Hübscher and West, unpublished) it has been found that exposure to EDTA modifies alkaline phosphatase in such a way that it becomes Zn^{++}-dependent, rather than Mg^{++}-dependent.
2. The homogenate was filtered through 9 N St. Martin's bolting cloth (a fine nylon mesh) before starting the fractionation. This removed some of the larger particles (e.g. connective tissue, blood vessels) which tend to carry down a large amount of non-nuclear particulate material into

the "nuclear" pellet. It will be shown later in this Thesis that the material filtered off by this procedure does not represent a selective removal of any particular subcellular fraction.

3. The nuclear pellet was twice washed at 1,000 x g for 10 min. and the washings added to supernatant 1 (see Figure 8).

The complete scheme is shown in Figure 8.

I should like to express my thanks to several members of this Department, particularly Dr. G.Hübscher, Mr. B.Sedgwick and Mr. D.N.Brindley for advice on cell fractionation procedures.

Assay of enzymes as markers for cell components.

The distribution of different structural components of the cell in the fractions prepared as described above were measured by the assay of marker enzymes known to be associated with specific cell components.

The use of RNA as a submicrosomal marker was based on the work of Dallner (1963).

The use of 5'-nucleotidase as a plasma membrane marker was based on the work of Emmelot et al. (1964), of Novikoff (1964) and of El-Aaser and Reid (1965).

The other markers are similar to those used by de Duve and his co-workers (de Duve et al. (1955), except that those workers assayed succinate dehydrogenase as succinate:cytochrome c reductase.

Succinate dehydrogenase was used as the mitochondrial marker and was assayed as succinate:INT* oxidoreductase (E.C. 1.3.99.1) by the method of Pennington (1961).

The distribution of lysosomes was traced using β-glucuronidase, (β-D-glucuronide glucuronohydrolase, E.C. 3.2.1.31.). This was measured as described by de Duve et al. (1955).

Microsomal distribution was measured using Swanson's (1955) assay for glucose-6-phosphatase (D-glucose-6-phosphate

* See abbreviations, p. 1.

phosphohydrolase, E.C. 3.1.3.9.), with the addition of an EDTA-potassium fluoride inhibitor mixture, as recommended by Hübscher and West (1965).

6-phosphogluconate dehydrogenase (6-phospho-D-gluconate NADP oxidoreductase (decarboxylating), E.C. 1.1.1.44.) was the marker for the soluble fraction. This was assayed by the method of Glock and McLean (1953).

The distribution of nuclei was measured using the assay of DNA. In submicrosomal fractionations RNA was used as a marker for rough-surfaced membranes (i.e. those with ribosomes attached to them).

5'-nucleotidase was used as a marker for plasma membranes. Its assay is described below.

Assay of 5'-nucleotidase.

5'-ribonucleotide phosphohydrolase (E.C. 3.1.3.5.) was assayed by a modification of the method of El-Aaser and Reid (personal communication, 1965) as follows. Incubations were in a 2 ml. system containing:

100 mM KCl; 10 mM $MgCl_2$;

50 mM Tris/HCl buffer, pH 7.4;

5 mM AMP; 10 mM sodium potassium tartrate.

Tissue preparations (less than 0.5 mg. protein per assay) were added to the assay system suspended in 0.3M sucrose (0.2 ml. or less). Incubation was for 15 min. at 37°, and the reaction was

stopped with 1 ml. of 25% (w:v) TCA. The precipitate was centrifuged off and P_i was assayed in a 2.0 ml. sample of the supernatant. Corrections were made for blanks in which the enzyme and substrate were omitted.

Using this method the release of P_i was proportional to protein concentration up to at least 0.8 mg. protein per assay. The release of P_i was linear with time for at least 30 min. Concentrations of AMP above 2mM were optimal. Activities were normally determined on freshly prepared cell fractions. However, the activities of these did not change during storage at 4^o for 24 hr. Assaying the activity of this enzyme in frozen and thawed preparations was normally avoided, due to activation of acid phosphatase by this procedure. The inclusion of tartrate in the assay medium, though, reduced the interference by this enzyme to a minimum.

Assays of enzymes other than those used as markers.

ATPase (ATP phosphohydrolase, E.C. 3.6.1.3.) was assayed according to Nielson and Lehninger (1955).

Respiration and oxidative phosphorylation in brain mitochondrial preparations were measured by the method of Aldridge (1957).

Assay of DPI labelling is separately treated in this section and in the results section.

Measurement of mitochondrial swelling and contraction.

The method used was a fall and rise in the optical density of a mitochondrial suspension during, respectively, swelling and shrinkage (see Lehninger, 1962, for a review). The details of the method were basically similar to those of Vignais et al. (1964 b), with some small modifications. The experiments reported here were performed at room temperature with 5 mM potassium phosphate or 10^{-5}M sodium oleate as the swelling agent. With preparations of mitochondria which had been kept in 0.3M sucrose for a day or more it was often necessary to add a substrate (7 mM sodium succinate) before swelling would occur (see Figure 9, curve 1). All measurements were made in a final incubation volume of 3 ml., containing approximately 0.5 mg. protein. This concentration gave absorbances at 520 mμ of about 0.25 (swollen) to 0.6 (contracted).

Contraction was expressed as the difference between the lowest absorbance and the highest absorbance measured after the addition of ATP. This method of expression was preferred to the change in absorbance during the 15 min. following ATP addition, the units used by Vignais et al. (1964 b), for two reasons. If contraction was rapid, a partial reversal from maximal contraction had sometimes occurred within 15 min. of ATP addition (Figure 9, curve 1). On the other hand, if the ambient temperature was low, maximal contraction had sometimes not been

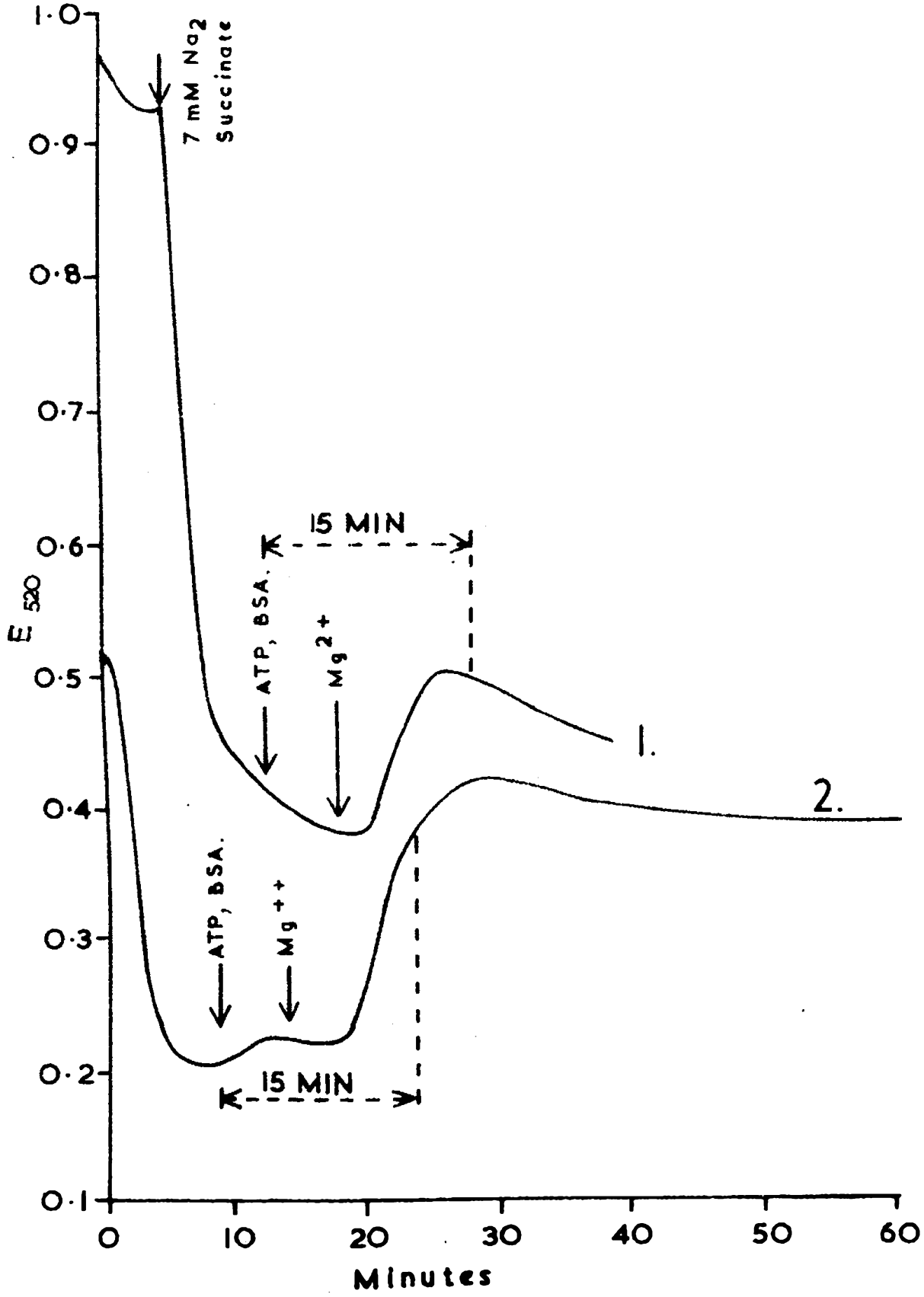


FIG. 9. TO ILLUSTRATE THE VARIABLE TIME-COURSE OF MITOCHONDRIAL CONTRACTION (CURVES 1 AND 2 ARE TAKEN FROM DIFFERENT EXPERIMENTS)

achieved within 15 min. (Figure 9., curve 2). Both of these effects tended to give low values for the amplitude of contraction.

Aging of mitochondria.

Either of the methods used by Vignais et al. (1964 b) was used. Where necessary, details are given in the text.

Assay of radioactivity incorporated into DPI.

Three methods were used:

a) Deacylation of the labelled phospholipid mixtures, followed by chromatography of the resulting phosphate esters on Dowex-1-formate (Ellis et al., 1963).

b) Separation of the labelled phospholipids intact on formaldehyde-impregnated papers (Hörhammer et al., 1959; Letters, 1964). The radioactivity of the separated DPI could then be assayed.

c) Extraction of the lipids, removal of contaminating water-soluble phosphates, and assay of the radioactivity of the total lipid extract.

a) Assay by ion-exchange chromatography.

The lipids were extracted by the method of Garbus et al. (1963). The resulting lipid solution was dried in vacuo and subjected to mild alkaline hydrolysis, (Hübscher et al. 1960).

The resulting water-soluble phosphate esters were made 5mM with respect to sodium tetraborate and loaded on to a column of Dowex 1 x 10, formate form, 200-400 mesh (Dowex 1 x 8, 200-400 mesh was used in a few experiments). Elution was usually by a simplified scheme. The columns (1 x 10-15 cm.) were washed with 0.18M ammonium formate:0.005M sodium tetraborate to remove the deacylation products of the diester phospholipids and of phosphatidic acid (GPC, GPE, GPS, GPI, GPGPG, 3-GP)*. This eluant also removes inorganic orthophosphate, which is not clearly separated from 3-glycerophosphate on these columns, when eluted by this method (Galliard, 1963).

After elution of the mixed P_i/3-GP peak, fractions were collected and counted until the activity of the eluate fell to the background level, when the eluant was changed to 0.2M ammonium formate:0.1M formic acid (Ellis et al., 1963). This eluted GPIP, the deacylation product of DPI. The peak appeared after approximately 100 ml. of this eluant had passed through the column. Fractions were collected either on an automatic fraction collector or by hand. When fractions were being collected by hand up to ten columns could be eluted simultaneously, the columns being mounted in a rack so that the eluate from the ten columns flowed into ten tubes mounted beneath them. Fractions were of 10 or 25 ml. The sum of the activities

* See abbreviations, p.1.

of the fractions making up the GPIP peak was taken as the activity of the labelled DPI in the original lipid sample.

In experiments when other esters were to be studied, elution was continued with 0.4M AF/0.1M FA*, 0.5M AF/0.1M FA (to elute IP_2 and $GPIP_2$ respectively) and 1N HCl (Ellis et al., 1963).

b) Chromatography of intact lipids on formaldehyde-impregnated papers.

The method, introduced by Hörhammer et al. (1959), has now been somewhat modified by Letters (1964). The technique used for the separations is described under "Paper chromatographic methods". After preparation of the lipid extracts (see a)), they were dried in vacuo and dissolved in 0.2 ml. chloroform. This was loaded on to the prepared paper and the vessel containing the lipid was twice washed with 0.1 or 0.2 ml. of 1:1 (v:v) chloroform:methanol. These washings were also applied to the same area. Normally the extracts from 5 or 6 incubations were applied to one 23 cm.-wide sheet of paper. The chromatograms were developed and the lipids detected as described elsewhere.

After detection, the areas corresponding to DPI were cut out and digested in 1.4 ml. 72% perchloric acid in the presence of one drop of 5% ammonium molybdate. The volume of the digest was made to 10 ml. with water and radioactivity determined.

* See abbreviations, p. 1.

c) Assay of ^{32}P incorporation into the total lipids of an incubation mixture.

This method was only used in the later stages of the work, when it was known that the predominant fraction of the labelled lipid produced was DPI.

The reaction was stopped and extraction and phase separation were performed as in a) and b). The lower phase was then removed and washed with 4.7 ml. of "synthetic" top phase from the same mixture of chloroform, methanol, water and KCl/phosphate, (Garbus et al., 1963). After removal of the top phase the lower phase was diluted to 10 ml. with 1:1 (v:v) chloroform:methanol and this extract was counted.

RESULTS

RESULTS

Part I

SYNTHESIS OF DPI AND TPI IN A BRAIN MITOCHONDRIAL FRACTION.

Introduction.

Wagner et al. (1961, 1962) and Ellis and Hawthorne (1961, 1962) showed that the monoesterified phosphate groups of DPI and TPI in brain were more rapidly exchanged in vivo with injected ^{32}P-orthophosphate than the diester phosphate groups of other phospholipids.

Brockerhoff and Ballou (1961, 1962) showed that a similar high labelling of these phosphate groups occurred in rabbit brain slices incubated with ^{32}P-orthophosphate.

The report of a lipid in liver mitochondria (Garbus et al., 1963) with similar properties to the brain phosphoinositides prompted its identification in this laboratory as DPI (Galliard and Hawthorne, 1963). At this stage it seemed that it might be profitable to attempt to show a system in a brain mitochondrial fraction similar to that found in liver. This work paralleled that already in progress in the liver system by T.Galliard.

Experiment 1.

Demonstration of the labelling of DPI and TPI by a rat brain "mitochondrial fraction".

A "mitochondrial fraction" was prepared from two rat

brains (approx. 2.5 g. tissue) by the method of Aldridge (1957). In a previous experiment (performed in collaboration with Dr. H.S.A.Sherratt) it had been shown that a fraction prepared by this method was capable of respiration (Q_{O_2} for succinate = 69 µl. O_2/mg. protein/hour, and for pyruvate + fumarate = 57), and oxidative phosphorylation (P:O, for succinate = 1.05, for pyruvate + fumarate = 1.35).

Mitochondria from 1 g. of tissue were added in 0.3M sucrose to a system containing 16 mM Tris/HCl buffer, pH 7.4, 12 mM potassium glutamate, 8 mM $MgCl_2$, 100 mM sucrose, (final concentrations in 2.4 ml. system). This mixture was equilibrated at 32° in a water bath, with shaking, for 2 min. 1 mM (final concentration)sodium phosphate, pH 7.4, containing approximately 250 µC of ^{32}P-orthophosphate, was added and incubation continued for 5 min. The reaction was stopped with 9 ml. 1:2 (v:v) chloroform:methanol and the total lipids extracted as described by Garbus et al. (1963) except that filtration was through glass wool instead of paper.

A sample of the Folch inositide fraction containing 660 µg. organic phosphate was added to the mitochondrial extract and the mixture subjected to mild alkaline methanolysis according to Hübscher et al. (1959). The phosphate esters released were isolated and loaded on to a Dowex 1 x 10 formate column (200-400 mesh, 1 x 25 cm.) as described by these authors. After elution

with 0.18M ammonium formate:0.005M sodium borate till little radioactivity was being eluted., the column was eluted as shown in Figure 10. Three major radioactive peaks were present. Two of these co-chromatographed with phosphate peaks derived from the hydrolysis products of Folch inositide fraction. These have been previously identified as GPIP (derived from DPI) and $GPIP_2$ (derived from TPI) by Ellis et al. (1963). The identity of peak III is not certain but it may well be ATP contaminating the lipid extract. This compound was found by Galliard (1963) in lipid extracts prepared from liver mitochondria under conditions similar to those described here.

In a zero-time incubation in which the chloroform: methanol mixture was added to the incubation medium immediately before the addition of the ^{32}P-orthophosphate, no radioactivity was found corresponding to either GPIP or $GPIP_2$.

Experiment 2.

Confirmation of labelling of DPI and TPI by brain mitochondrial fraction.

An experiment similar to Experiment 1 was performed, with the following changes.

The final volume of the incubation was 8 ml., containing 500 μC ^{32}P-orthophosphate at a final phosphate concentration of 4 mM. The mitochondrial preparation from 6.5 g. rabbit brain was used and incubation was for 5 min. at 37°.

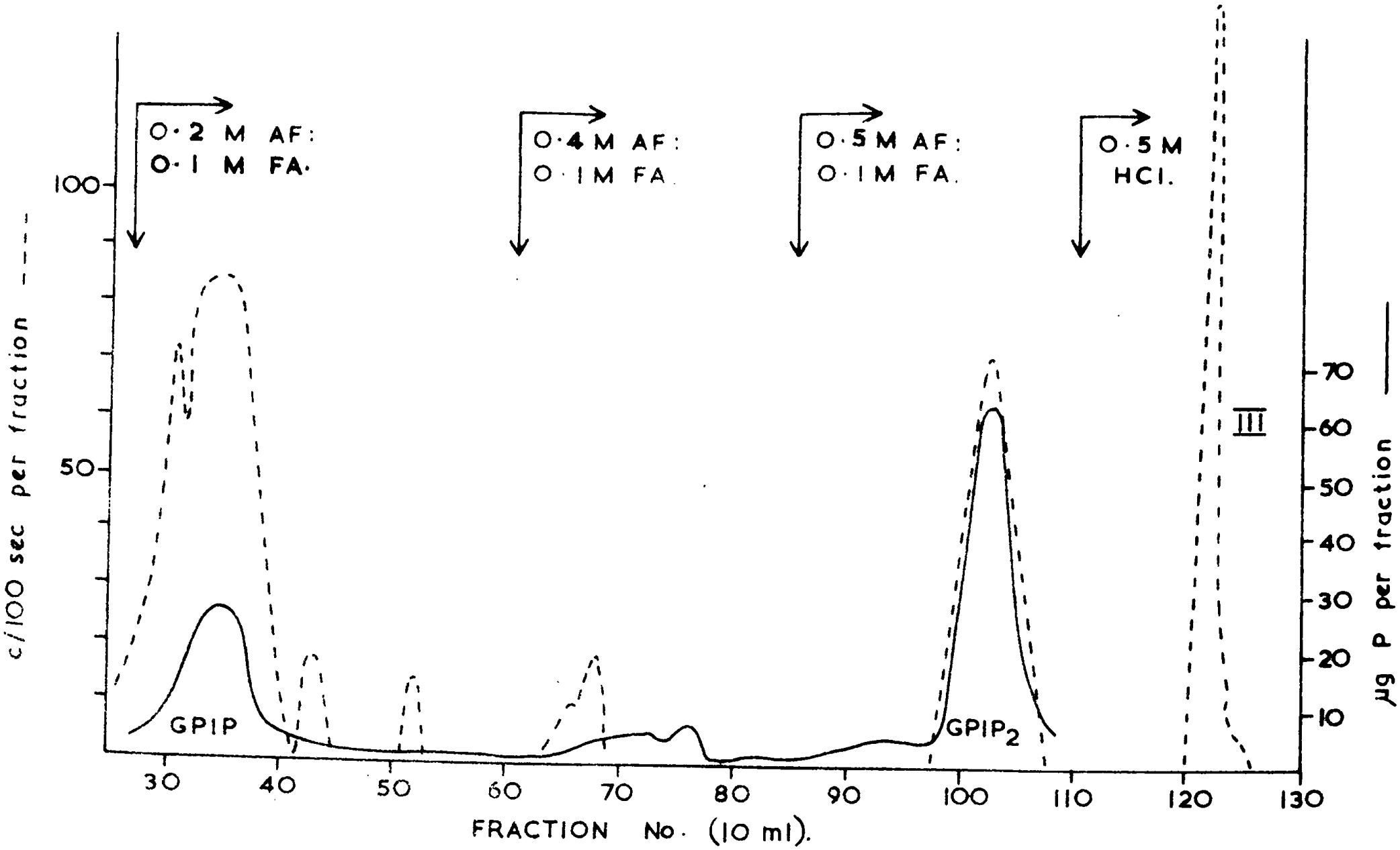


FIG. 10. SEPARATION OF HYDROLYSIS PRODUCTS OF RAT BRAIN "MITOCHONDRIAL" LIPIDS ON DOWEX 1 X 10

The lipids were extracted, deacylated and chromatographed as in Experiment 1, except that no Folch inositide was added before hydrolysis.

Separation of the radioactive products showed GPIP and $GPIP_2$ peaks eluted in the same positions as in Experiment 1, with an intermediate peak (peak II) eluted in 0.4M AF/0.1M FA. This was not seen in Experiment 1 when rat brains were used. The elution pattern is shown in Figure 11. Peak II corresponds in elution position to that expected for inositol diphosphate (Ellis et al., 1963) but no further evidence was obtained of its identity.

Time course of inositide labelling in the brain mitochondrial fraction.

One of the unexplained findings in the work of Garbus et al., (1963) on liver mitochondrial DPI labelling was that the peak activity of the DPI occurred after only 5 min. at 30° and that the activity fell steadily after this time. Galliard (1964) confirmed this and it seemed to be of interest whether brain "mitochondria" showed the same pattern.

Experiment 3.

"Mitochondria" were prepared from 2 rat brains (approx. 2.5 g. tissue) and suspended in 3 ml. 0.3M sucrose for addition to incubations. Incubation conditions were as in

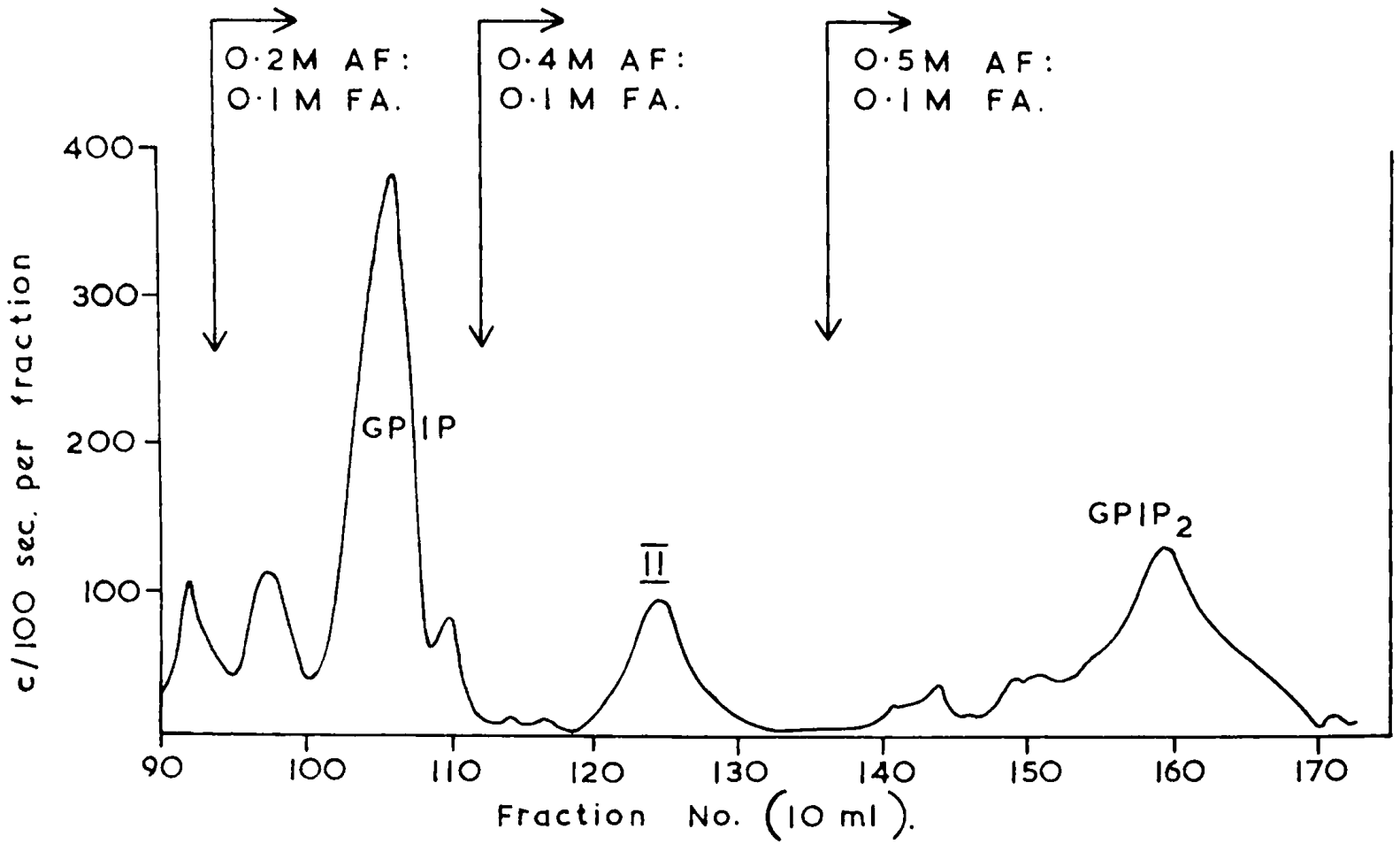


FIG. 11. CHROMATOGRAPHY OF ^{32}P-LABELLED RABBIT BRAIN "MITOCHONDRIAL" LIPIDS ON DOWEX 1 X 10

Experiment 1, except that the temperature used was 30° and the time for which incubation was continued was varied (2 min., 5 min., 20 min.).

Experiment 4.

Four rat brains (approx. 5 g. tissue) were used. Incubation conditions were as in Experiment 3, except that the final phosphate concentration was decreased to 2×10^{-4}M (0.48 µmoles in 2.4 ml.), and that incubations were allowed to proceed for from 0 to 40 min.

Experiment 5.

Three rat brains (~ 4 g.) were used.

Conditions were as in Experiment 4, except that approximately 150 µC ^{32}P-orthophosphate were added to each incubation, rather than the 250 µC previously used. Incorporations were measured over periods from 1 to 15 min.

Assay of radioactivity.

The lipids from incubations in Experiments 3, 4 and 5 were extracted and the radioactivity of DPI determined as described in the Methods section under method a) (p. 73).

Results.

Figure 12 shows a time curve obtained by scaling the results of Experiments 3, 4 and 5, on to one graph. In Experiment 5 the incorporation in the two-minute incubation was

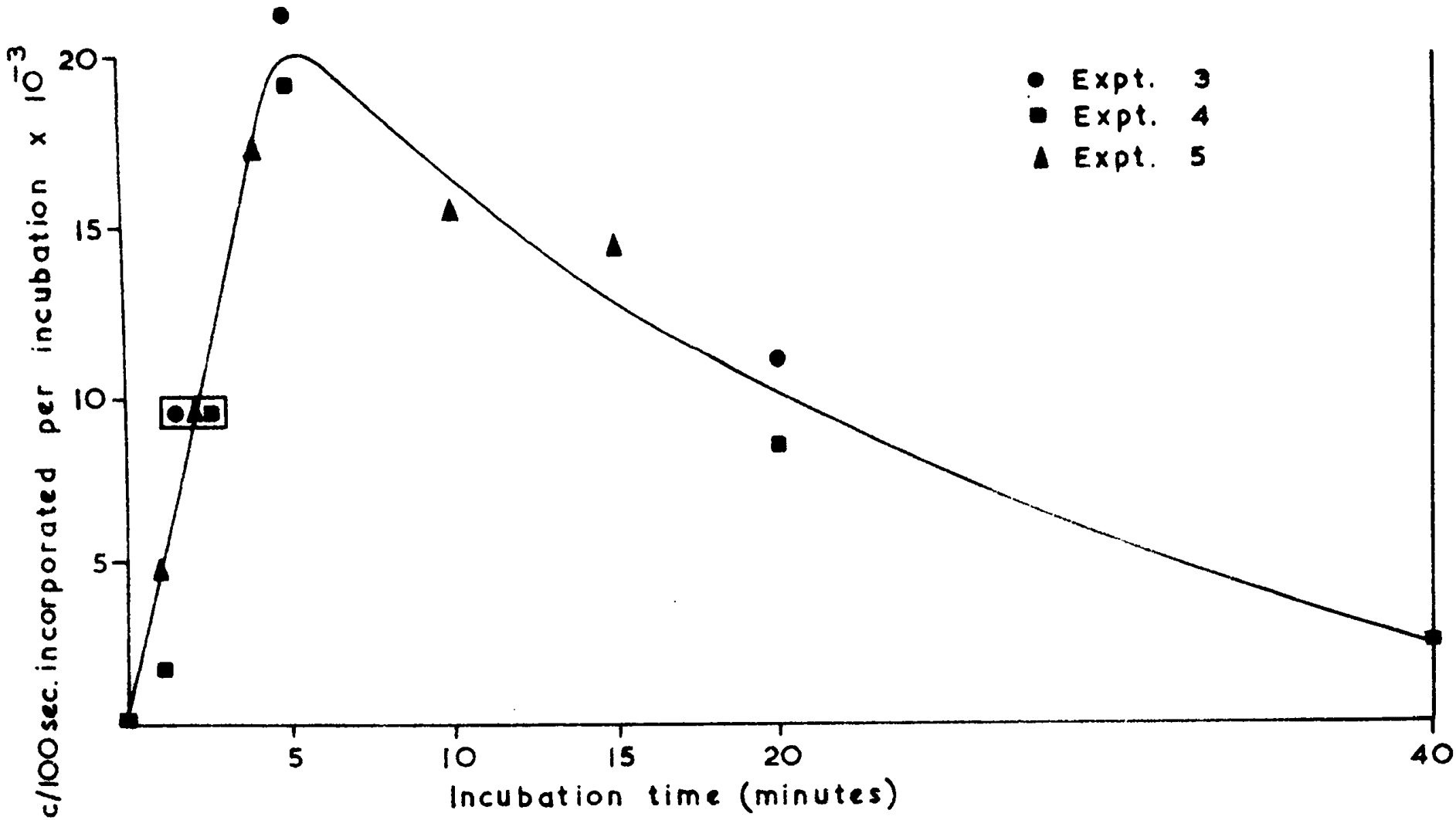


FIG. 12. TIME COURSE OF $^{32}P_i$ INCORPORATION INTO DPI IN BRAIN "MITOCHONDRIA."

9,700 counts/100 sec. To plot the results of the other experiments on to the same graph, the 2 min. figures were adjusted to 9,700 counts/100 sec. and the figures for incorporation at other times were multiplied by the ratio

$$\frac{9{,}700}{\text{counts/100 sec. at 2 min.}} .$$

The behaviour of the system was very similar to that reported by Garbus et al., with a peak of activity after only about 5 min., followed by a steady decline.

RESULTS

Part II

STUDIES ON SYNTHESIS OF DPI FROM ^{32}P-ORTHOPHOSPHATE IN LIVER.

Evidence is given in section I for the synthesis of DPI and TPI in brain "mitochondria". However, it was thought that it would be easier to work out DPI synthesis and function in liver mitochondria and then apply the results in a further attempt to elucidate the brain system. There were several reasons for this decision.

1) The cell fractionation procedures applicable to brain were less likely to give clearly defined fractions and easily interpretable results than those available for liver.

2) The observation that oligomycin did not inhibit DPI labelling in liver mitochondria (Garbus et al., 1963) suggested that the phosphorylating species in this reaction was derived from a high-energy intermediate (HEI) of oxidative phosphorylation, rather than from ATP, (see Figure 13).

3) Other processes utilising this HEI as their energy supply are accumulation of divalent cations (accompanied by P_i), and ATP-induced contraction of swollen mitochondria. The divalent cation accumulation had been studied with mitochondria from heart (Brierley et al., 1962, 1963), liver (Rossi and Lehninger, 1963; Chappell and Greville, 1963),

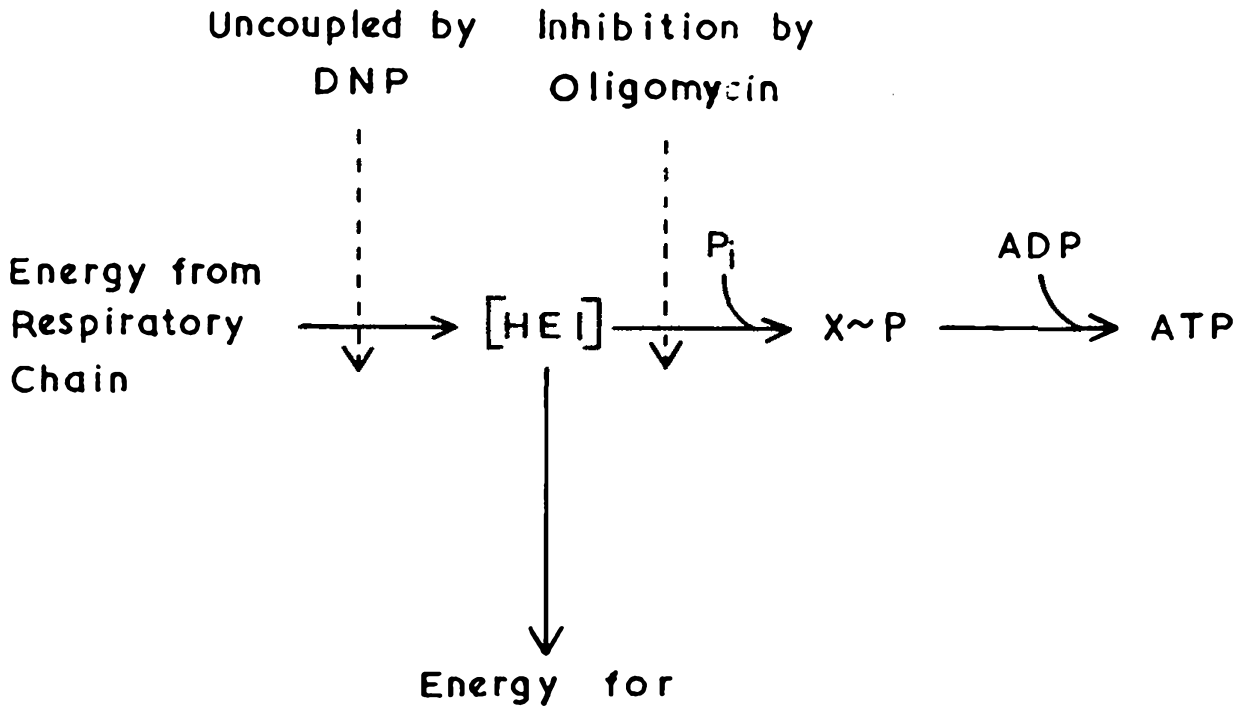


Fig 13. Energy supply pathway to processes which are

a. Oligomycin – sensitive when ATP is the energy source.

b. Oligomycin – insensitive when substrate oxidation is the energy source.

and kidney (DeLuca and Engstrom, 1961; Vasington and Murphy, 1962) but no data were available on any similar process in brain mitochondria. The mitochondrial contraction had been investigated almost exclusively in liver mitochondria (see Review by Lehninger, 1962) and it was in this system that the specific phosphatidylinositol requirement for the contraction of "aged" mitochondria was described (P.M. Vignais et al., 1963, 1964). Brain mitochondria do not show a high-amplitude swelling and contraction cycle similar to that which can be induced in liver or kidney mitochondria and are therefore not suitable for the study of this process.

The experiments described in this section were carried out (in collaboration with Drs. J.N.Hawthorne and T.Galliard) to ascertain whether the DPI labelling was in any way connected with these energy-linked functions of the mitochondrion. Unfortunately we had no oligomycin during part of this study.

Experiment 6.

Effects of the tonicity of the incubation medium on DPI labelling.

Garbus et al. (1963) used a hypotonic incubation medium for their incorporations. As mitochondria are greatly affected by osmotic changes, it was felt wise, if possible, to work at a tonicity comparable to that of the cell.

Incubations were in two different media:

a) That of Garbus et al., the final concentrations being 16.6 mM Tris/HCl buffer, pH 7.4; 12.5 mM potassium glutamate; 8.4 mM $MgCl_2$; 100 mM sucrose; 1 mM potassium phosphate buffer, pH 7.4 containing approximately 40 µC $^{32}P_i$; 3.0 mg. mitochondrial protein per ml. in a total volume of 2 ml.

b) An approximately isotonic medium containing 20 mM Tris/HCl buffer, pH 7.4; 10 mM potassium glutamate; 10 mM $MgCl_2$; 250 mM sucrose; 1 mM potassium phosphate buffer, pH 7.4, containing approximately 40 µC $^{32}P_i$ per incubation; 3.0 mg. mitochondrial protein per ml. in a total volume of 2 ml.

The incubations were prepared, with the omission of the P_i. After equilibration of these mixtures for 2 min. at 30^o on a shaking water bath, the P_i was added. Incorporations were stopped by the addition of 7.5 ml. 1:2 chloroform:methanol after another 5 min. Incorporations were measured by method a). They were:

a. Hypotonic - 3,800 ; 3,900 counts/100 sec.

b. Isotonic - 3,950 ; 4,330 counts/100 sec.

As there was very little difference between the two systems, the isotonic being, if anything, slightly the better, this isotonic system was used in the subsequent experiments. In most experiments the incubation volume was reduced to 1 ml., but the composition of the medium was unchanged.

Effects of the extraction procedure used on the activity of the DPI recovered.

The extraction procedure used here (that of Garbus et al., 1963) involves washing of the lipid extract with a KCl solution of high ionic strength. As the sodium (and probably the potassium) salts of DPI and TPI are more soluble in water than the calcium salts, it seemed necessary to check that an alternative procedure using $CaCl_2$ solution to wash the lipid extract would not yield higher recoveries of DPI.

Experiment 7.

All incubations were of 1 ml. total volume. The conditions used were the same as in Experiment 6 (isotonic medium). The protein concentration was 7.4 mg. per incubation. All incorporations except No. 5 were stopped after 5 min. by the addition of 3.75 ml. chloroform:methanol (1:2, v:v) giving a monophasic system. Extractions were continued as follows, using two incubation mixtures in each case.

1. The usual extraction procedure was followed; 1.25 ml. chloroform and 1.25 ml. of 2M KCl:0.5M phosphate buffer, pH 7.4 were added and the mixture shaken. After centrifugation the lower phase was taken and the top phase was washed with 2.6 ml. chloroform. The lower phases were combined and DPI labelling measured in them.

2. Procedure was as in 1., except that the KCl/phosphate

solution was replaced by 0.02M $CaCl_2$.

3. Procedure as in 1., except that the monophasic mixture was filtered through glass wool prior to the additions of the chloroform and wash solution. These were added through the glass wool containing the protein precipitate. The lower phase is called 3A in Table 3. The protein residue and the glass wool were extracted at 37° for 20 min. with 2.5 ml. acidified chloroform:methanol. This was chloroform:methanol: conc. HCl, 200:100:0.75 (v:v:v). After washing with 0.5 ml. 0.02M $CaCl_2$, this extract was taken as 3B.

4. Procedures for the preparation of 4A and 4B were similar to those for 3A and 3B, except that all washings were with 0.02M $CaCl_2$.

5. Incorporation was stopped after 5 min. by the addition of 3 ml. acetone. After centrifugation the precipitate was washed with 3 ml. acetone and the acetone extracts combined. These were dried <u>in vacuo</u> and the residue obtained was extracted with 2.5 ml. acidified chloroform:methanol (see above) and washed with 0.5 ml. 0.02M $CaCl_2$. This yielded 5A. The precipitate from the acetone extractions was extracted and washed similarly. This extract is designated 5B.

DPI labelling in all of these fractions was assayed by method b). The results are given in Table 3.

TABLE 3 Extraction of labelled DPI by different methods

Method	Counts/100 sec. Extract A	Extract B	Total	Total (mean)
1.			967 792	880
2.			73 83	78
3.	586 590	260 260	846 850	848
4.	610 650	115 320	725 970	848
5.	75 50	890 850	965 900	933

The total yields of labelled DPI in 1, 3, 4 and 5 were similar. However, separation of the phases in the presence of both $CaCl_2$ and the mitochondrial protein (Method 2) resulted in the loss of most of the labelled lipid. This suggested that in the presence of these components some sort of binding of DPI was removing the lipid into the aqueous phase. If the protein was filtered off prior to the addition of $CaCl_2$ to the system this effect was considerably reduced. Thus some form of calcium-dependent binding of DPI to protein was probably occurring.

Experiment 8.

Incubations were similar to those in the previous experiment. The protein concentration was 6.1 mg. per incubation. All incubations were stopped with chloroform: methanol, and the phase separation conditions were varied.

1. Conditions as for 1. in Experiment 7.

2. Conditions as for 2. in Experiment 7.

3. Conditions as for 3A in Experiment 7.

4. As for 2., except that the mixture was filtered after the addition of the reagents to separate the phases, but before centrifugation and removal of the lower phase.

5. For this and sample 6, the extracts of the incubations were isolated by the usual procedure (i.e. as for No. 1 above) and were then washed to study the partition of the lipid extracted by the Garbus procedure. 5. was washed with 2.5 ml. methanol plus 2.25 ml. water.

6. As for 5., but washed with 2.5 ml. methanol plus 2.25 ml. 0.02M $CaCl_2$.

The results (Table 4) lent further support to the idea of a calcium-dependent DPI binding to protein. They also showed that the extracted DPI was in a form in which it was very readily partitioned into methanol:water. As the washing procedure used in its extraction utilised a high ionic strength

mixture of potassium chloride and phosphate this form is presumably the potassium salt. Surprisingly, the presence of 0.02M $CaCl_2$ in the solution used to wash the lipid extract only partially prevented the loss of the DPI into the aqueous phase.

TABLE 4 Effects of $CaCl_2$ on the extraction and partition of labelled DPI

Method	Counts/100 sec.	
	Duplicates	Mean
1.	676 : 677	676
2.	190 : 216	203
3.	242 : 498	370
4.	228 : 204	216
5.	7 : 9	8
6.	259 : 267	263

As the extraction procedure used by Garbus et al. consistently gave a DPI yield as high as that obtained by any of the other methods tested it was retained unchanged for the rest of the work.

Effects of unlabelled adenine nucleotides.

ATP, ADP and AMP were added to mitochondria incubated as in Experiment 6 (isotonic medium). In each experiment the incorporations are expressed as percentages of the incorporations

found in the experiment in the absence of added nucleotides.

Experiments 9 - 12.

9. Incubations were of 2 ml. Assay of radioactivity by method a).

10. Conditions were as in Experiment 9.

11. Incubations were of 1 ml. Assay was by method a).

12. Incubations were of 1 ml. Assay was by method b).

The results are given in Table 5.

TABLE 5 Effects of unlabelled adenine nucleotides.

Nucleotide Conc^{n}	DPI labelling as % of control		
	0	1mM	5mM
Experiment 9.			
Control	100		
AMP			38
ATP			18
Experiment 10.			
Control	100		
AMP			34.5
ADP			17.5
ATP			16
Experiment 11.			
Control	100		
ADP		202	
ATP		44	
Experiment 12.			
Control	100		
ADP		35	19
ATP		7	0
CTP		52	9
GTP		30	
UTP		32	13

These show inhibition of labelling by all adenine nucleotides except in one case, ADP. ATP was always the most effective inhibitor. Figures for CTP, GTP and UTP are also given and show that these compounds also inhibited strongly, but none was nearly as effective as ATP.

Measurement of the DPI content of mitochondria.

Galliard and Hawthorne (1963) reported that the level of DPI in rabbit liver was very low. Mitochondria were prepared in large quantities, incubated with $^{32}P_i$ and their DPI content assayed.

Experiment 13.

Three rabbit livers (260 g. tissue) were homogenised in 0.3M sucrose/0.002M EDTA, pH 7.4, the homogenate was diluted to approximately 2 litres, and mitochondria were isolated, using the 6 x 250 ml. rotor of the M.S.E. "18" centrifuge, the speeds used being,

1) 3,000 revs./min. for 7 min. to sediment nuclei.
2) 6,000 revs./min. for 10 min. to sediment mitochondria, which were washed and resedimented at the same speed.

These mitochondria, suspended in 50 ml. 0.3M sucrose, were added to a medium containing 20 ml. 0.2M Tris/HCl buffer, pH 7.4; 20 ml. 0.1M $MgCl_2$; 10 ml. 0.2M sodium succinate; 35 ml. 1M sucrose and 55 ml. water. The mixture was warmed to 30°. This took about 5 min. Ten ml. 20 mM sodium phosphate buffer,

pH 7.4, containing approximately 1 mC $^{32}P_i$ were then added. During the incubation the medium was aerated by blowing oxygen through it. After 5 min. at 30° the incorporation was stopped by the addition of 2 volumes of acetone at -20°.

After the mixture had been kept at -20° for 60 min., the precipitate was centrifuged off at -30°. The sediment was extracted with 2 x 200 ml. chloroform:methanol (2:1, v:v) and the extracts filtered through glass wool. The combined filtered extracts were washed with 0.2 volume of 0.002M $CaCl_2$.

The lower phase was dried in vacuo and the lipids hydrolysed (Hübscher et al., 1959) and chromatographed on Dowex 1 x 10 in the formate form (see Methods section: Assay of DPI labelling, method a)). The fractions comprising the radioactive DPI peak were pooled, ammonium ions removed with Dowex 50W, 50-100 mesh, H^+ form, and the resulting solution dried in vacuo. The residue was hydrolysed for 16 hr. in 6N HCl at 110° in a sealed tube. Inositol was assayed in the hydrolysate by the microbiological assay of Norris and Darbre (1956), using Schizosaccharomyces pombe as the test organism.

The quantity of inositol in the GPIP was approximately 17 µg., derived from mitochondria containing 2.1 g. protein, i.e. DPI content of mitochondria was approximately 45 mµ moles per g. protein. 4.05 mg. lipid P were extracted per gram of protein. This recovery, which is lower than would be expected

for the total mitochondrial phospholipid, is discussed after the next experiment.

Experiment 14.

Mitochondria were prepared from 350 g. ox liver as described in Experiment 13 for rabbit liver.

The mitochondria were incubated with $^{32}P_i$ as in Experiment 13, except that the medium contained 10 ml. 0.2M sodium glutamate in place of the succinate.

The lipids were extracted, washed, hydrolysed and the products separated and analysed as in Experiment 13, except that 0.005M $CaCl_2$ was used for washing the lipid extract.

16.5 mg. lipid P were extracted from mitochondria containing 4.04 g. protein. The GPIP contained approximately 25 µg. inositol, i.e. DPI content of mitochondria was approximately 34 mµ moles per g. protein.

The quantity of lipid phosphorus recovered in the acetone insoluble fraction of the mitochondrial lipids, was much lower than that expected for the total phospholipid content of the mitochondria. This low figure probably reflected considerable extraction of phospholipid into the acetone. However, as negligible quantities of radioactive lipid were present in the acetone extracts, it was assumed that DPI recovery was quantitative.

Experiment 15.

Confirmation that MPI will restore the ATP-induced contraction of aged mitochondria.

The mitochondria used in this experiment were aged at 0^{o} for 3 hours in 0.6M KCl.

They were allowed to swell in the presence of 5 mM potassium phosphate, pH 7.4 (see Methods section). When there was little further change in the optical density at 520 mμ, ATP (to give 5 mM) and bovine serum albumin (to give 2 mg./ml.) were added, followed after 5 min. by $MgCl_2$ (to give 3 mM). The changes in optical density were recorded. A typical response of aged mitochondria is shown in Figure 14 a.,(in this figure the optical density changes at 520 mμ are plotted from a common point when ATP was added to the system).

In those samples where lipid was added, the PI was added to the swelling medium prior to the addition of the mitochondria and both swelling and shrinkage occurred in the presence of exogenous PI.

In Figure 14b. the results of varying the quantity of PI added are shown. Half-maximal restoration of contraction occurred at a concentration of approximately 3×10^{-6}M (indicated by arrow on Fig. 14b.). The PI used in this experiment was a sample which contained approximately 30% phosphatidylserine and the concentrations plotted in the figure are corrected for this

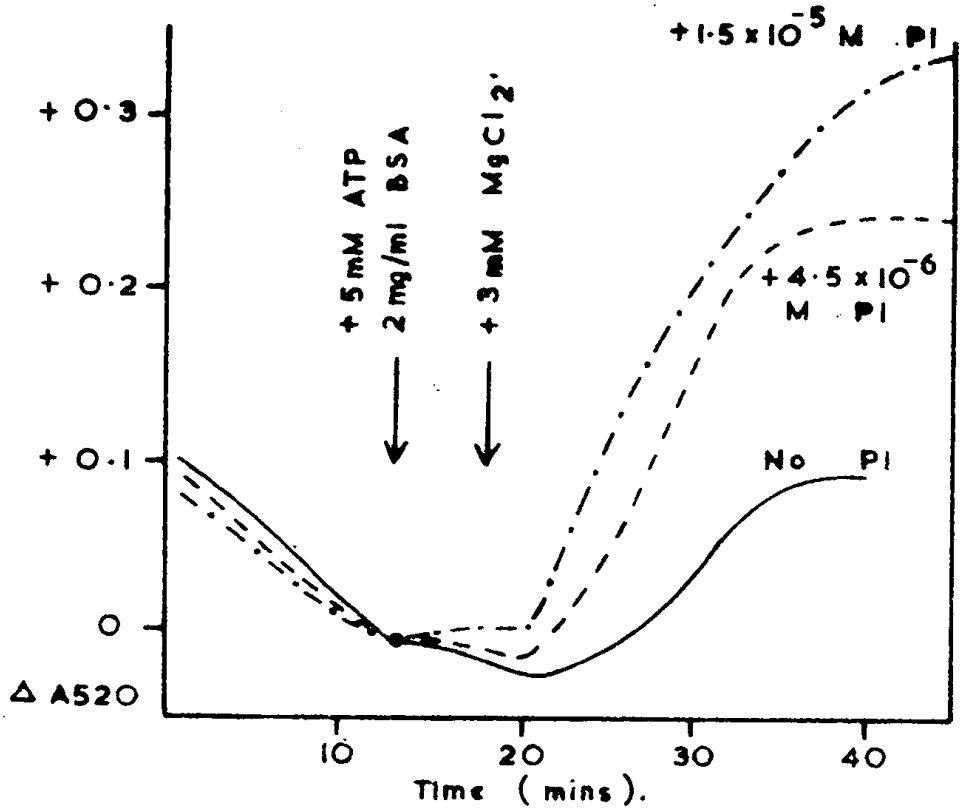


FIG. 14 a. RESTORATION OF ATP-INDUCED CONTRACTION IN AGED MITOCHONDRIA BY PI.

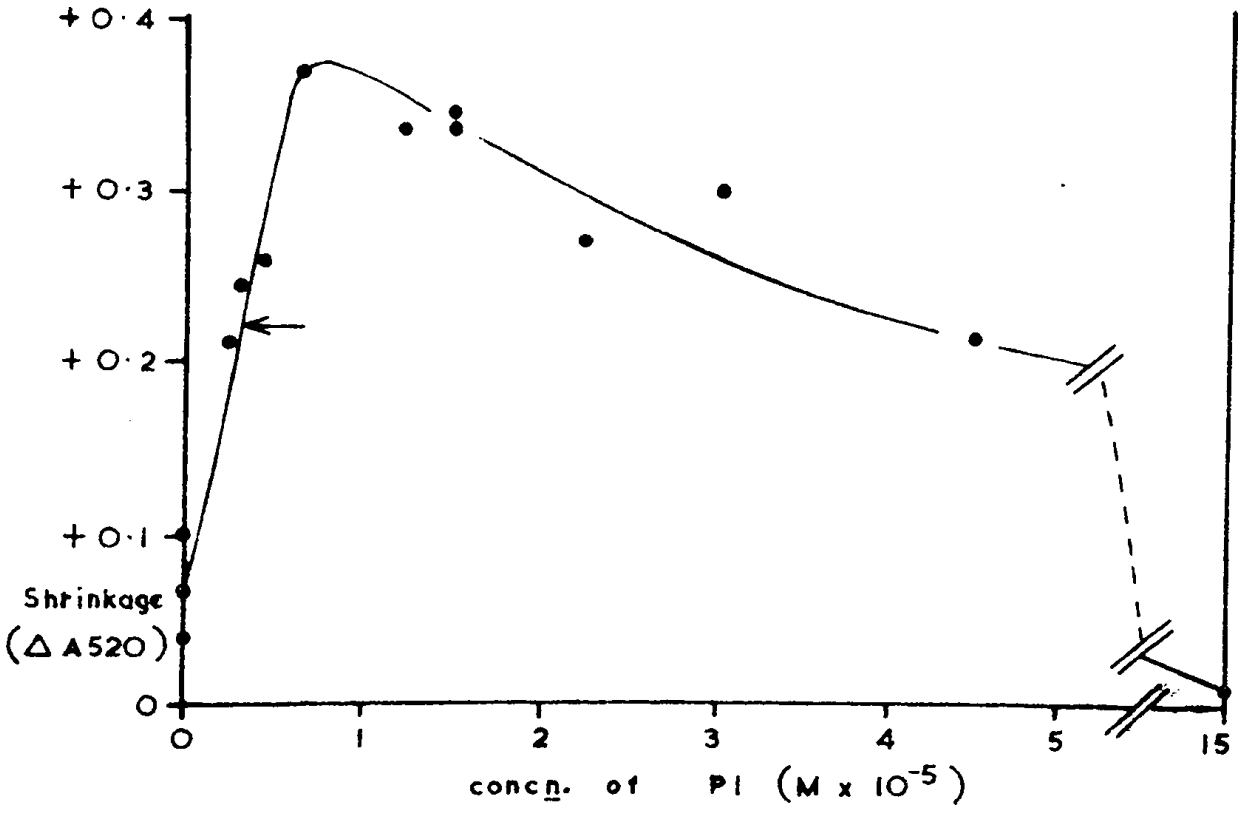


FIG. 14 b. EFFECT OF PI CONCENTRATION ON AMPLITUDE OF ATP-INDUCED CONTRACTION IN AGED MITOCHONDRIA.

contamination.

In subsequent experiments using this sample of PI to restore shrinkage, concentrations of between 8×10^{-6} and 2×10^{-5}M were used (usually 1×10^{-5}M).

Experiment 16.

Effect of ATP concentration on mitochondrial shrinkage.

As experiments were planned using ^{32}P-labelled ATP as a phosphate donor for the study of lipid labelling during contraction, it was hoped that a final ATP concentration of less than 5 mM might be adequate for optimal shrinkage. Mitochondria had been aged for 1 day in 0.3M sucrose. All incubations contained 1×10^{-5}M PI. The results are shown in Figure 15. They show that although contraction was maximal at 5 mM, a plateau existed from 1.25 mM - 5.0 mM. For future incubations a final ATP concentration of 2.5 mM was used. This caused only a 10% decrease in the amplitude of shrinkage, and allowed a doubling of the specific activity of ^{32}P-ATP in incorporation experiments.

DPI labelling from $^{32}P_i$ and from γ-^{32}P-ATP during mitochondrial shrinkage.

Experiment 17.

Mitochondria were isolated from a liver homogenate in 0.3M sucrose. The washed mitochondrial pellet was suspended in 0.6M KCl for 3 hr. (Vignais et al., 1964 b) and mitochondria

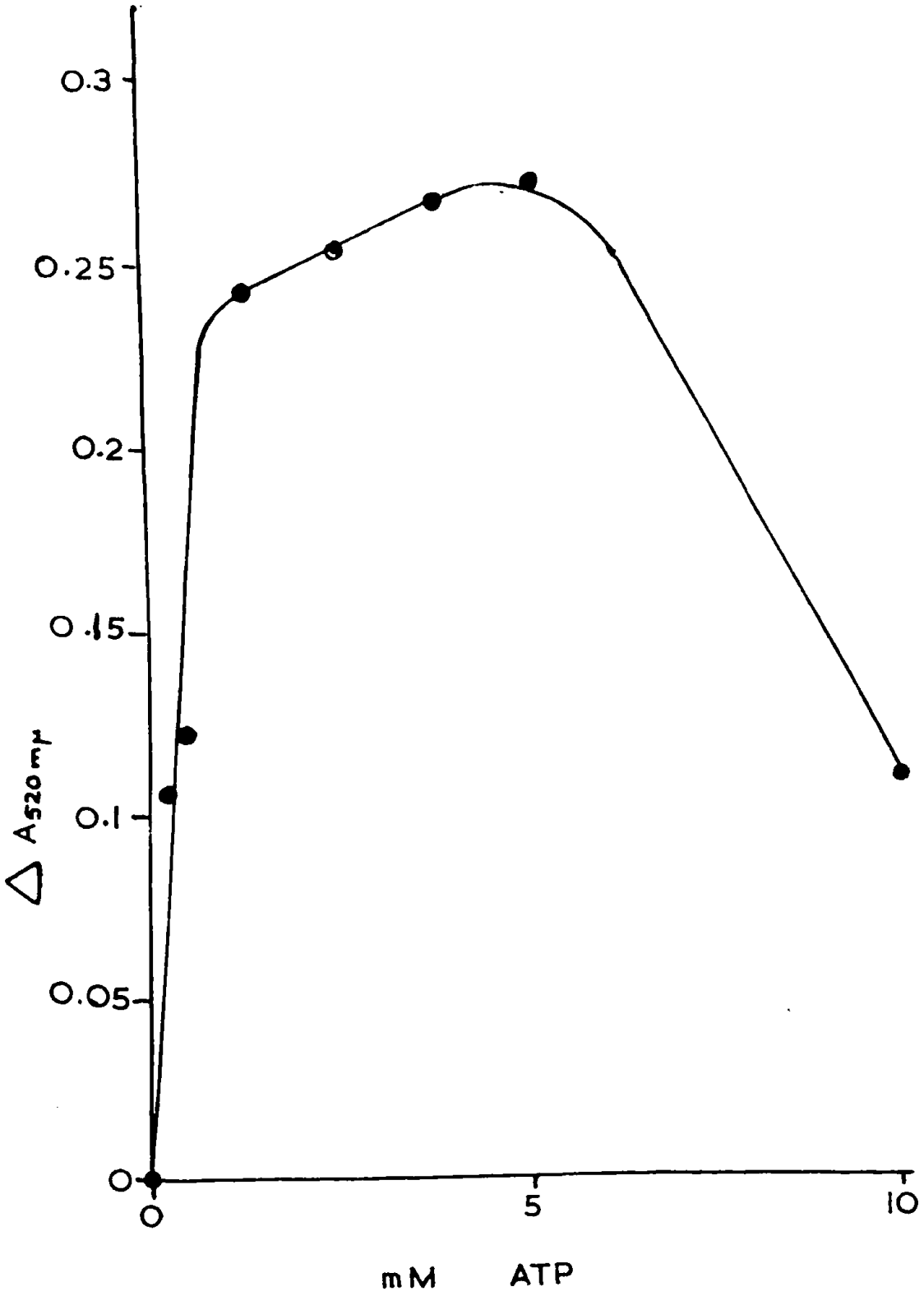


Fig.15. Dependence of mitochondrial shrinkage on ATP.

were sedimented from the KCl suspension at 10,000 x g for 10 min.

Mitochondria were allowed to swell in buffered KCl (see Methods section) with 5 mM potassium phosphate, pH 7.4 as the swelling agent, in the presence of 2 x 10^{-5}M PI. Incubation media were 2 ml. in volume, containing 8.3 mg. protein and were kept at 30°.

Swelling was initiated in one tube before the others and swelling and shrinkage in this tube were followed. 0.1 ml. samples were transferred at intervals into 3 ml. of 20 mM Tris/HCl, pH 7.4: 0.125M KCl, kept at 0°. The optical densities of these were measured at 520 mμ, immediately after the transfer. The behaviour of the mitochondria in this tube guided the assay of the others.

When the swollen mitochondria had reached a steady optical density the following additions were made to five pairs of tubes.

a. 40 μC $^{32}P_i$ (carrier-free) and 4 mg. bovine serum albumin, followed after 5 min. by 6 μmoles $MgCl_2$.

b. 40 μC $^{32}P_i$ (carrier-free), 5 μmoles ATP and 4 mg. bovine serum albumin, followed after 5 min. by 6 μmoles $MgCl_2$.

c. 40 μC $^{32}P_i$ (carrier-free), 5 μmoles ATP, 4 mg. bovine serum albumin and 10 μg. oligomycin, followed after 5 min. by 6 μmoles $MgCl_2$.

d. 5 μC γ-^{32}P-ATP in 2.5 μmoles ATP and 4 mg. bovine serum albumin, followed after 5 min. by 6 μmoles $MgCl_2$.

e. 5 μC γ-^{32}P-ATP in 2.5 μmoles ATP, 4 mg. bovine serum albumin and 10 μg. oligomycin, followed after 5 min. by 6 μmoles $MgCl_2$.

In all cases the total volume of these additions was less than 0.2 ml.

The samples taken from the control tube indicated that contraction occurred, with a rise in optical density at 520 mμ from 0.166 before the addition of $MgCl_2$ to 0.252 17 min. later. At this time the shrinkage was still continuing. The incorporations were stopped with chloroform:methanol 17 min. after the addition of $MgCl_2$. The lipids were extracted and assay of lipid labelling was by method a). The columns were eluted, after the removal of the P_i, with 0.2M ammonium formate: 0.1M formic acid, followed by 0.5M ammonium formate:0.1M formic acid. In the incubations with γ-^{32}P-ATP (d. and e.) no radioactivity was detected in either eluate. The activities of the products labelled from $^{32}P_i$ were:

		Counts/ 100 sec.			Counts/ 100 sec.
a.	0.2 AF : 0.1 FA	Zero	c.	0.2 AF : 0.1 FA	30
	0.5 AF : 0.1 FA	12		0.5 AF : 0.1 FA	110
b.	0.2 AF : 0.1 FA	60			
		80			
	0.5 AF : 0.1 FA	440			
		640			

None of these radioactive materials was adsorbed on to charcoal in 0.1N HCl, suggesting that they were not nucleotides contaminating the lipid extracts.

These results suggested that DPI (to a very small degree) and TPI (to a greater degree) were labelled from $^{32}P_i$ during ATP-induced mitochondrial shrinkage. They further suggested that this labelling was ATP-dependent and oligomycin-sensitive.

Experiments 18 - 20.

Two attempts to repeat these incorporations were unsuccessful, as was an attempt to incorporate ^{32}P from labelled ATP during shrinkage, this time using 20 μC ATP. This left the possibility of the involvement of DPI or TPI in mitochondrial shrinkage an open question.

Effects of oligomycin on P_i-dependent DPI labelling.

As any theories of DPI activity in mitochondria as a factor in ion-translocation were dependent on the observation that its labelling was not inhibited by oligomycin (Garbus et al., 1963) it was imperative to check this observation.

Experiment 21.

Incubation conditions were as in Experiment 6 (isotonic medium). Oligomycin was added in solution in ethanol (maximum volume added was 25 μl.), which had no effect on the

incorporations at the concentrations used. Assay was by method a).

Figure 16 gives the results of this experiment. Oligomycin was shown to be a very effective inhibitor of DPI labelling, giving maximal inhibition at 2.5 µg. per ml. and half-maximal inhibition at 0.7 µg. per ml. In four experiments, all assayed at 5 µg. per ml. of oligomycin, the inhibitions were 96.5%, 75%, 82.5% and 71%. The incorporations were all assayed by ion-exchange chromatography (method a)).

The conditions of these assays differed from those of Garbus et al. in two respects. The method of assay used by those workers was a measurement of the radioactivity of the total lipid extract. Their hypotonic incubation conditions differed from our isotonic ones. However, assay of incorporation into DPI under the conditions used by Garbus et al. showed a 77% inhibition of both total lipid labelling and DPI labelling by 12.5 µg. per ml. oligomycin, the concentration which they used.

Experiment 22.

Effects of oligomycin on the labelling of DPI and of water-soluble acid-labile phosphate esters.

Incubations were under the same conditions as in Experiment 6 (isotonic medium), with the addition of 5 µg. per ml. of oligomycin. Mitochondria were also incubated in the hypotonic medium of Garbus et al., with the addition of 5 µg.

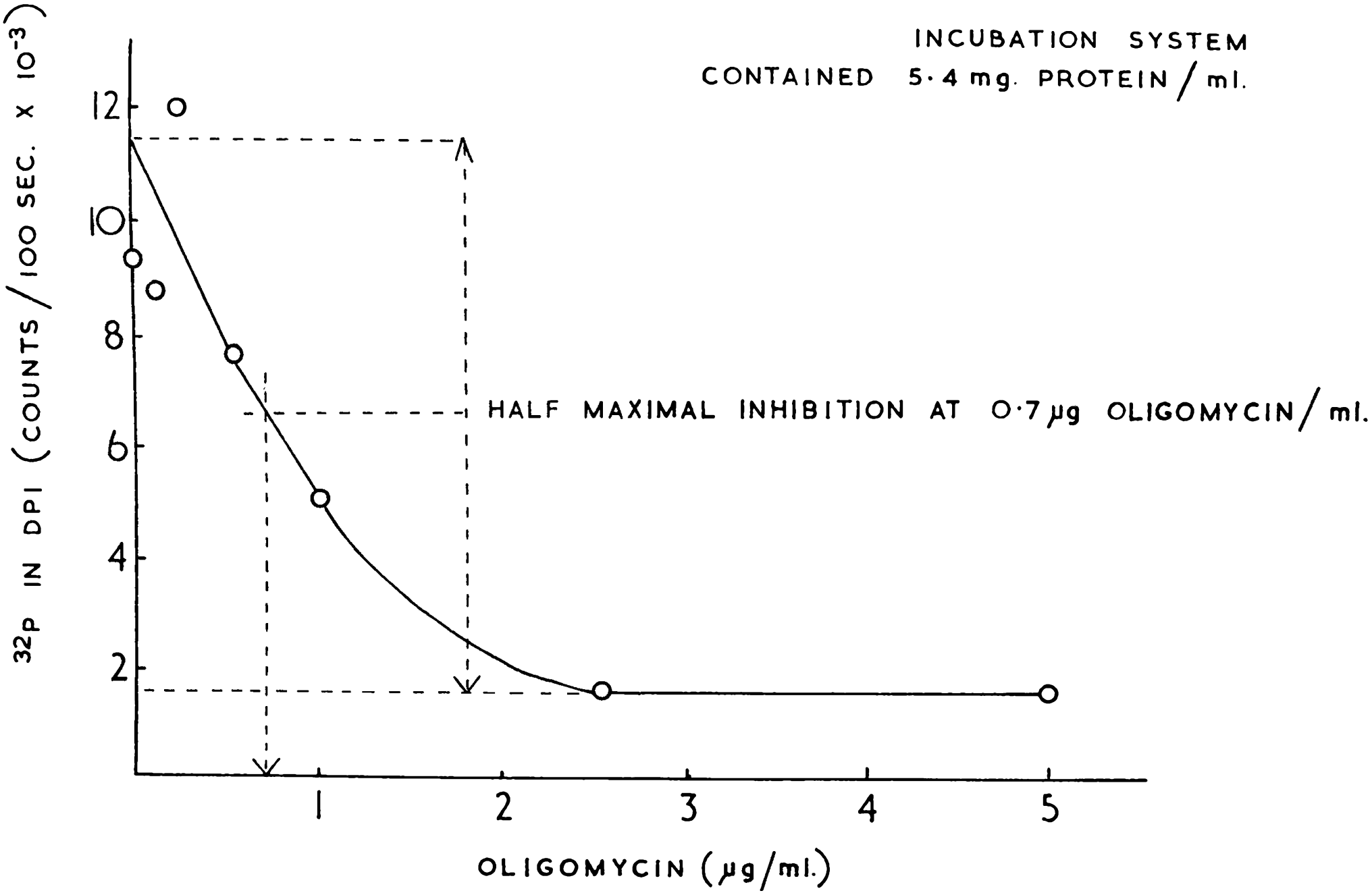


FIG. 16 INHIBITION OF $^{32}P_i$-DEPENDENT DPI LABELLING BY OLIGOMYCIN.

per ml. of oligomycin.

After the reaction had been stopped and the phases separated the lower phase was assayed for DPI labelling by method a), and the upper phase for labelling of acid-labile phosphate esters.

One ml. of the top phase was taken. This was extracted with iso-butanol:benzene in the presence of ammonium molybdate and sulphuric acid (Martin and Doty, 1949) several times, until no further radioactivity could be extracted from the aqueous phase. This indicated that all the $^{32}P_i$ had been removed from the sample. 0.5 ml. of this extracted lower phase was then added to 7 ml. of N HCl and heated at 100^{o} for 7 min. The radioactivity released as P_i by this procedure was assayed by the method of Martin and Doty. Under the conditions used for incubation this acid-labile phosphate represents mainly ATP (Garbus et al.).

The results (Table 6) show that the inhibition of DPI labelling and of acid-labile phosphate labelling by oligomycin was the same, both in isotonic and hypotonic conditions. This was further evidence for a relationship between the labelling of DPI and of ATP.

TABLE 6 Effect of oligomycin on labelling of DPI and of acid-labile phosphates.

Incubation system	% inhibition of labelling by 5 µg./ml. oligomycin	
	GPIP	Acid-labile P
Hypotonic (Garbus et al.)	75	70
Isotonic	71	69

These results, showing a clear inhibition of DPI labelling under conditions which would have permitted optimal transport of divalent cations into the mitochondria, preclude DPI from a carrier role in the oligomycin-insensitive, substrate-dependent cation accumulation.

Subcellular localisation of P_i-dependent DPI labelling.

Experiment 23.

A mitochondrial fraction was prepared and washed four times, rather than the usual two or three. At each stage a sample was removed for enzyme assay.

Assays for DPI labelling (incubation conditions as in Experiment 6 (isotonic medium) and analysis of products by method b)), succinate dehydrogenase (a mitochondrial enzyme) and glucose-6-phosphatase (a microsomal enzyme) were made on each sample of mitochondria. Figure 17 shows the effects of

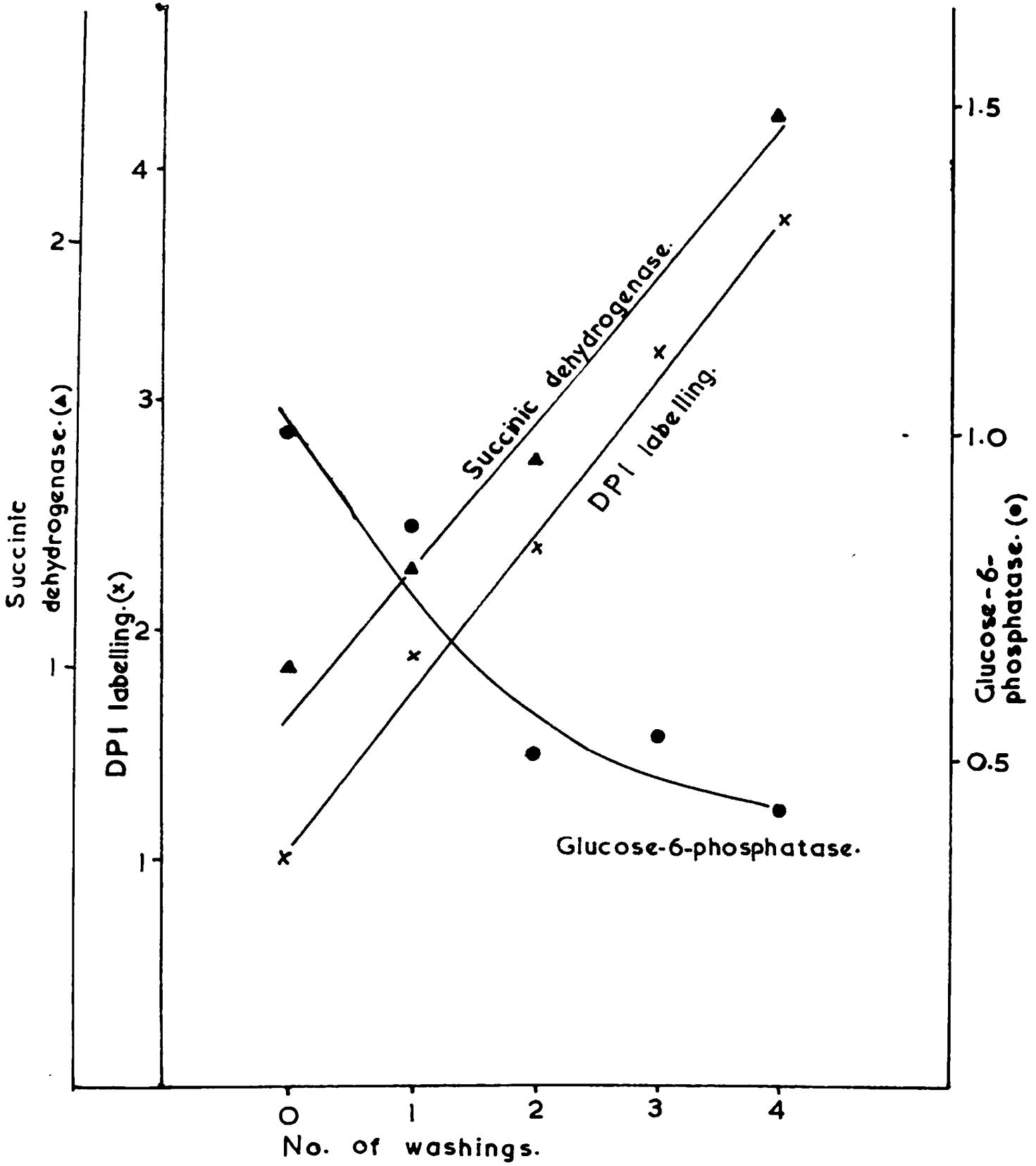


Fig 17. Effect of washing on the enzyme activities of the liver mitochondrial fraction.

Figure 18.

Specific activities of marker enzymes and of DPI labelling from $^{32}P_i$ in rat liver subcellular fractions.

The fractions were designated:

Mt: Mitochondrial

L: Lysosomal

Mc: Microsomal

PFS: Particle-free supernatant

All marker enzymes are plotted as μmoles substrate converted per mg. protein per hour.

DPI labelling is plotted as mμmoles $^{32}P_i$ incorporated into DPI per gram protein per 5 min.

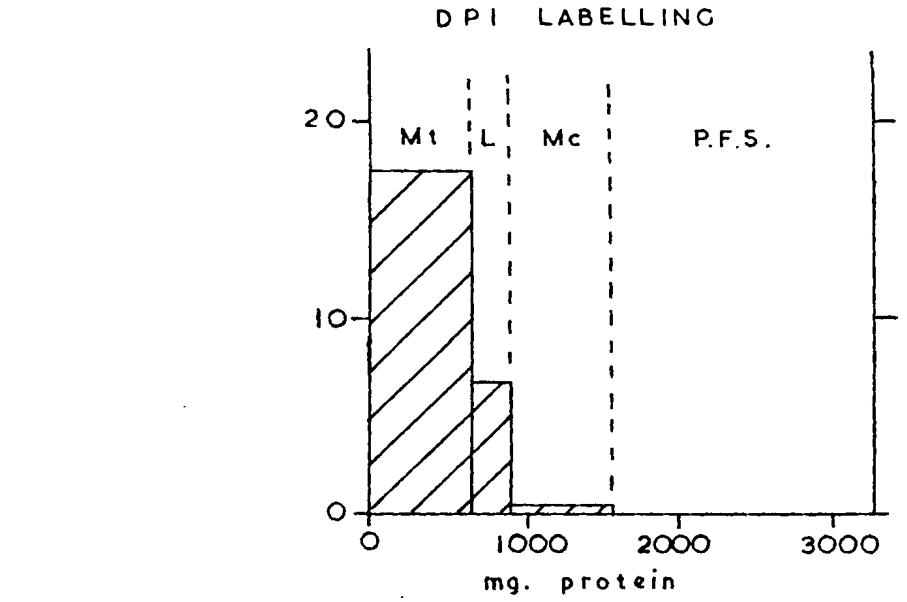
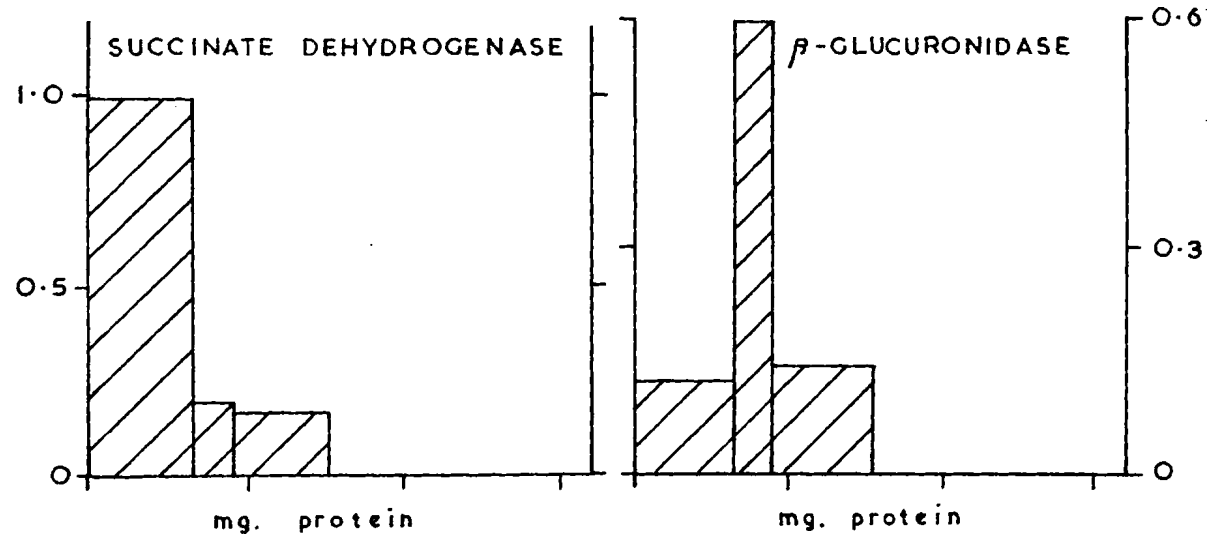
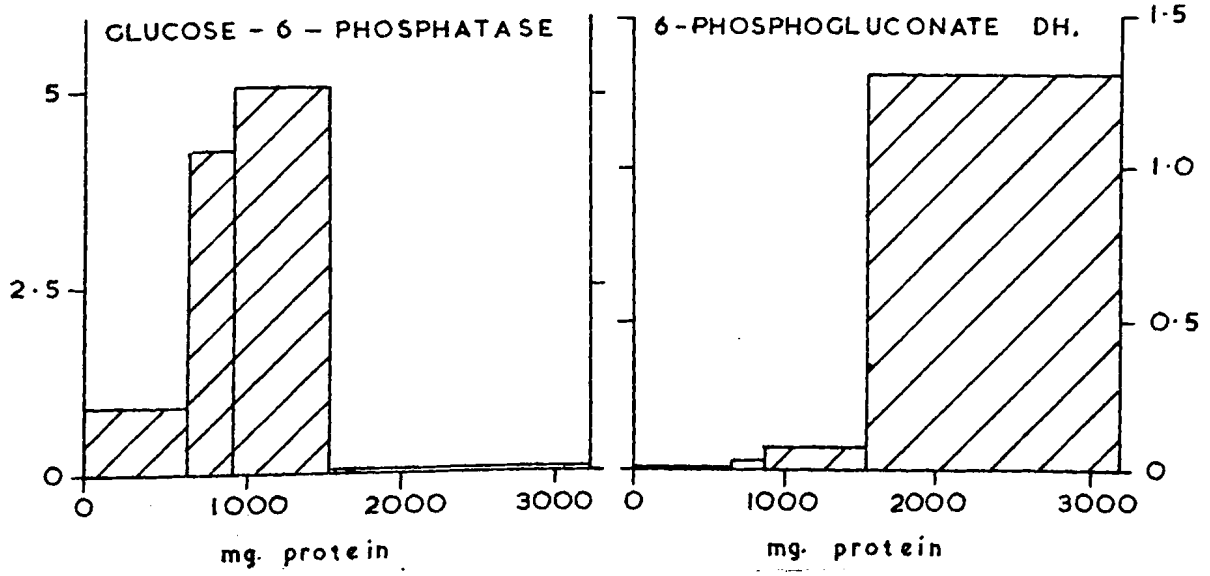

DPI LABELLING
20
10
0
Mt
L
Mc
P.F.S.
0
1000
2000
3000
mg. protein

FIGURE 18.


SUCCINATE DEHYDROGENASE
1·0
0·5
0
mg. protein
β-GLUCURONIDASE
0·6
0·3
0
mg. protein


GLUCOSE - 6 - PHOSPHATASE
5
2·5
0
0
1000
2000
3000
mg. protein
6-PHOSPHOGLUCONATE DH.
1·5
1·0
0·5
0
0
1000
2000
3000
mg. protein

The specific activity of DPI labelling in the nuclei-free homogenate was 4.3 mμmoles of P_i incorporated per gram of protein in 5 min.

Experiments 24 a, b, c.

Three attempts were made to show a dependence on added PI for the formation of DPI, using the same conditions as those used in Experiment 6 (isotonic medium). The volumes of the incubations were 1 ml. each and PI (prepared from brain and approximately 70% pure) was added as an emulsion in water at concentrations of from 0.1 to 1.0 mM. Assay of the radioactivity was by method b). In no case was any stimulation observed. Inhibitions of up to 80% (at 0.5 mM PI) were seen in two experiments.

TABLE 7 Subcellular localisation of DPI labelling from P_i.

Fraction	Protein	Succinate dehydrogenase.	β- glucuronidase	Glucose-6-phosphatase	6-phosphogluconate dehydrogenase	DPI labelling
Nuclear	-	(28%)	-	(37.0%)	-	(3.4%)
Nuclei-free homogenate	(100)	(100)	(100)	(100)	(100)	(100)
Mitochondrial	16.5	63.5	18.3	10.1	0.2	70.8
Lysosomal	5.7	4.5	31.0	16.5	0.4	8.7
Microsomal	16.9	1.1	28.2	58.6	2.6	0.9
Supernatant	42.6	0.0	0.0	0.2	98.3	0.4
% Recoveries	81.7	69.1	77.5	85.4	101.5	80.8

Footnote: Activities are expressed as the percentage of the activity of the nuclei-free homogenate recovered in each fraction. The recoveries are the sums of these figures for the four fractions derived from the nuclei-free homogenate.

RESULTS

Part III

SYNTHESIS OF DPI FROM ^{32}P-ATP IN LIVER.

Experiment 25.

Labelling of mitochondrial DPI from ^{32}P-ATP.

Although several unsuccessful attempts had been made previously to show labelling of DPI using labelled ATP as the phosphate donor, circumstantial evidence at this stage still pointed very strongly to its being involved. In the experiments described below the concentration and specific activity of the ATP used was higher than those used in the previous experiments.

The basic incubation medium for the first experiment contained 80 mM Tris/HCl, pH 7.4; 1 mM ATP containing 2.51×10^6 counts per 100 sec. per µmole; 60 mM sucrose; 10 mM $MgCl_2$; mitochondria (10.2 mg. protein). Incubations were for 5 min. at 30° in a total volume of 1 ml. The incorporations were started by the addition of the mitochondria, suspended in 0.3M sucrose. They were stopped as usual with 1:2 (v:v) chloroform:methanol.

The extracted lipids were chromatographed on paper (method b)) and the chromatograms radioautographed. A sample of the Folch insoitide fraction was chromatographed on each sheet of paper with the labelled samples. Each lipid sample showed a clear radioactive area which corresponded to the DPI of the Folch fraction.

Table 8 lists the variations of incubation conditions from those given above, together with the incorporations. As the duplicates were rather poor, figures for individual incubations are given, with the means.

TABLE 8 Labelling of mitochondrial DPI from γ-^{32}P-ATP

Additions	Omissions	mμmoles P incorp./ mg. protein	mμmoles P (mean)	% of control
-	-	.00126 .00231	.00178	100)
200 mM sucrose	-	.00105 .00205	.00155	89
100 mM KCl	-	.00188 .00198	.00193	108
1 mM PI	-	.00417 .00795	.00606	339
5 mM P_i	-	.00251 .00239	.00245	138
5 mM ADP	-	.00065 .00046	.00055	31
10 mM KF	-	.00137 .00152	.00145	81
-	No $MgCl_2$	.0022 .0034	.0028	157

This was the first direct evidence that PI could be phosphorylated by ATP to DPI in liver mitochondria, the process being inhibited by ADP (a probable product) and stimulated by phosphatidylinositol,

the substrate for the reaction. The stimulation by P_i might be due to inhibition of ATPases. Mg^{++} ions did not seem to be necessary for the reaction.

Experiment 26.

General characteristics of ^{32}P-ATP-dependent labelling of mitochondrial DPI.

Following the results of the previous experiment a set of incubations was made, under a variety of conditions, in the absence of any added divalent cations. Under none of the conditions (varying concentrations of PI and of ATP, and varying pH) was any labelling of DPI observed. Assay was by the resin column technique (method a)).

Experiment 27.

In this experiment, $MgCl_2$ was again included as a standard component of the incubation system and was shown to be necessary for activity. The reason for its apparent ineffectiveness in the previous experiment is unknown.

Table 9 shows the incorporations, using the following standard incubation conditions. The variations from these are given in the table.

100 mM KCl; 50 mM Tris/HCl, pH 7.4; 1.32 mM ATP containing 2.67×10^6 counts per 100 sec. per μmole; 60 mM sucrose; 10 mM $MgCl_2$; mitochondria (10.6 mg. protein). Incubations were at 30° for 5 min. The final volume was 1 ml.

TABLE 9 Conditions for DPI labelling from γ-^{32}P-ATP

Additions	Omissions	mμmoles P incorp./ mg. protein	mμmoles P (mean)	% of control
-	-	.048 .044	.046	(100)
0.6 mM PI	-	.11 .138	.124	270
10 mM P_i	-	.0575 .0565	.057	124
-	No $MgCl_2$	.0082	(.0082)	18
1 mM CTP	-	.033	(.033)	72
1 mM GTP	-	.037 .032	.0345	75
1 mM UTP	-	.028 .026	.027	59
Mitochondria frozen and thawed.	-	.041 .036	.0385	83
0.25% deoxycholate	-	.074 .108	.091	198

Assay was by paper chromatography and autoradiography (method a)). Where specified the mitochondria were frozen and thawed in suspension in sucrose. The suspension was immersed in ether and the ether rapidly evaporated by blowing over it with a hair dryer. The incubations were performed as soon as the suspension, which was frozen for about 5 min., had been thawed. Deoxycholate was used as a neutral solution of the

sodium salt.

This experiment confirmed that ^{32}P-ATP could be used as a phosphate source for the mitochondrial synthesis of DPI. It also confirmed that the reaction was stimulated by added PI. Inorganic orthophosphate also stimulated the reaction again, as did deoxycholate. Contrary to Experiment 25, Mg^{++} ions were found to be almost essential. The inhibitions by nucleotide triphosphates other than ATP, though considerable, were insufficient to suggest that any of the three tried was the true phosphate donor, rather than ATP. However, they may be alternative phosphate donors. Clarification of this point must wait for experiments using labelled PI. Finally, it was found that even brief freezing and thawing of the mitochondrial suspension diminished its capacity to catalyse the phosphorylation of PI.

Experiment 28.

Effect of freezing and thawing on the capacity of mitochondria to catalyse the phosphorylation of PI.

Six days after the experiment described immediately above fresh mitochondria were prepared and their activity assayed together with an assay of the mitochondria from the above experiment, which had been stored at -20°. The experimental conditions were identical with those above, except that in the incubations using the fresh mitochondria the protein concentration was 8.5 mg. in the 1 ml. incubation. The results,

including some taken from table 9, are given in table 10.

Preparation A was that used for the previous experiment, Preparation B the fresh one. The figures given are the means of duplicates.

TABLE 10. Effects of freezing and thawing on ATP-dependent DPI labelling.

Treatment of mitochondria	mμ moles P incorporated per mg. protein per 5 min.	
	A	B
Fresh	.046 (100)	.141 (307)*
Fresh + 0.6 mM PI	.124 (270)	.164 (356)
Frozen/Thawed	.0385 (83)	
Frozen 6 days	.013 (28)	
Frozen 6 days + 0.6 mM PI	.022 (48)	

* Figures in parentheses relate the activities to that of fresh preparation A, taken as 100.

The capacity of the mitochondria to phosphorylate PI continued to fall off during storage at -20°. However, they were still stimulated by the addition of PI to approximately the same extent as in the fresh preparation. This suggests that the inactivation of the enzyme is a general property, and

not a feature of a PI-dependent or -independent fraction.

The difference in properties of fresh preparations A and B in the absence of added PI may have reflected a difference in concentration of available endogenous PI in the two preparations. This is supported by the fact that their activities became very similar in the presence of 0.6 mM exogenous PI.

It now seemed appropriate to check that the compound being studied was identical with brain DPI and the labelled material first identified in liver mitochondria by Galliard and Hawthorne as DPI.

Experiment 29.

Identification of the labelled phospholipid produced during incubation of mitochondria with γ-^{32}P-ATP and PI.

The incubation medium contained: approximately 0.6 mM brain PI; 100 mM KCl; 50 mM Tris/HCl buffer, pH 7.4; 1.4 mM γ-^{32}P-ATP, containing 29,800 counts/sec./µmole; 10 mM sodium phosphate buffer, pH 7.4; 10 mM $MgCl_2$; 60 mM sucrose. The total volume was 10 ml. and incubation was at 30° for 5 min. after the addition of 44 mg. mitochondrial protein.

The reaction was stopped and the lipids extracted by the usual procedures, except that the procedure was scaled

up ten times. Ninety per cent of the lipid extract was then combined with a sample of the Folch inositide fraction containing approximately 5 mg. of organic phosphate. The mixture was hydrolysed by the method of Hübscher et al. (1960). The aqueous phase, which contained 99% of the radioactivity of the hydrolysed lipid, was made 5 mM with sodium tetraborate and was chromatographed on a 1 x 10 cm. column of Dowex 1 x 10, 200-400 mesh, in the formate form (see Methods section).

The elution pattern is given in Figure 19 and shows that one of the peaks, that eluted with 0.2M ammonium formate: 0.1M formic acid, co-chromatographed precisely with GPIP derived from the brain inositide. The labelled material eluted in 0.5M ammonium formate:0.1M formic acid (peak IV) did not, however, correspond to $GPIP_2$ derived from the brain triphosphoinositide.

Of the two peaks which emerged in the first eluate (0.18M ammonium formate:0.005M sodium tetraborate) the first was inorganic orthophosphate. The second appears in the position expected for glycerophosphate and is probably derived from a trace of labelled phosphatidic acid. The quantities of radioactivity observed represented the synthesis of the quantities of each component given in Table 11.

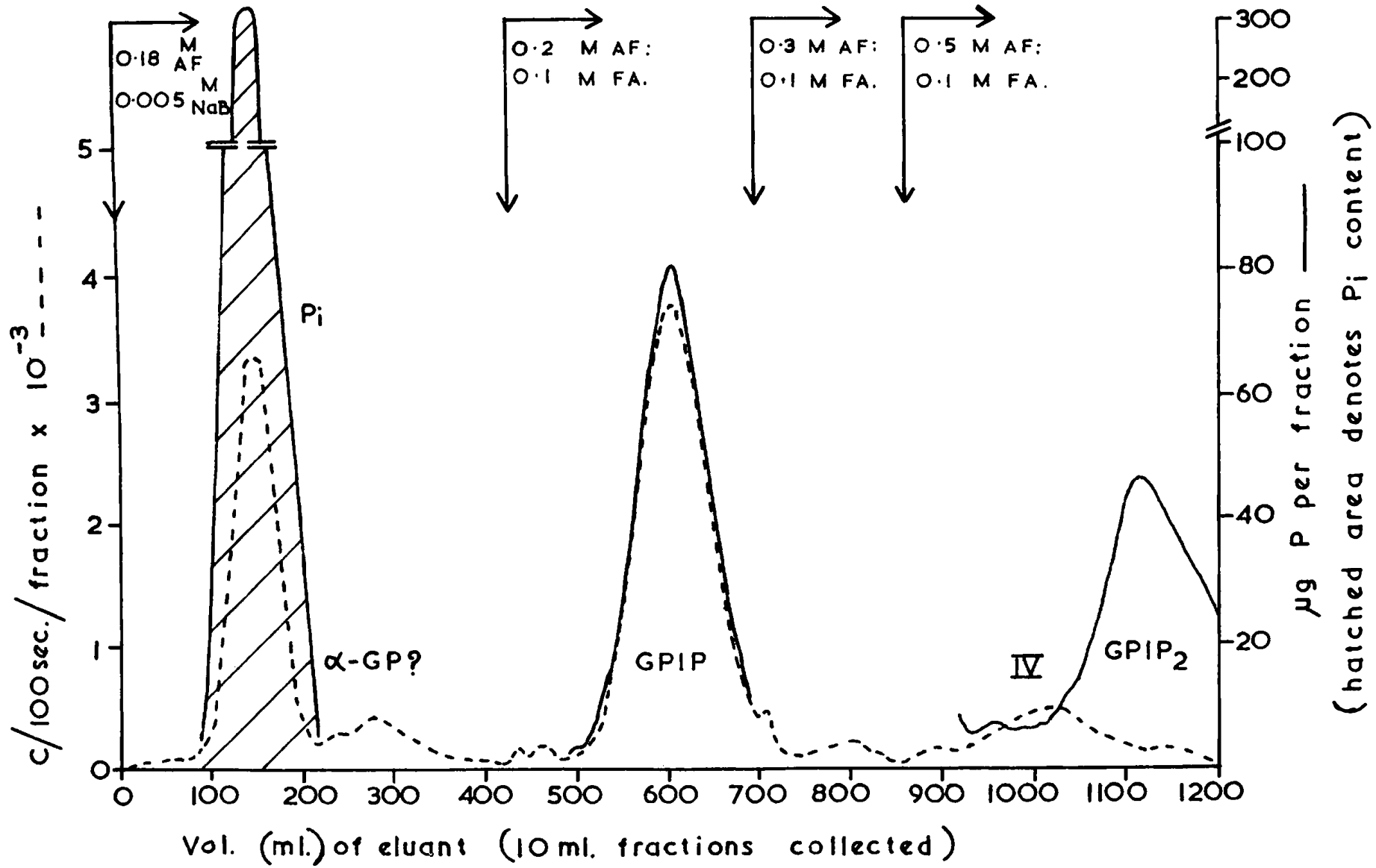


FIG. 19. SEPARATION OF DEACYLATION PRODUCTS OF LABELLED RAT LIVER MITOCHONDRIAL LIPIDS ON DOWEX 1×10 - FORMATE.

TABLE 11. Labelled compounds synthesised from ^{32}P-ATP in liver mitochondria.

Compound	Eluant Volume (ml.)	Counts/ 100 sec.	mμmoles P incorporated	
			Total	Per mg. protein
Glycero-phosphate	250-400	4,600	1.55	0.032
GPIP	500-730	41,000	13.8	0.28
Peak IV	920-1,150	8,800	2.95	0.06

These figures are corrected for the fact that only 90% of the lipid was hydrolysed and they represent the total incorporation.

Identity of the intact lipid with the lipid which yields GPIP

A pair of duplicate incubations, of the same composition as the above, but each in a final volume of 1 ml., were run simultaneously with that described above. The lipids from these were extracted by the usual method and the individual components were separated on formaldehyde-impregnated papers (method b) for assay of DPI labelling), and the papers radioautographed. The only major radioactive spot other than P_i had a mobility identical with the DPI component of the Folch inositide fraction. As expected from the column separation, no activity was detected corresponding with either PI or TPI. When the activity of the separated DPI was measured it was found that

in the 2 incubations the incorporations represented 1.40 and 1.41 mμmoles of phosphate in 5 min. These figures corresponded well to the incorporation of 13.8 mμmoles calculated from the activity of the separated GPIP derived from a tenfold larger incubation. On neither column nor paper was there any other radioactive material present of similar activity. This indicated that the DPI separated intact by paper chromatography was identical with the precursor of the labelled phosphate ester eluted with GPIP from Dowex-1-formate.

Rechromatography of the radioactive GPIP from the Dowex-1-formate column on Dowex-1-chloride.

Fractions from the beginning (550-600 ml. of eluant) and end (630-680 ml.) of the GPIP peak separated on Dowex-1-formate were combined. Ammonium ions were removed by passing the solution through a column containing approximately 20 ml. of Dowex 50W, 50-100 mesh, in the H^+ form. The column was washed with 100 ml. water and this added to the eluate.

The eluate was passed through a column of Dowex-1 x 10, 200-400 mesh (16 x 1 cm.) in the chloride form. The column was washed with 100 ml. of water. Elution was with a lithium chloride gradient. The mixing vessel contained 100 ml. of water and the reservoir 500 ml. of 4M LiCl. Ten-ml. fractions were collected. Figure 20a shows the close correspondence in elution pattern of the GPIP and the radioactive phosphate ester.

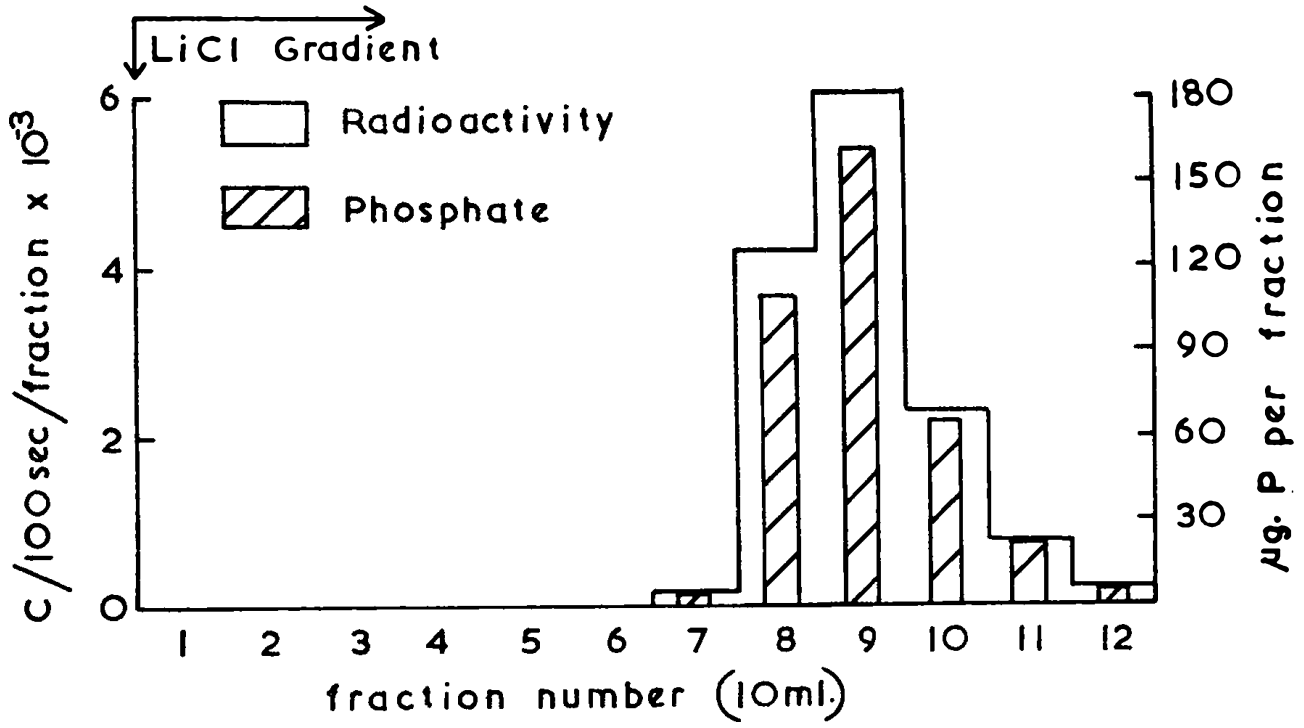


FIG.20a. CO-CHROMATOGRAPHY OF BRAIN GPIP AND ^{32}P-LABELLED MITOCHONDRIAL GPIP ON DOWEX-1-Cl^-

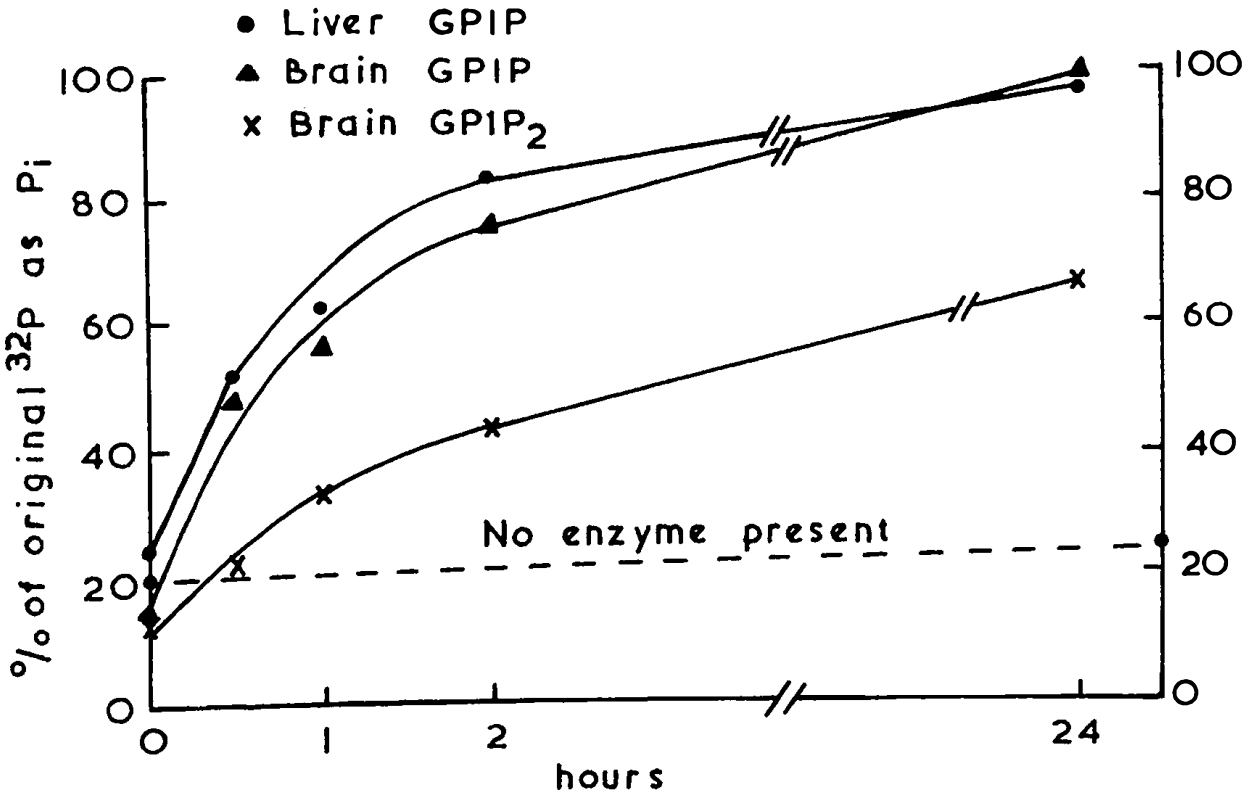


FIG.20b. RELEASE OF ^{32}P—ORTHOPHOSPHATE FROM DEACYLATED DPI AND TPI BY ALKALINE PHOSPHATASE.

From Table 12 it can be seen that both activity and phosphate were quantitatively eluted from the column in the GPIP peak.

TABLE 12 Recovery of a phosphate ester from a Dowex-1-Cl^- column.

	Counts per 100 sec.	µg. P
Loaded on to column	14,250	360
Recovered in GPIP peak	14,100	379
Recovery	99%	105%

Experiment 30.

Comparison of the properties of brain GPIP and $GPIP_2$ with those of GPIP from mitochondrial lipid.

Labelled GPIP and $GPIP_2$ from brain were kindly provided by Dr. M.Kai, the method of preparation being as follows:

The brain of a 3-week old rat was homogenised in 15 ml. 0.32M sucrose. Four ml. of the homogenate was incubated for 5 min. at 37° in the following medium: 35 mM NaCl; 7 mM $MgCl_2$; 60 mM potassium phosphate, pH 6.9; 1.6 mM ATP, containing 1.26×10^5 counts/sec. ^{32}P per µmole; 65 µg. P (organic) per ml. of the Folch inositide fraction. The final incubation volume was 10 ml.

Incorporation was stopped with 10 ml. 10% TCA and the precipitate washed with 10 ml. 5% TCA. The residue was extracted

with 25 ml. acidified chloroform:methanol (Folch, 1952) at 37° for 30 min., the mixture filtered and the filtered extract washed with 5 ml. 0.01M $CaCl_2$. The lower phase was rewashed with a synthetic upper phase from the same system. This lipid extract was mixed with unlabelled Folch inositide fraction. Hydrolysis and Dowex-1-formate chromatography were performed according to Ellis et al. (1963) and the radioactive peaks of GPIP and $GPIP_2$ pooled separately. They co-chromatographed precisely with the corresponding phosphate peaks derived from the Folch fraction.

Preparation of the phosphate esters for chromatography.

Three samples were taken for analysis:

1) Labelled GPIP derived from the mitochondrial lipid, (Experiment 29, centre of peak eluted from Dowex-1-formate, 600-630 ml. of eluate) and its accompanying unlabelled GPIP derived from brain DPI. This will be referred to hereafter as GPIP (M).
2) and 3) Labelled GPIP and $GPIP_2$ from brain (see above), hereafter referred to as GPIP (B) and $GPIP_2$ (B), respectively.

Each fraction was treated with Dowex-50, H^+ form, until it was free of ammonium ions (negative to Nessler's reagent). Each batch of Dowex-50 was washed with 2 volumes of water and the three fractions were dried off in vacuo below 50°. Each

residue was dissolved in 1 ml. of water and neutralised with concentrated ammonia solution. At this stage the samples gave the analyses shown in Table 13.

TABLE 13 Properties of phosphate esters before analysis.

Sample	µg. P/ml.	Counts/ 100 sec./ml.	Specific activity (counts/100 sec./µg.P)
GPIP(M)	170	8,400	49
GPIP(B)	269	16,700	62
$GPIP_2$(B)	203	16,600	82

Chromatography of the intact esters.

The following samples were chromatographed in the n-propanol solvent (solvent a, p.52) for 14 hr.

1) GPIP(M), 0.2 ml.
2) GPIP(B), 0.1 ml.
3) $GPIP_2$(B), 0.2 ml.
4) 0.05 ml. of a Hübscher et al. (1960) hydrolysate of the Folch inositide fraction, containing 1,580 µg. P per ml.

The mobilities of the components detected by the phosphate spray of Hanes and Isherwood (1948) are given in Table 14.

TABLE 14 Mobilities of phosphate esters in solvent a.

	R_f		
GPIP(M)	---	---	0.50
GPIP(B)	---	---	0.49
$GPIP_2$(B)	---	0.43	---
Folch inositide hydrolysate	Streak to 0.39	0.45	0.50
Probable identity	P_i	$GPIP_2$	GPIP

Each of the samples being analysed ran as a single spot and corresponded in mobility with the expected component of the hydrolysate of the Folch fraction. The phosphate-containing areas were excised and analysed for phosphate and radioactivity. The results are given in Table 15.

TABLE 15 Properties of phosphate esters separated in solvent a.

	Phosphate		Radioactivity		Specific activity (c/100sec./µg. P)	
	µg. P	Recovery	c/100 sec.	Recovery	Loaded	Recovered
GPIP(M)	32	94%	1,440	86%	49	45
GPIP(B)	26	96%	1,300	78%	62	50
$GPIP_2$(B)	38	96%	2,880	87%	82	76

Each sample contained a single unlabelled phosphate ester derived from DPI or TPI, which contained approximately 95% of the phosphorus applied to the paper. In each case 78% or more of the applied radioactivity co-chromatographed with this

phosphate ester, yielding a spot with a specific activity very close to that of the applied sample.

Determination of ^{32}P distribution in the GPIP and $GPIP_2$ molecules

a. Study of the products of strong alkaline hydrolysis

Samples of the Folch inositide fraction, GPIP(M), GPIP(B) and $GPIP_2$(B) (0.2 ml. of each of the diester solutions) were hydrolysed for 75 min. at 100° in aqueous N NaOH. The samples were cooled and Na^+ ions removed by the addition of an approximately equal volume of Dowex-50, H^+ form.

The hydrolysate of the Folch fraction contained 1.08 mg. P per ml. and 0.05 ml. of this was applied to Whatman No. 1 paper. The hydrolysates of the other fractions were approximately 0.4 ml. each, of which 0.2 ml. was applied to Whatman No. 1 paper.

Development was with the n-propanol:ammonia solvent (solvent a) for 14 hours. The chromatograms were stained for phosphate and the spots tentatively identified by comparison with the data published by Ballou and his co-workers, (Grado and Ballou, 1961; Brockerhoff and Ballou, 1961). The R_f's and probable identities are given in Table 16.

TABLE 16 Chromatography of hydrolysis products of inositol derivatives in solvent a.

Probable Identity	Inositol triphosphates	Pi	Inositol diphosphates	Inositol monophosphate	GP
Folch inositide hydrolysate	0.19 0.22	0.22- 0.33	0.36	---	0.51
GPIP(M)	---	---	(0.13)*	(0.18)*	0.52
GPIP(B)	---	---	0.34	0.43	0.52
$GPIP_2$(B)	0.18 0.22	---	0.30	---	0.50

* Footnote: in GPIP(M) 2 spots appeared at R_f's 0.13 and 0.18. They had run as crescents with the point towards the origin, almost overlapping, and there appeared to be a salt spot running at an R_f of approx. 0.2-0.4. In no other sample was the salt spot apparent, and in each case a clear separation was obtained. It is suggested that the two spots observed at low R_f's in GPIP(M) were inositol phosphates, retarded by a salt spot. Unfortunately, insufficient material was available for this part of the experiment to be repeated.

The phosphate-containing areas listed in Table 16 were cut out and their phosphate content and radioactivity assayed, with the results given in Table 17. They are identified as suggested in Table 16.

TABLE 17. Properties of the products of alkaline hydrolysis of deacylated inositides.

	Probable Identity	µg.P	c/100 sec.	c/100 sec./ µg. P
GPIP (M)	IP_2	7.6	444	58
	IP	4.0	264	66
	GP	1.7	11	6
	Total Recovered	13.3	719	53
	Approximate Total Loaded	17	840	49
GPIP (B)	IP_2	8.6	440	51
	IP	7.5	500	66
	GP	7.2	80	11
	Total Recovered	23.3	960	42
	Approximate Total Loaded	27	1,670	62
$GPIP_2$ (B)	$IP_{3's}$	17.2	1,570	91
	IP_2	3.7	410	112
	GP	2.3	44	19
	Total Recovered	23.2	2,424	106
	Approximate Total Loaded	20.3	1,660	82

Although the poor running of the GPIP(M) sample on the chromatogram throws some doubt on the interpretation of the results, two features of the results in Table 17 are worthy of mention.

1) In each sample, the activity of the phosphate of glycerophosphate (derived from the "phosphatidyl" phosphate group) was far less than that of the inositol phosphates. This suggested that in each sample the diester phosphate group was less radioactive than the monoesters.

2) The inositol phosphates which did not include the diesterified phosphate (i.e. IP from GPIP and IP_2 from $GPIP_2$) always had a higher specific activity than those containing these groupings. This was further evidence of the low radioactivity of the diester phosphate group.

Degradation of the phosphate esters by alkaline phosphatase

A method similar to that of Ellis et al. (1963) and Galliard (1963) was used for these assays.

0.2 ml. samples of GPIP(M), GPIP(B) and $GPIP_2$(B) were each added to a tube containing 0.2 ml. 0.1 M ethanolamine/HCl, pH 9.5; 0.05 ml. 0.1 M $MgCl_2$; 0.55 ml. water; 0.2 mg. calf intestine alkaline phosphatase (orthophosphoric monoester phosphohydrolase, E.C.3.1.3.1.). The resulting mixtures were incubated at 37°.

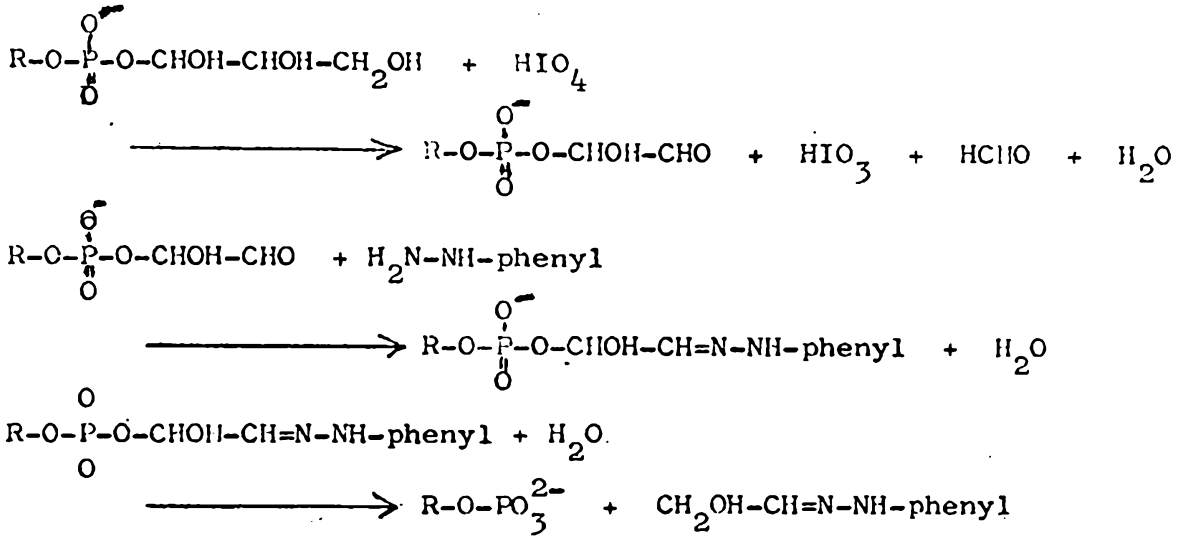
Samples were removed at intervals and the proportion of the total ^{32}P in the form of ^{32}P-orthophosphate was determined using the partition method of Martin and Doty (1949). The results are shown in Figure 20 b. Clearly the time-courses of $^{32}P_i$ release from GPIP(M) and GPIP(B) were similar, whereas release from $GPIP_2$(B) was much slower. In previous work with the same preparation of phosphatase Ellis et al. (1963) found it to catalyse no release of P_i from GPI, and to promote the release of 54% and 67% respectively of the phosphate of GPIP and $GPIP_2$ as P_i. It thus contained negligible phosphodiesterase activity.

In both GPIP(M) and GPIP(B) almost all of the ^{32}P was released as P_i, showing the labelling of the diesterified phosphate group to be very low. The similarity in the time-course of the release from the two samples was further evidence of their identity.

Study of the products of periodate oxidation.

Brown et al. (1959) described the use of limited periodate oxidation (1), followed by phenylhydrazine treatment (2) for the isolation of unchanged inositol phosphate from GPI. Ballou and his co-workers have extended the use of this method to allow the isolation of unchanged inositol di- and triphosphate, respectively, from GPIP and $GPIP_2$. An attempt was made to apply

the method to the samples being studied.



A second sample of GPIP(M), designated GPIP(M2), was prepared from the eluate from the Dowex-1-Cl column (Experiment 29). Tubes 8 - 11, containing 282 µg. P and 9,750 counts/100 sec. were dried <u>in vacuo</u> at 60°, acetone and ethanol being used to remove the last traces of water. The material in the flask was twice extracted with ethanol and was then dissolved in water. The ethanol extracts were pooled, centrifuged and the precipitate twice washed with ethanol. This was also dissolved in water and the two aqueous solutions were pooled and made up to 1 ml. Recoveries were 79% for phosphate and 70% for radioactivity. The solution was acidified

with a few beads of Dowex-50, H^+ form, filtered and neutralised with ammonia.

A sample of acid-dialysed Folch inositide was hydrolysed according to Hübscher et al. (1960), the water-soluble esters separated, treated with Dowex-50, H^+ form and neutralised with ammonia.

Samples of GPIP(M2), GPIP(B), $GPIP_2$(B) and the inositide hydrolysate were taken. These were adjusted to approximately pH 6 with formic acid and treated with periodic acid (in equimolar quantities) for 1 hr. at room temperature. Equimolar quantities of phenylhydrazine were then added and the mixtures held at 37° for 15 hr. After treatment with Dowex-50, H^+ form, samples were applied to Whatman No. 1 paper and chromatographed.

The following were separated in solvent a.

1. The periodate and phenylhydrazine-treated Folch inositide hydrolysate.
2. Periodate/phenylhydrazine-treated GPIP(B).
3. Periodate/phenylhydrazine-treated $GPIP_2$(B).
4. An alkaline (1 N NaOH at 100° for 1 hr.) hydrolysate of Folch inositide.

The resulting pattern of phosphate esters is shown in Figure 21. The identities given are tentative, based on the data of Ballou and co-workers. Sample 1 gave the expected

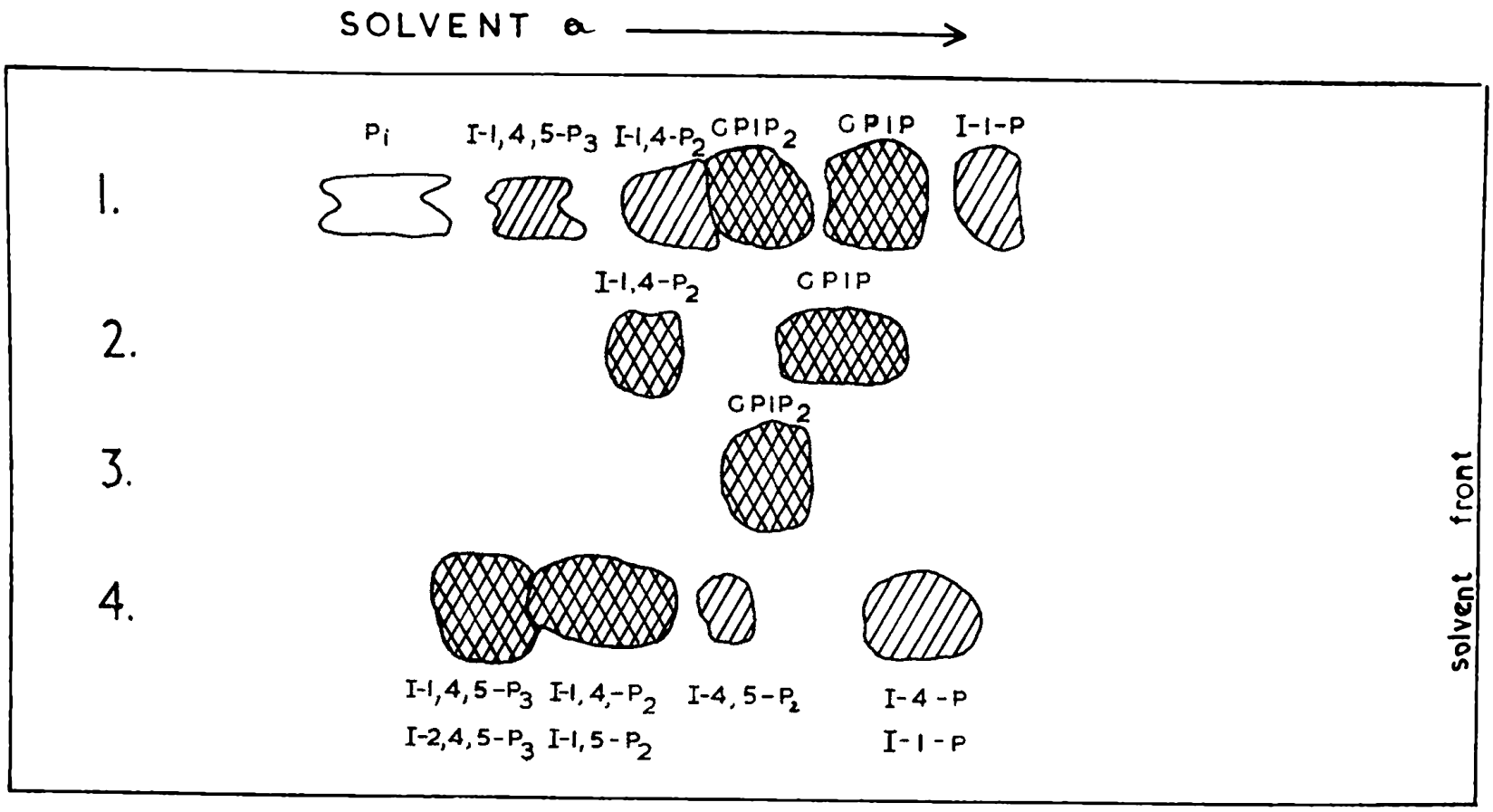


FIG. 21. SEPARATION OF PHOSPHATE ESTERS OF INOSITOL ON PAPER IN SOLVENT a (n-PROPANOL - AMMONIA - WATER). SAMPLES AS DETAILED IN THE TEXT.

pattern of phosphate esters derived from the inositide mixture. Sample 2 contained unchanged GPIP and, as expected, inositol-1,4-diphosphate. With sample 3 the periodate treatment had been unsuccessful and the sample only contained unchanged $GPIP_2$. Sample 4 contained the expected pattern of esters. In the figures the intensity of the phosphate reaction on the paper is approximately represented by the amount of cross-hatching.

The following were separated in solvent b.

1. 1 N NaOH hydrolysate of undialysed Folch inositide.
2. 1 N NaOH hydrolysate of GPIP(M).
3. Periodate/phenylhydrazine treated GPIP(M2).
4. Mild alkaline hydrolysate (Hübscher et al. 1960) of Folch inositide (undialysed).
5. GPIP(M).
6. GPIP(B).
7. P_i + 3-GP.

The main points to be noted from the results of this separation, shown in Figure 22, are:

a. GPIP(M) and GPIP(B) showed the same mobility. They also migrated with the faster-running of the two major components derived from the Folch inositide (Sample 4).

b. Strong alkaline hydrolysis of GPIP(M) gave 3 components, almost certainly glycerophosphate and inositol mono- and diphosphate.

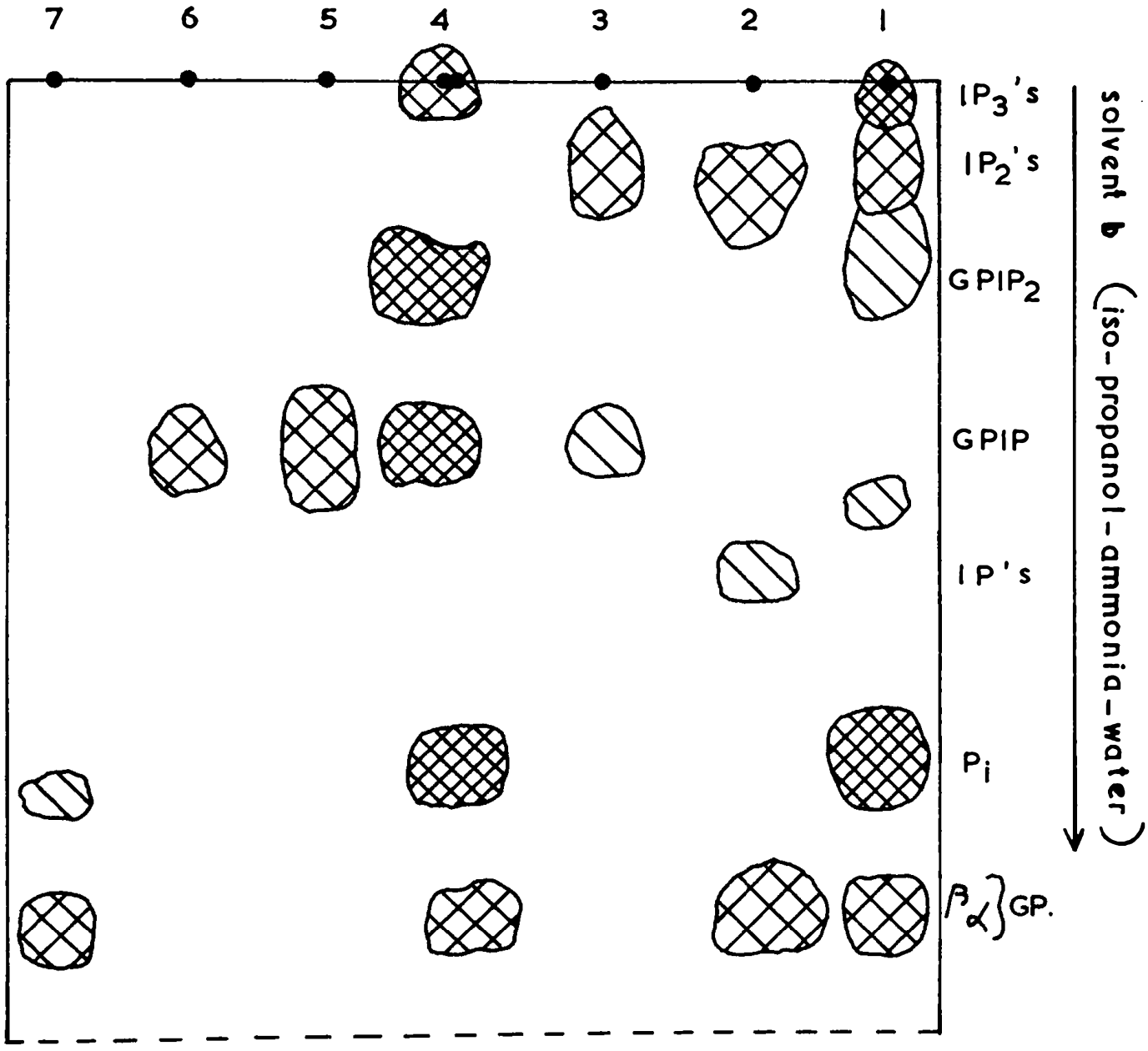


Fig.22. Chromatography of inositide hydrolysis and oxidation products in iso-propanol - ammonia - water (solvent b). Details of the samples applied are given in the text.

c. Periodate/phenylhydrazine treatment of GPIP (M2) released only one compound, with the mobility of an inositol diphosphate.

On neither chromatogram was any radioactivity detected, other than that associated with the phosphate-containing areas.

Summary of data on the effects of various chromatographic techniques and chemical degradation procedures on mitochondrial DPI, and on GPIP derived from it.

1. Mitochondrial DPI co-chromatographed with brain DPI on formaldehyde-impregnated papers.

2. Incorporation studies produced mainly one radioactive lipid, similar to DPI. Methanolysis of the lipid mixture released mainly one radioactive water-soluble phosphate ester, similar to GPIP. The recovery of radioactivity from DPI in the ester was quantitative.

3. Mitochondrial GPIP co-chromatographed on Dowex-1-formate with brain GPIP.

4. The mitochondrial GPIP from the dowex-1-formate column co-chromatographed with brain GPIP on Dowex-1-chloride. Both radioactivity and phosphate were quantitatively eluted in this single peak.

5. Mitochondrial GPIP co-chromatographed with brain GPIP on paper in two solvent systems. Almost all of the radioactivity of the material applied to the paper was recovered from the single phosphate-containing areas detected on the papers.

6. Degradation of the mitochondrial GPIP with alkali produced three products, tentatively identified as glycerophosphate

and inositol mono- and diphosphates.

7. Treatment of mitochondrial GPIP with periodate, followed by phenylhydrazine, released a single product, tentatively identified as inositol diphosphate.

8. Treatment of mitochondrial GPIP with alkaline phosphatase released virtually all of its radioactivity as inorganic phosphate. Thus the labelling is confined to the mono-esterified phosphate group. Assuming the parent DPI to be identical with that from brain, this would be the phosphate group esterified to the 4- position of the inositol ring.

Experiment 31.

Effect of ATP concentration on mitochondrial DPI labelling.

Incubation conditions were: 100 mM KCl; 1 mM PI; 25 mM potassium phosphate buffer, pH 7.4; 10 mM $MgCl_2$; mitochondria (4.4 mg. protein); 60 mM sucrose; ATP (9.14×10^5 counts/100 sec. per µmole) at concentrations of from 0 to 10 mM. Incubations were for 5 min. at 30^o in a final volume of 1 ml. The incorporations were assayed by paper chromatography and radioautography, followed by measurement of the radioactivity in the localised DPI (method b)). The results, shown in Figure 23, show an ATP optimum at approximately 5 mM.

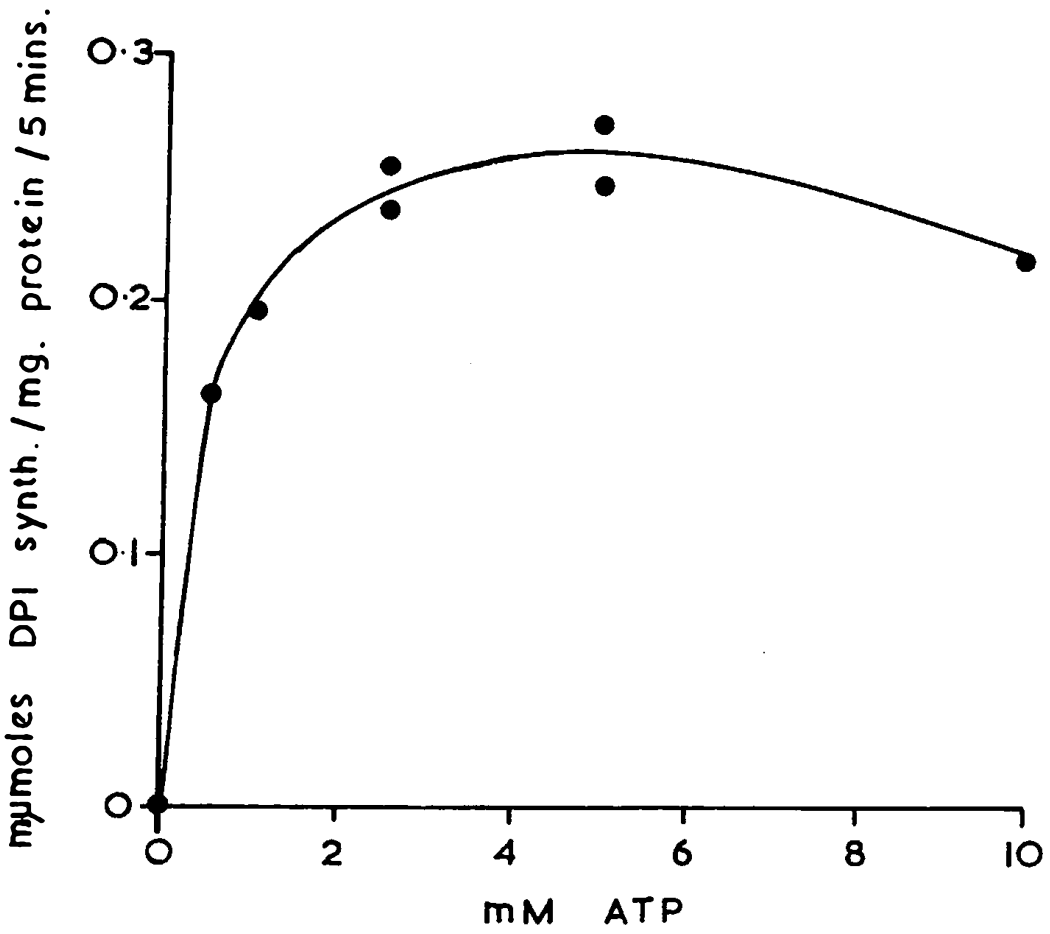


Fig. 23. Effect of ATP concentration on mitochondrial DPI synthesis.

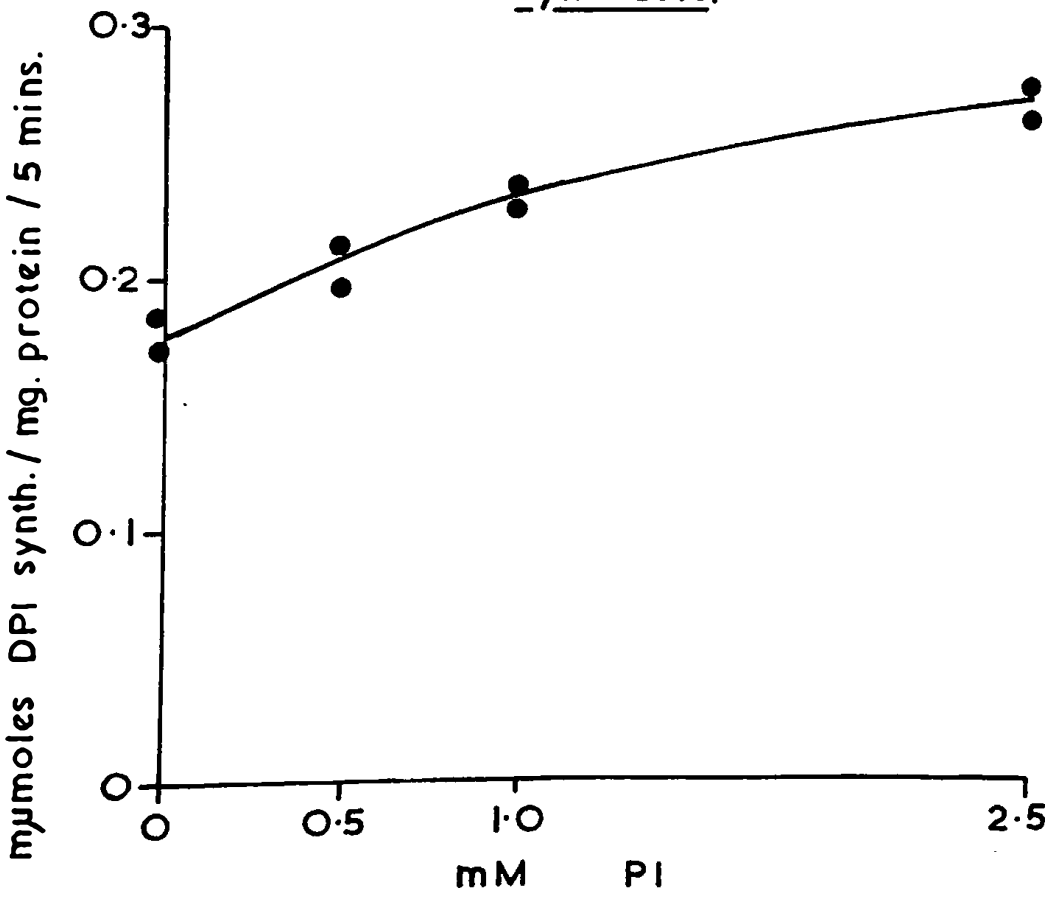


Fig. 24. Effect of PI concentration on mitochondrial DPI synthesis.

Experiment 32.

Effect of PI concentration on the mitochondrial DPI labelling.

The incubation conditions were the same as those for Experiment 31, except that the ATP concentration was constant at 2.5 mM and the PI concentration varied from 0 to 2.5 mM. The results, shown in Figure 24, show that, although maximal stimulation had not been attained, this mitochondrial preparation was not very markedly stimulated by PI. In this respect it resembled preparation B of Experiment 28.

Experiment 33.

Incubation conditions were similar to those for the previous two experiments, except that no PI was present, and other phospholipids were added, as emulsions in water, at a concentration of 1 mM. The results are given in Table 18 and include those for PI as a comparison. No lipid other than PI stimulated the reaction; all the others tested inhibited quite markedly.

TABLE 18 Effects of added phospholipids on DPI labelling.

Phospholipid (1 mM)	mμmoles P incorporated/ mg. protein	mμmoles P incorporated (mean)	% of control
None	.171 .181	.176	(100)
Phosphatidyl-inositol	.233 .241	.237	135
Phosphatidyl-choline	.121 .125	.123	70
Phosphatidyl-ethanolamine	.055 .095	.075	44
Phosphatidic acid	.116 .134	.125	71

Experiment 34.

Distribution of ^{32}P-ATP-dependent DPI labelling in liver subcellular fractions.

In Experiment 24 it was found that P_i-dependent DPI labelling was essentially confined to the mitochondrial fraction. A similar experiment was performed using labelled ATP as phosphate donor.

Incubation conditions: 100 mM KCl; 25 mM potassium phosphate buffer, pH 7.4; 10 mM $MgCl_2$; 1 mM PI; 2.5 mM ATP (9.14×10^5 counts per 100 sec. per μmole); 60 mM sucrose (150 mM for supernatant). Incubations were for 5 min. at 30°

in a final volume of 1 ml. Assay was by method b. (paper chromatography and autoradiography). The results, given in Table 19, show that most of the activity was non-mitochondrial, but was not clearly localised in any one fraction. Surprisingly, the largest portion of the activity occurred in the mixture generally known as the nuclear fraction. As the final assay conditions for the ATP-dependent reaction had not been established at this stage, the result could only be taken as a guide, and no marker enzyme assays were attempted. Furthermore, in this fractionation the quantity of protein recovered in the nuclear fraction, about 45% of the total, was very high, indicating severe contamination with other fractions. Nevertheless, it was clearly unlikely that the ATP-dependent activity was confined to the mitochondrial, lysosomal or microsomal fraction and, if a single site in the cell was assumed, an alternative had to be sought. The only structure thought likely to show the observed spread through the fractions was the fragmented plasma membrane.

TABLE 19. Distribution of ATP-dependent DPI labelling.

Fraction	mg. protein per incubation	mg. protein in total fraction	mμmoles DPI synthesised/ mg. protein	mμmoles DPI synthesised/ fraction	% of recovered activity
Nuclear	10.00	2,900	0.152	438	54.3
Mitochondrial	4.36	371	0.209	77.5	9.6
Lysosomal	4.06	203	0.455	92.5	11.4
Microsomal	6.18	309	0.240	74	9.2
Supernatant	7.85	2,980	0.042	125	15.5

Experiment 35.

Cation requirements for DPI labelling in homogenates.

Incubation conditions: 100 mM KCl; 20 mM Tris/HCl, pH 7.4; 1.9 mM PI; 60 mM sucrose; 4.0 mM ATP (4.65×10^5 counts/100 sec./µmole; a filtered homogenate (19.8 mg. protein per incubation). Incubations at 30° for 5 min. in a final volume of 1 ml. Detection was by co-chromatography with Folch inositide fraction and Nile Blue staining (method b)). $MgCl_2$, $MnCl_2$, $CaCl_2$ or $FeCl_3$ concentrations were varied as shown in Figure 25.

Clearly divalent cations are required for the reaction, Mn^{++} being the most efficient at low concentrations, Mg^{++} becoming considerably more efficient at higher concentrations.

A study was next undertaken to determine the optimal conditions for ATP-dependent DPI labelling in liver cell fractions, initially by varying the concentrations of ATP, PI, $MgCl_2$ and $MnCl_2$.

Experiment 36.

Study of optimum conditions for DPI labelling in cell fractions.

The basic medium consisted of: 1.3 mM PI; 100 mM KCl; 20 mM Tris/HCl, pH 7.4; 4.2 mM ATP (5×10^5 counts per 100 sec. per µmole); 10 mM $MgCl_2$; 60 mM sucrose.

Concentrations of PI, $MgCl_2$, ATP and $MnCl_2$ were varied as shown in the figures. When $MnCl_2$ was present $MgCl_2$

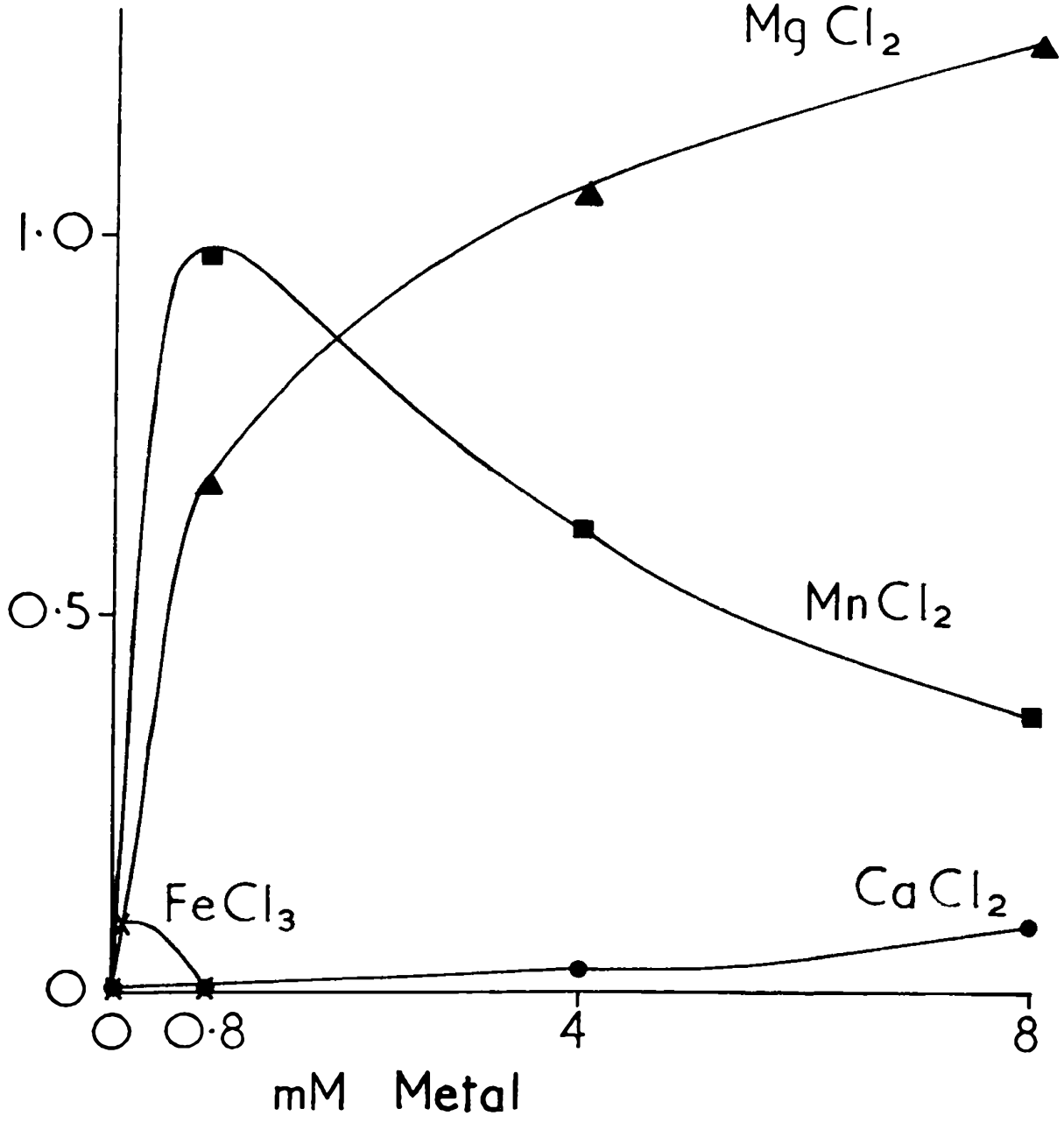


FIG. 25. EFFECT OF CATIONS ON DPI LABELLING IN LIVER HOMOGENATE

was omitted. Protein contents were (mg. per incubation): Homogenate, 7.4; Nuclear, 4.3; Mitochondrial, 4.5; Lysosomal, 3.7; Microsomal, 4.2; Supernatant, 3.1.

Incubations were at 30° for 5 min. in a final volume of 1 ml. Assay was by chromatography and radioautography, (method b.). Results are plotted on Figures 26 - 29. Figure 29 also includes results from the subsequent experiment.

Optimal concentrations were taken from these curves where possible and used in the subsequent experiments. These optima were:

For ATP: 5 mM in all fractions except microsomal and possibly lysosomal.

For PI: all fractions were still sub-optimal at 2.6 mM.

For $MgCl_2$: for mitochondria 10-20 mM (15 mM used subsequently); for other fractions 20 mM was still sub-optimal.

For $MnCl_2$: for all fractions except supernatant 1.0 mM was optimal. However, optimal activities with $MnCl_2$ were lower than the highest activities measured with $MgCl_2$. $MgCl_2$ was used in subsequent experiments. The effects of $MnCl_2$ and $MgCl_2$ were not additive (see below).

Before either $MnCl_2$ or $MgCl_2$ could be used alone the possibility of their being additive or synergistic was investigated.

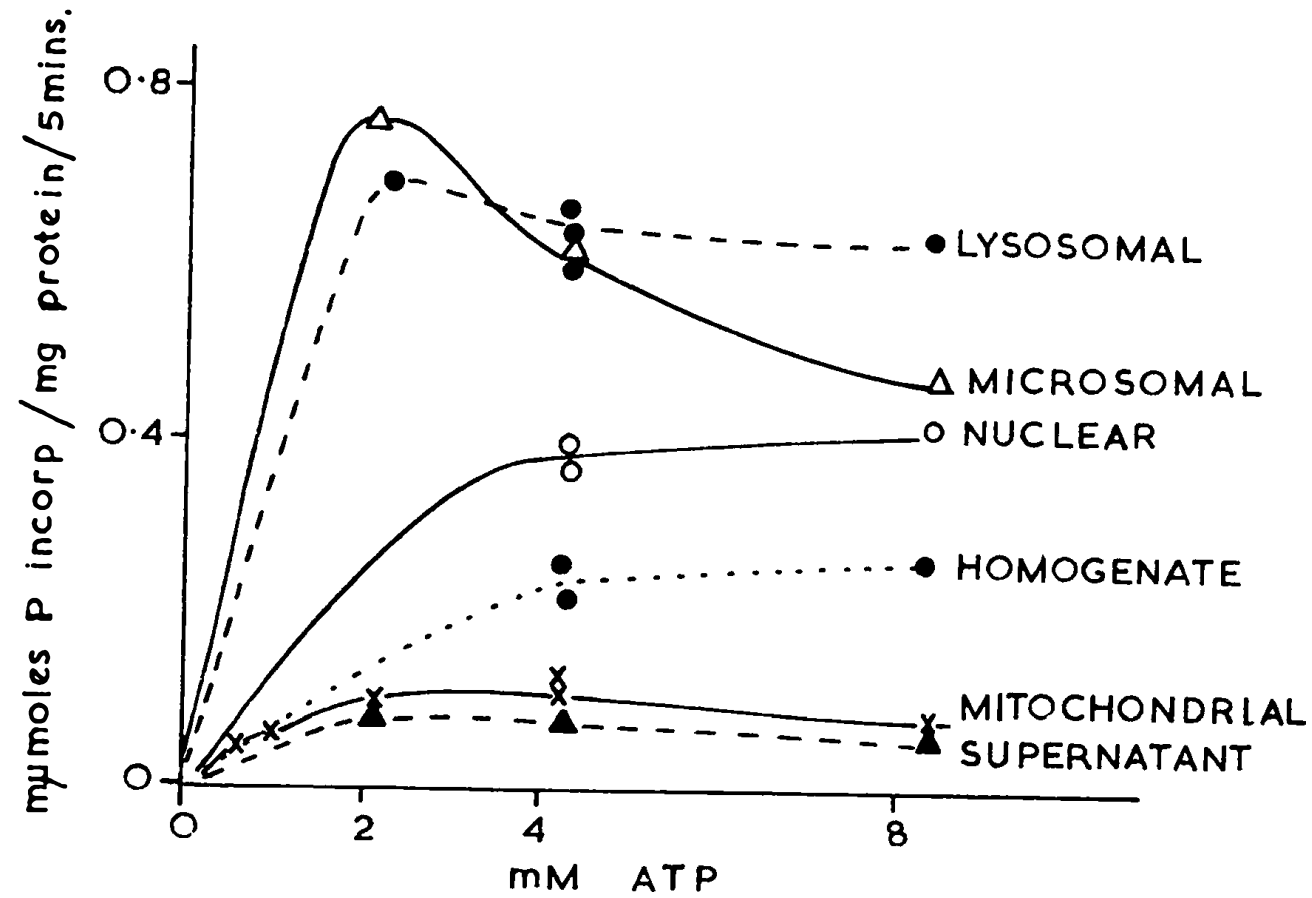


Fig. 26 Effect of ATP on DPI labelling.

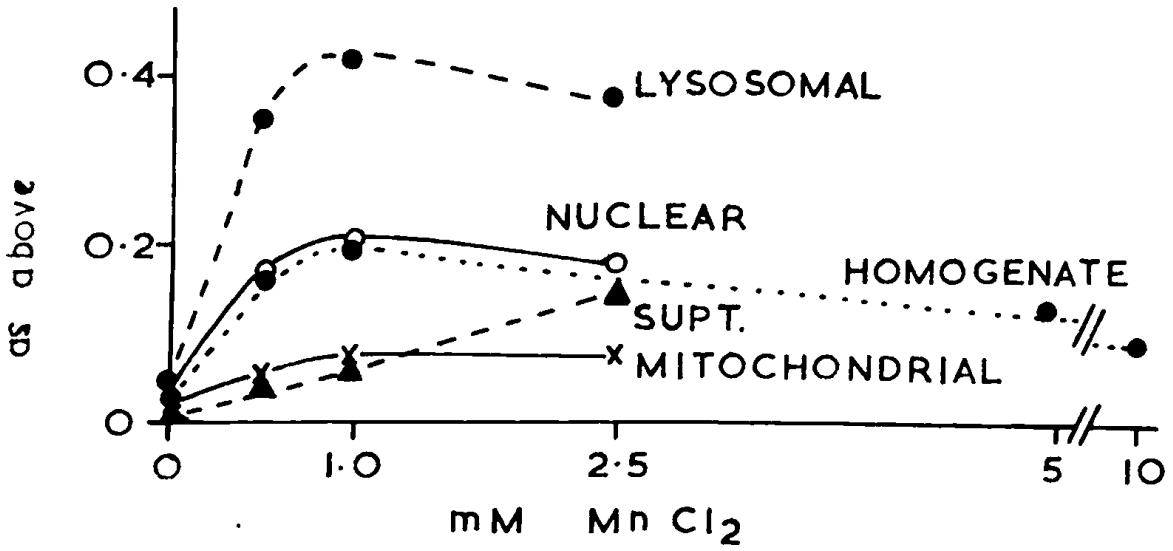


Fig. 27 Effect of $MnCl_2$ on DPI labelling.

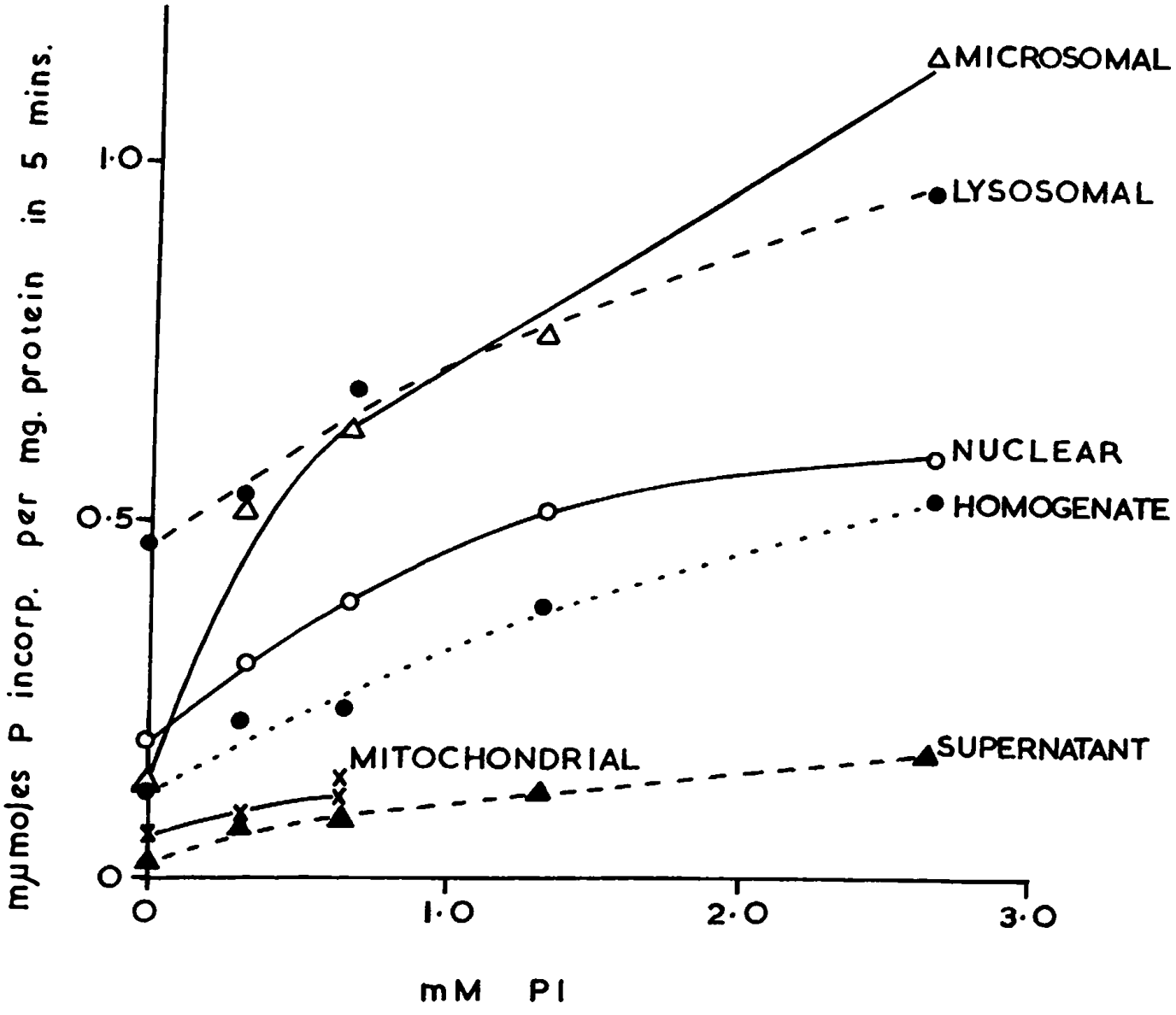


FIG. 28 EFFECT OF PI ON DPI LABELLING.

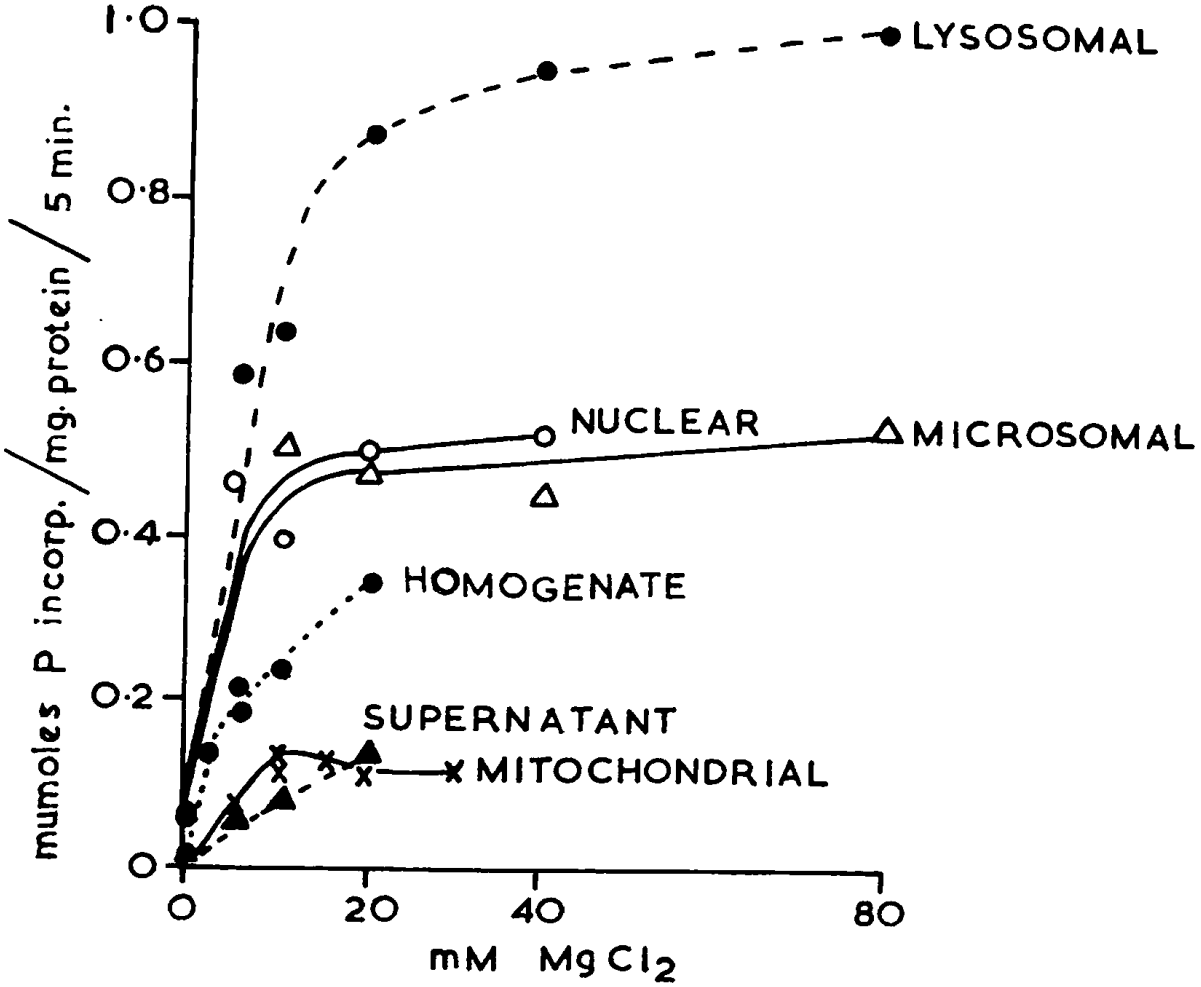


Fig. 29. $MgCl_2$ concentration and DPI labelling.

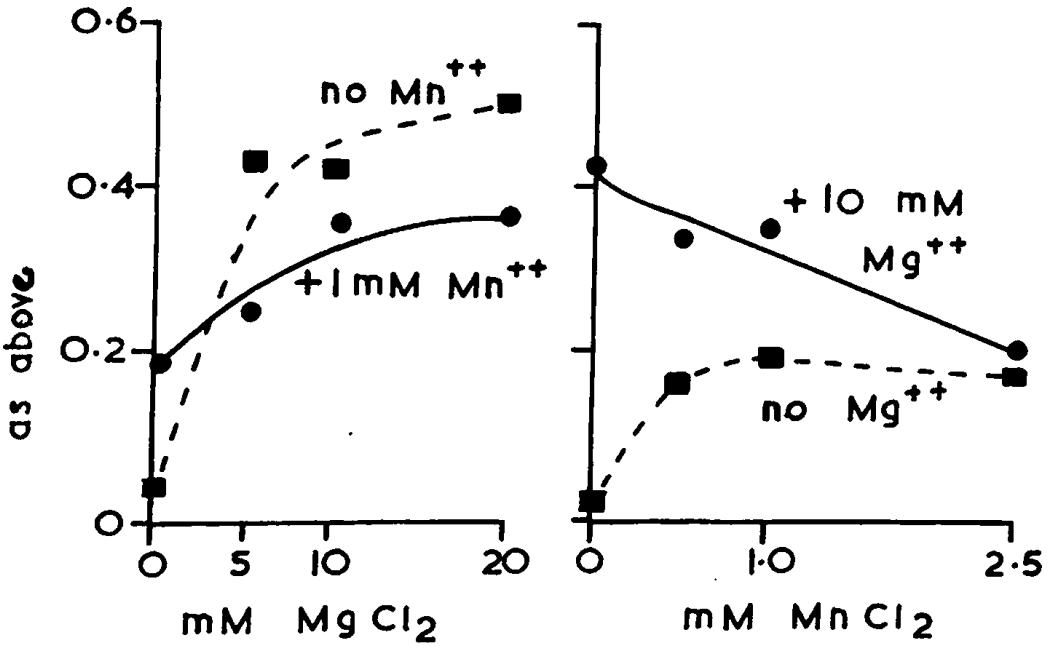


Fig. 30. Effects of Mg^{++} and Mn^{++} in combination on "nuclear" DPI labelling.

The results for the nuclear fraction given in Figure 30 were typical of those obtained. They showed that up to 2.5 mM $MnCl_2$ in the presence of 10 mM $MgCl_2$, or up to 20 mM $MgCl_2$ in the presence of 1 mM $MnCl_2$, could never stimulate the system to a higher activity than that obtained with 10 mM $MgCl_2$ alone.

Experiment 37.

Establishment of optimal $MgCl_2$ concentrations.

A further experiment was performed under similar conditions to the above, except that the quantities of protein in the 1 ml. incubations were:

Nuclear, 5.7 mg.; Mitochondrial, 4.8 mg.; Lysosomal, 3.8 mg.; Microsomal, 5.7 mg.; Supernatant, 3.5 mg.

The results of this experiment were scaled on to the same graphs as those obtained previously. The combined results are given in Figure 29. In this second experiment no activity was detected in the supernatant fraction.

$MgCl_2$ concentrations for later experiments were: mitochondrial, 15 mM; all other fractions, 40 mM.

Experiment 38.

Optimal concentration of PI.

The same cell fractions as had been used for the previous experiment were studied at PI concentrations of up to

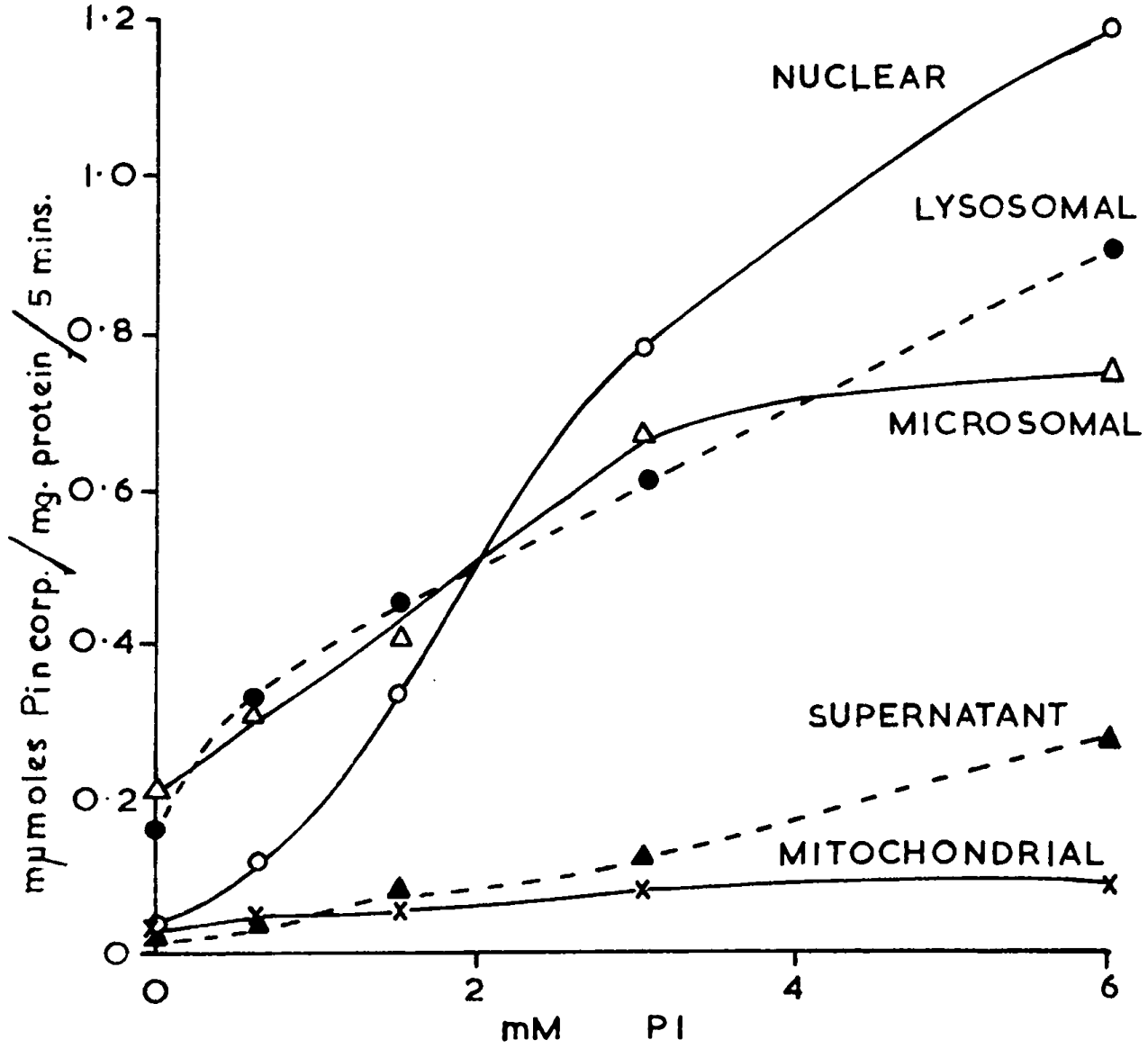


Fig. 31. PI concentration and DPI labelling in liver subcellular fractions.

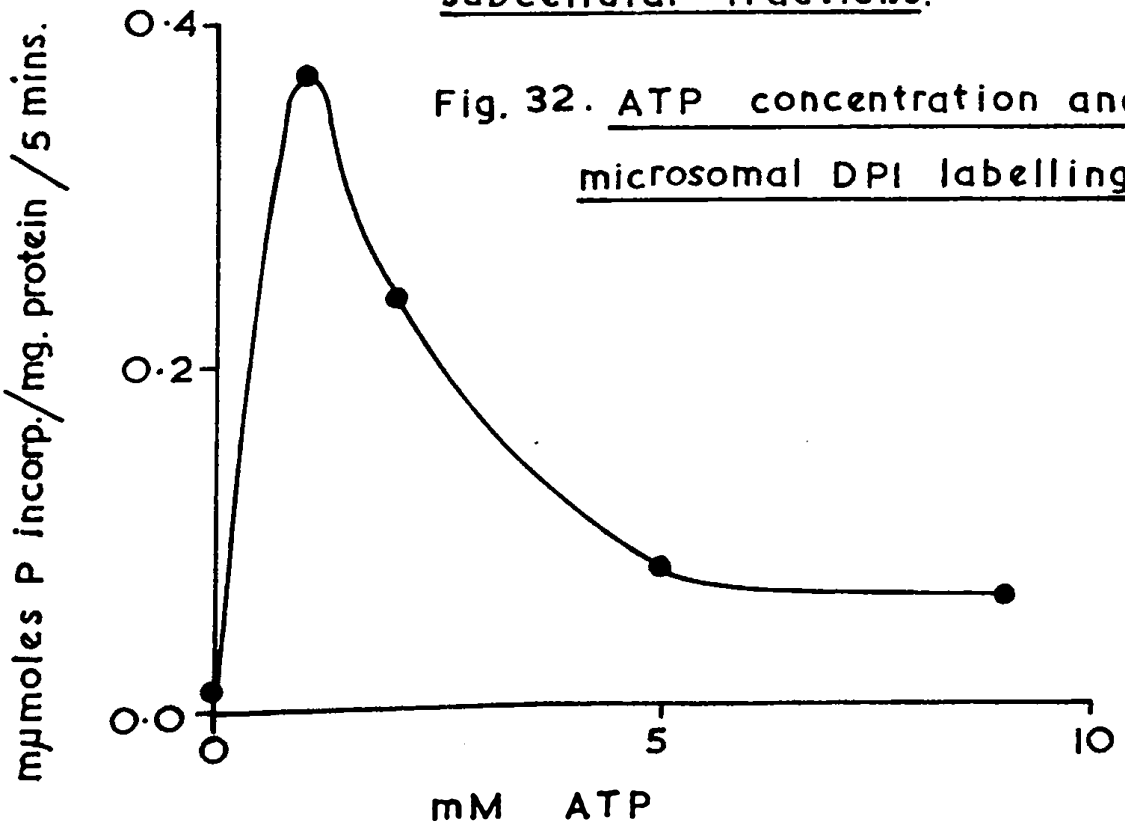


Fig. 32. ATP concentration and microsomal DPI labelling

6 mM, under conditions otherwise identical to those for Experiment 36. Only the mitochondrial and microsomal fractions seemed certainly to have reached their optimum at 6 mM (see Figure 31).

A comparison of the activities measured with 5 mM ATP, 10 mM $MgCl_2$ and 6 mM PI in this experiment was of interest, as the protein distribution through the fractions followed the usual pattern rather better than in Experiment 34. Table 20 gives the specific activity (mμmoles DPI formed/5 min./mg. protein), protein content and total activity of each fraction. KCl, Tris/HCl, sucrose, and protein concentrations were as in the previous two experiments, as were the temperature, time and total volume of the incubations.

TABLE 20 Subcellular distribution of ATP-dependent DPI labelling.

Fraction	Specific activity*	Protein (mg.)	Total Activity (mμmoles DPI formed/5 min.)	% of total recovered activity
Nuclear	1.2	338	409	45
Mitochondrial	0.093	240	22	2.4
Lysosomal	0.92	94	87	9.5
Microsomal	0.725	260	188	20.5
Supernatant	0.29	725	210	22.9

* mμmoles DPI formed/5 min./mg. protein

At near optimal conditions it was found that very little activity was present in the mitochondrial fraction, and that there was a fairly even distribution through the other fractions, with nuclear, microsomal and lysosomal fractions showing much the highest specific activities.

Experiment 39.

ATP optimum for microsomal fraction.

As the microsomes had shown an unusual response to ATP concentration, this co-factor was assayed at lower concentrations than those used previously. Results are shown in Figure 32. The optimum was far lower than that for the other fractions, and fell at about 1 mM. The conditions were: 100 mM KCl; 20 mM Tris/HCl, pH 7.4; 60 mM sucrose; 40 mM $MgCl_2$; 2.2 mM PI; 5 mg. microsomal protein; ATP (2.24×10^5 counts/100 sec./µmole) at concentrations of 0 to 10 mM; total volume 1 ml. Tubes were incubated for 5 min. at 30°. Assay was by determination of the radioactivity of the lipid extract after washing (method c)).

In subsequent microsomal assays, both 1 and 5 mM ATP were used.

Experiment 40.

Effect of PI emulsion concentration on PI concentration curve.

It was thought that the very high apparent PI optimum ($>$ 5 mM) might be due to the high concentration of the emulsions

added to the incubation media (20 - 50 mM). This type of effect had been observed by Dr. M.I.Gurr when studying the concentration of diglyceride required by intestinal CDP-choline: 1,2-diglyceride cholinephosphotransferase (E.C. 2.7.8.2.). If emulsion concentrations were decreased the substrate optimum also decreased.

A nuclear fraction which had been frozen for 1 week was used in the incubations. PI was added as emulsions of from 2.6 to 26 mM, and concentration curves were plotted. Incubations (final volume 1 ml.) were for 5 min. at 30° in 20 mM Tris/HCl, pH 7.4; 40 mM $MgCl_2$; 30 mM sucrose; 5 mM ATP (2.24×10^5 counts/100 sec./µmole); 2.86 mg. homogenate protein. Assay was by counting of the washed lipid extract (method c)). The results are plotted in Figure 33 and show that the incorporations were dependent on the final PI concentration, but not on the concentration of the added lipid emulsion.

Later incubations were performed at a final concentration of 5 or 6 mM PI unless otherwise specified. Due to the high quantity of lipid required for incubations under these conditions some of the later studies of conditions were first made on homogenates, and then checked in individual cell fractions, thus effecting an economy of PI.

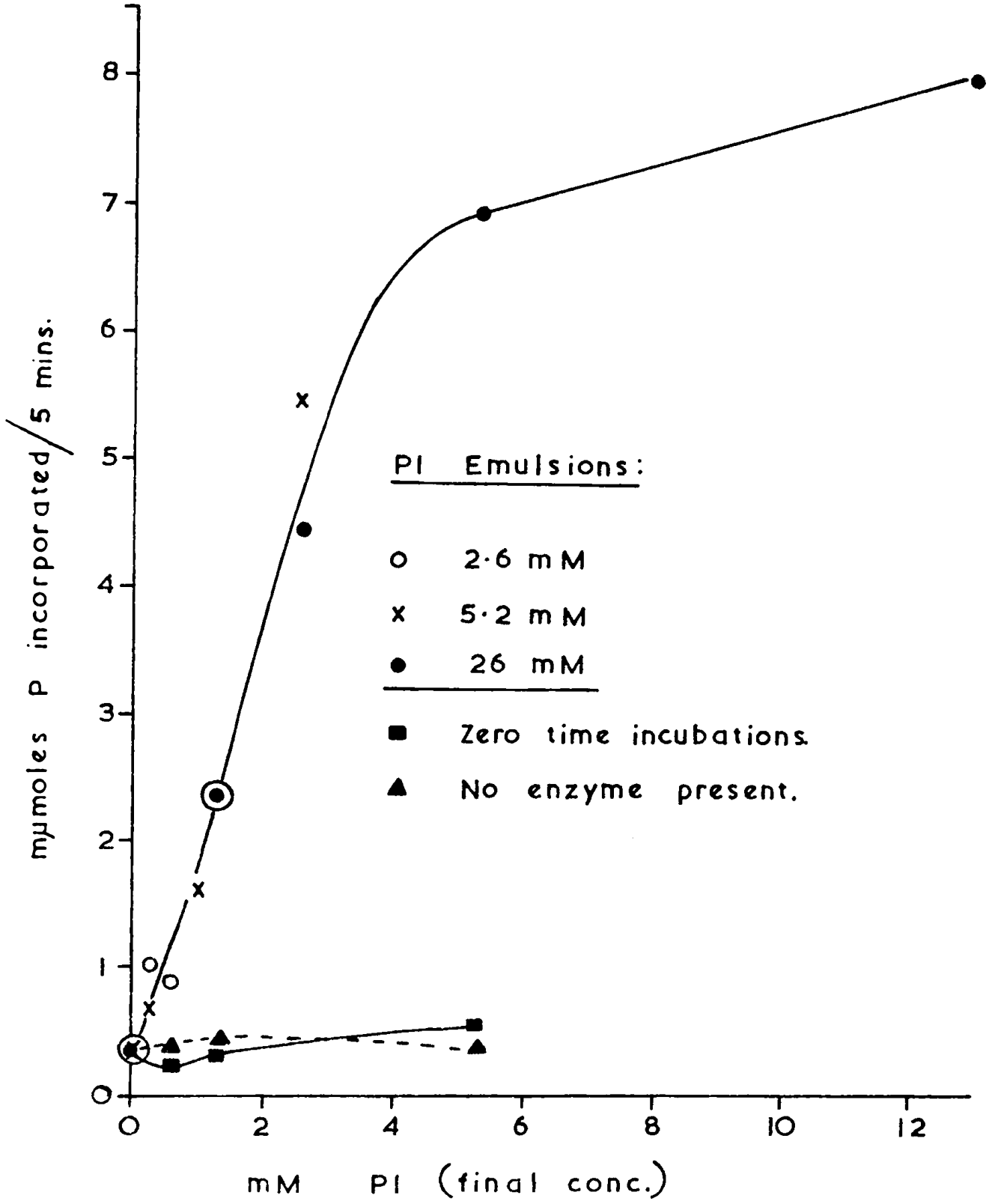


Fig. 33 Effect of PI emulsion concentration on its efficiency as substrate for DPI production.

Experiments 41 and 42.

Time-course of DPI labelling.

Conditions of incubation for Experiment 41 were as in Experiment 40, except that incubations contained 6.24 mg. protein (homogenate), the ATP had a specific activity of 1.72×10^5 counts/100 sec./μmole and the period of incubation was varied. In Experiment 42 the specific activity of the ATP was 2.18×10^5 counts/100 sec./μmole and the protein used was 7.9 mg. of homogenate. Assay of the radioactivity of the DPI was by method c).

Figure 34 shows the combined results of these two experiments. As in the previous time-study (Experiments 3-6), a common point was taken from the two experiments, in this case that at three minutes.

Incorporation of ^{32}P into DPI was linear with time for at least 3 min., but after about 5 min. the rate of incorporation of radioactivity into the lipid decreased considerably. Subsequent experiments used an incubation period of 2 min., rather than the 5 min. used previously. It was then certain that the activity being measured was proportional to the time of incubation.

Experiment 43.

Subcellular distribution of ^{32}P-ATP-dependent DPI labelling.

Fractionation was as described under Methods. One rat liver was used. The assay conditions were: 100 mM KCl;

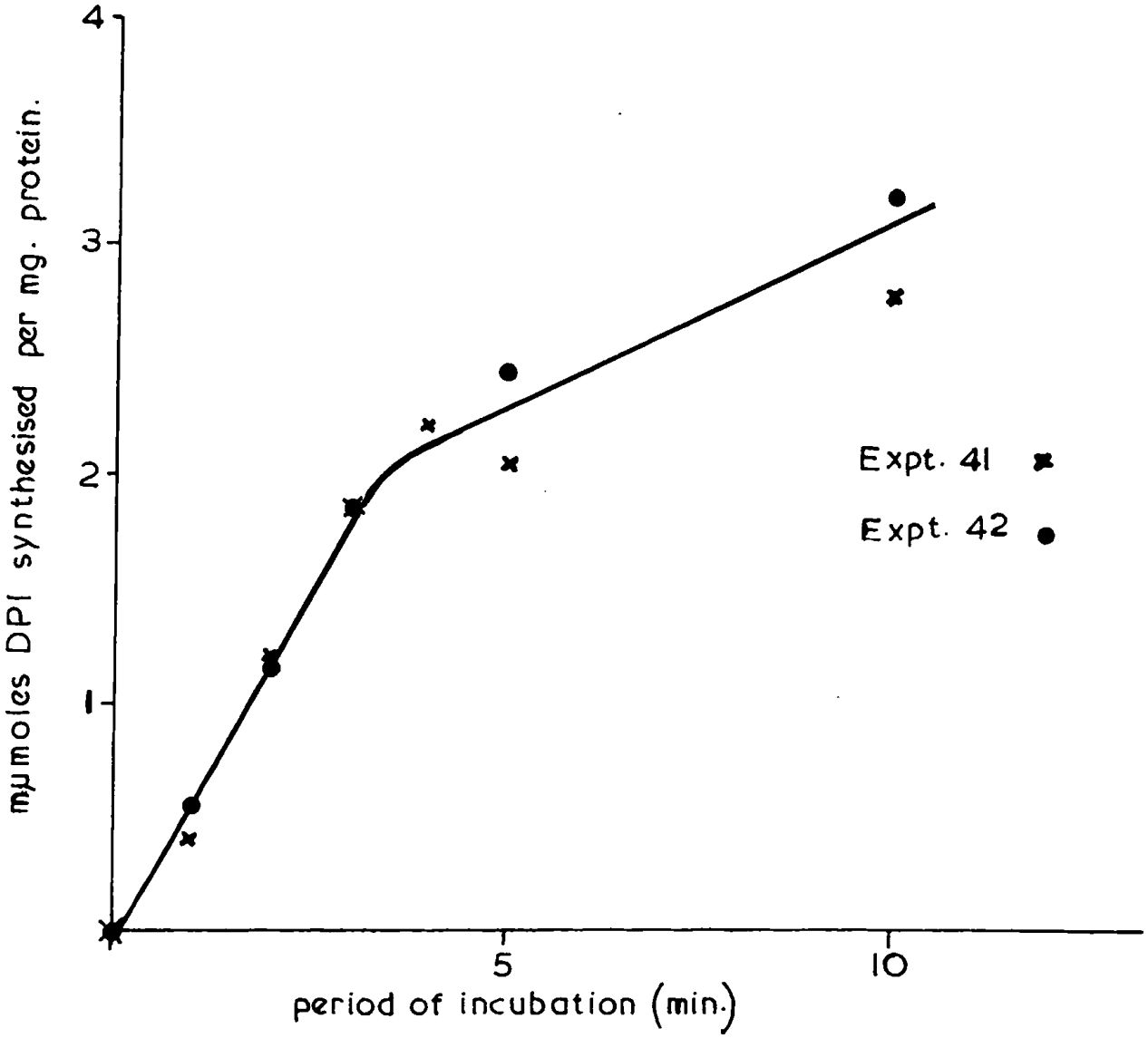


Fig. **34**. Time-course of DPI labelling in homogenate.

20 mM Tris/HCl buffer, pH 7.4; 40 mM $MgCl_2$ (15 mM for mitochondrial fraction): 5 mM ^{32}P-ATP containing 3.27×10^5 counts/100 sec./µmole (the microsomal fraction was also assayed at 1 mM); 5 mM soya-bean PI. Assays were in a total volume of 1 ml., for 2 min. at 30°. The protein concentrations were: homogenate, 3.9 mg.; nuclear, 1.65 mg.; mitochondrial, 1.43 mg.; lysosomal, 1.55 mg.; microsomal, 1.77 mg.; supernatant, 1.77 mg. Extraction and measurement of DPI labelling were by method b).

The results are given in Table 21. They are expressed as "specific activities", these being activities relative to that of the filtered homogenate:

i.e. $\frac{\text{Enzyme activity (or µg. DNA) per mg. protein of fraction}}{\text{Enzyme activity (or µg. DNA) per mg. protein of homogenate}}$.

The total activity of each fraction is also given, as the percentage of the total activity of the filtered homogenate which was recovered in that fraction.

Assay of each fraction at double the protein concentration quoted gave a doubled incorporation of ^{32}P into DPI. Assay of labelling with the homogenate at 7.5 mM PI gave only a 6% higher incorporation than that at 5 mM PI. Assay of the microsomal fraction at 1 mM ATP gave a lower level of DPI labelling than with 5 mM ATP. The reason for this difference from the previous result (Experiment 39) is unknown and requires further investigation.

The homogenate incorporated 0.43 mμmole of phosphate into DPI per min. per mg. protein.

Experiment 44.

A second fractionation was performed with one modification. The material which was filtered out of the homogenate before fractionation was scraped off the nylon cloth, homogenised in sucrose, and treated as a further cell fraction, designated debris (D).

Incubation conditions were as in Experiment 43. Again assays were made at two protein concentrations, the lower being taken for the calculation of the results given in Table In all fractions except the nuclear the incorporation was linear with protein concentration over the range tested. In the nuclear fraction, increasing the protein content from 1.62 to 3.24 mg. in the 1 ml. assay only raised the incorporation by 52%, so the recorded activity for this fraction may be somewhat low. The protein quantities for the experiments quoted were:

Debris, 1.88 mg.; homogenate, 2.31 mg.; nuclear, 1.62 mg.; mitochondrial, 1.85 mg.; lysosomal, 2.11 mg.; supernatant, 0.81 mg.

The results are given in Table 22, in the same form as those in the previous experiment. The homogenate incorporated 1.68 mμmoles of phosphate into DPI per mg. protein per min.

Figure 35 shows a plot of the mean specific activity distributions of DPI labelling and the marker enzymes in the above two experiments. The figures for the debris fraction (D) are those of Experiment 44 alone.

In these fractionations the DPI labelling did not appear to be localised in any single fraction. Neither did its distribution closely parallel that of any of the conventional marker enzymes. However, it did closely parallel the activity of 5'-nucleotidase, an enzyme which appears to be a characteristic constituent of the plasma membrane of the liver cell.

TABLE 21 Subcellular distribution of ATP-dependent DPI labelling (Experiment 43).

	Protein		DPI Labelling		5'-nucleotidase		DNA	
	mg.	% of H	S.A.	% of H	S.A.	% of H	"S.A."	% of H
Homogenate (H)	(2020)	(100)	(1.0)	(100)	(1.0)	(100)	(1.0)	(100)
Nuclear	497	24.7	1.77	45.2	1.67	41.0	3.95	110
Mitochondrial	222	11.0	0.3	3.5	0.18	2.0	0.09	1.0
Lysosomal	139	6.9	0.09	0.7	0.95	6.6	0.08	0.5
Microsomal	222	11.0	1.23	14.1	1.43	15.7	0.05	0.7
Supernatant	656	32.5	0.49	16.7	0	0	0.07	2.0
Recoveries %		86.1		80.2		65.3		114.2

	Succinate dehydrogenase		β-glucuronidase		Glucose-6-phosphatase.		6-phosphogluconate dehydrogenase	
	S.A.	% of H	S.A.	% of H	S.A.	% of H	S.A.	% of H
Homogenate (H)	(1.0)	(100)	(1.0)	(100)	(1.0)	(100)	(1.0)	(100)
Nuclear	1.36	34.4	1.05	25.8	0.94	22.9	0.01	1.6
Mitochondrial	6.0	67.9	2.1	23.1	0.7	7.9	0	0
Lysosomal	0.54	3.9	5.66	39.5	0.13	9.3	0	0.1
Microsomal	0.07	0.7	1.26	13.7	3.7	40.0	0.01	1.04
Supernatant	0.01	0.3	0.16	5.2	0.08	2.5	2.56	84.0
Recoveries %		107.2		107.3		82.6		86.8

TABLE 22. Subcellular distribution of ATP-dependent DPI labelling (Experiment 44).

	Protein		DPI Labelling		5'-nucleotidase		DNA	
	mg.	% of H	S.A.	% of H	S.A.	% of H	"S.A."	% of H
Debris	(150)	(8.1)	(0.82)	(6.7)	(1.64)	(13.8)	(1.88)	(15.3)
Homogenate (H)	(1850)	(100)	(1.0)	(100)	(1.0)	(100)	(1.0)	(100)
Nuclear	278	15.6	3.3	49.6	3.31	51.5	5.54	86.5
Mitochondrial	302	16.3	0.17	2.8	0.39	6.6	0.12	2.0
Lysosomal	169	9.1	1.1	9.1	0.50	4.7	0.12	1.1
Microsomal	267	14.4	0.76	11.1	1.1	16.3	0.08	1.1
Supernatant	610	33.0	0.29	9.5	0.44	14.4	0.23	7.9
Recoveries %		88.4		82.1		93.5		98.6

	Succinate dehydrogenase		β-glucuronidase		Glucose-6-phosphatase		6-phospho-gluconate dehydrogenase	
	S.A.	% of H	S.A.	% of H	S.A.	% of H	S.A.	% of H
Debris	(0.85)	(6.7)	(1.77)	(14.3)	(1.29)	(10.5)	(0.44)	(3.6)
Homogenate (H)	(1.0)	(100)	(1.0)	(100)	(1.0)	(100)	(1.0)	(100)
Nuclear	0.98	14.7	0.98	14.9	0.66	9.9	0.02	3.5
Mitochondrial	3.76	61	0.89	14.5	0.38	6.3	0	0
Lysosomal	0.58	5.3	2.9	26.4	1.95	18.0	0	0
Microsomal	0.004	0.6	1.55	22.2	3.06	44	0.08	1.1
Supernatant	0.002	0.6	0.5	16.4	0	0	3.32	109.4
Recoveries %		82.2		94.4		78.2		114

Fig. 35.

Distribution of marker enzymes and of DPI labelling from γ-^{32}P-ATP in rat liver subcellular fractions.

The fractions are designated:

D: Debris

N: Nuclear

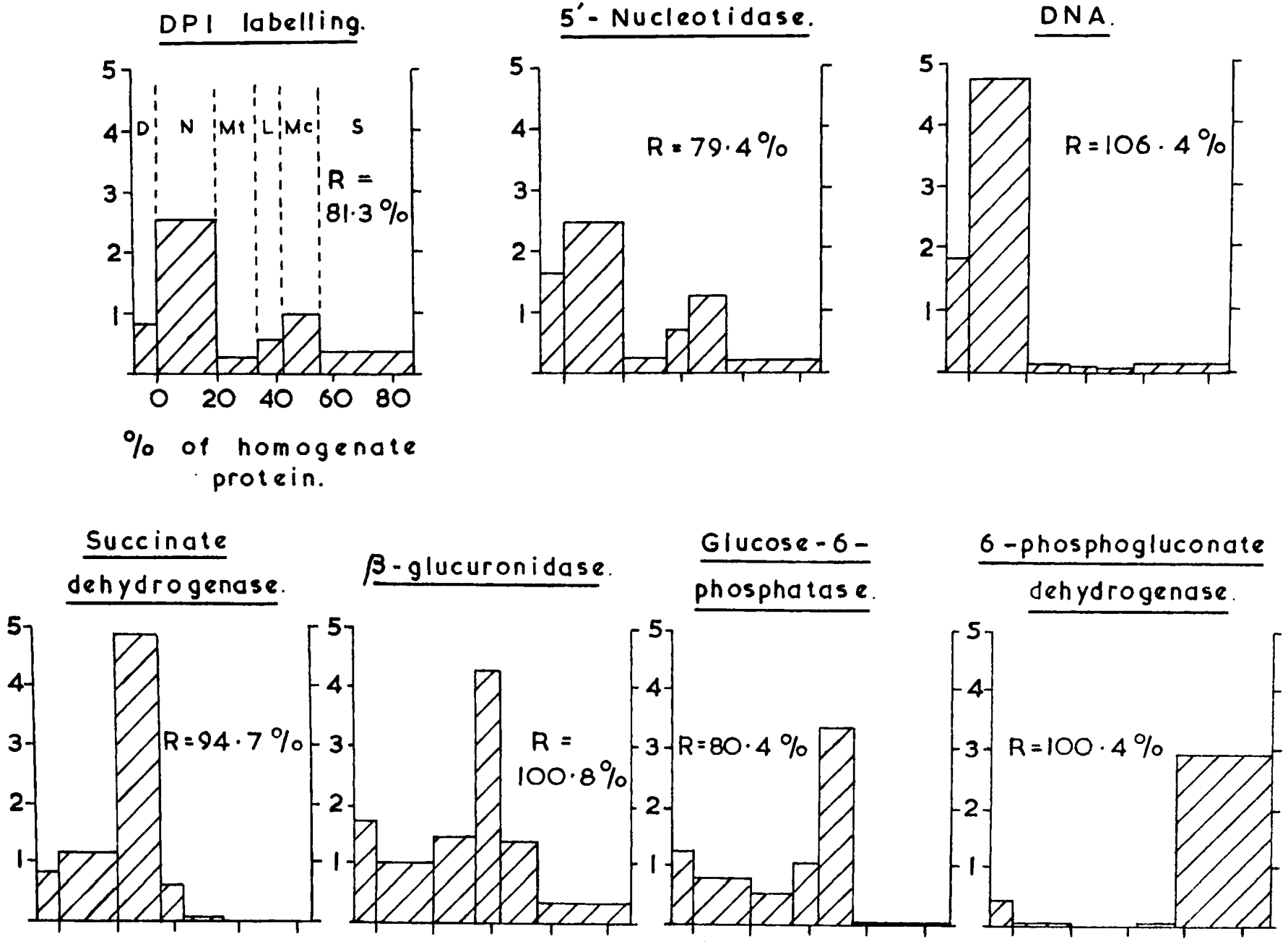
Mt: Mitochondrial

L: Lysosomal

Mc: Microsomal

PFS: Particle-free supernatant

Specific activities relative to that of the homogenate are plotted on the ordinates (see explanation in the text) Protein distribution in the fractions (as percentage of the protein of the filtered homogenate) is shown on the abscissae.


DPI labelling.
D
N
Mt
L
Mc
S
R = 81·3 %
0 20 40 60 80
% of homogenate protein.
5′- Nucleotidase.
R = 79·4 %
DNA.
R = 106·4 %
Succinate dehydrogenase.
R = 94·7 %
β-glucuronidase.
R = 100·8 %
Glucose-6-phosphatase.
R = 80·4 %
6-phosphogluconate dehydrogenase.
R = 100·4 %

DISCUSSION

DISCUSSION

When this work was initiated our knowledge of di- and triphosphoinositide biosynthesis was very scanty. The structures of these lipids had just been unravelled and knowledge of their metabolism was confined to information on their turnover in slices of brain (Brockerhoff and Ballou, 1962 a, b) and *in vivo* in brain (Wagner *et al*. 1961, 1962; Ellis and Hawthorne, 1962). These studies had suggested that the following sequential phosphorylations occurred:

$$PI \xrightarrow{X - P} DPI \xrightarrow{X - P} TPI$$

The occurrence of DPI and TPI outside the nervous system was still a matter of doubt.

The identification of a labelled lipid in liver mitochondria (Garbus *et al*., 1963) as DPI (Galliard and Hawthorne, 1963) resolved this doubt and provided the first method for the study of DPI turnover in a cell-free system.

DPI and TPI synthesis in a brain "mitochondrial" fraction.

The first experiments (1 - 5) described here showed that if the incubation system used by Garbus *et al*. was applied to a crude "mitochondrial" fraction derived from brain, two or three lipids were labelled. Co-chromatography of the deacylated lipids with GPIP and $GPIP_2$, derived from brain DPI and TPI, respectively, identified two of these lipids as DPI and TPI. The third labelled compound, which was eluted in an intermediate

position from a Dowex-1-formate column, was clearly seen in the rabbit brain "mitochondrial" lipids, but not in those from rat brain. Its elution position was close to that of inositol diphosphate, but it was not further characterised.

The crude mitochondrial fraction used in these studies is quite similar to the fraction which Nyman and Whittaker (1963) have shown, by density gradient centrifugation, to contain mitochondria, pinched-off nerve-endings and myelin fragments. Recent studies by Kai and Hawthorne (unpublished) would suggest that the observed labelling of DPI probably occurred mainly in the nerve-endings and that of TPI mainly in the myelin. This suggestion is based on their observations on the distribution of the ATP-linked phosphorylations of PI to DPI and DPI to TPI in these subcellular fractions of rat brain.

The time-course of the labelling in the crude mitochondrial fraction of brain was very similar to that reported for rat liver mitochondria by Garbus et al. i.e. a linear rise for a few minutes followed by a rapid decline. The reason for this behaviour is still unknown, but a similar time-course pattern was observed when ^{32}P-ATP was used as the phosphate donor (Kai and Hawthorne, unpublished). This suggested that the reason is not a fall in the rate of synthesis of ATP. It may be a reflection of the high "phosphoinositidase" activity of both liver and brain (Kemp et al., 1961 a, b; Thompson and Dawson, 1964). Due to the uncertainties involved in the interpretation of data obtained from

any enzyme studies on the whole brain, whether intact, in slices, or homogenised, this work was temporarily shelved. It was hoped that a clearer picture of the enzyme involved in DPI labelling could be obtained from work on liver preparations, these having the advantage that the tissue is predominantly composed of a single cell type. This decision was reinforced by our feeling at the time that DPI turnover might have been in some way connected with mitochondrial energy-linked functions, which had been most thoroughly studied in liver and heart mitochondria.

Extraction procedures.

The extraction method used by Garbus et al. (1963) was used for the early experiments. This method was similar to that of Bligh and Dyer (1959), except that 2M KCl/0.5M potassium phosphate, pH 7.4, and not water, was used for washing the extracted lipids. Garbus et al. had reported that their method gave the same extraction of labelled DPI from rat liver mitochondria as the method of Folch et al. (1957). However, as polyphosphoinositides are frequently extracted from tissues tightly bound to protein, study was made (Experiments 7 and 8) to ascertain whether labelled DPI was being lost due to binding to the protein present in the extraction medium. Dittmer and Dawson (1961) found that the sodium salt of TPI was soluble in water. However, the Ca^{++}/Mg^{++} salt of the Folch inositide fraction was very hydrophobic (Kerr et al., 1963).

This suggested that the use of $CaCl_2$ in the wash solution might ensure the quantitative partition of DPI into the non-polar phase of the extraction system. Experiments 7 and 8 showed that this was not the case. In fact, the reverse was observed. The presence of Ca^{++} caused a loss of more than two-thirds of the DPI. This could be recovered by extraction of the precipitated "protein" with acidified chloroform:methanol. Thus it seems that in the presence of low concentrations of P_i (from the incubation mixture) and Ca^{++}, the DPI was complexed out of the chloroform phase and bound to the interfacial protein. Kerr et al. (1963) have reported that polyphosphoinositides bind approximately 1 mole of P_i and 1 equivalent of Mg^{++} or Ca^{++} per mole of monoesterified phosphate. Furthermore, Eichberg and Dawson (1965) have found that in purified myelin there was a close correlation between the total equivalents of (Ca^{++} + Mg^{++}) and of (TPI + DPI). This, they suggest, was due to the presence of the lipids as complexes with alkaline-earth metals. This was supported by the observation (Dawson and Eichberg, 1965) that the addition of cyclo-hexane-1,2-diaminotetra-acetate, a chelator of alkaline-earth metals, to chloroform:methanol facilitated the extraction of DPI and TPI from brain tissue.

In Experiment 8 it was found that if a solution containing labelled DPI, prepared by the Garbus procedure, was washed with water almost quantitative transfer of the DPI into

the aqueous phase occurred. This suggests that after the first washing with high ionic strength KCl/phosphate the DPI was probably present as the water-soluble potassium salt. This solubility was partially reversed by the presence of $CaCl_2$. The partition of the DPI in the organic phase in the presence of the KCl/phosphate is presumed to have been a result of the insolubility of the water-soluble potassium form in aqueous solutions of high ionic strength. The initial breakage of the bonding between inositides and protein was also presumably a result of the high ionic strength of the medium.

As the procedure of Garbus et al. consistently gave recoveries of DPI as high as any obtained with other procedures, including extraction with acidified chloroform:methanol (discussed by Dawson and Eichberg, 1965), it was retained unchanged throughout the rest of the work.

DPI and mitochondrial ion-accumulation.

The inhibitor studies of Garbus et al. suggested to us that there might be a connection between DPI labelling and mitochondrial ion-accumulation. This resulted in the suggestion, put forward by Hawthorne (1964a) that DPI might act as an ion-carrier across the mitochondrial membrane (see Figure 36). It was suggested that DPI, known to have a strong affinity for divalent cations (Kerr et al., 1963; Folch, 1949), would pick these up from the medium around the mitochondria and in some way

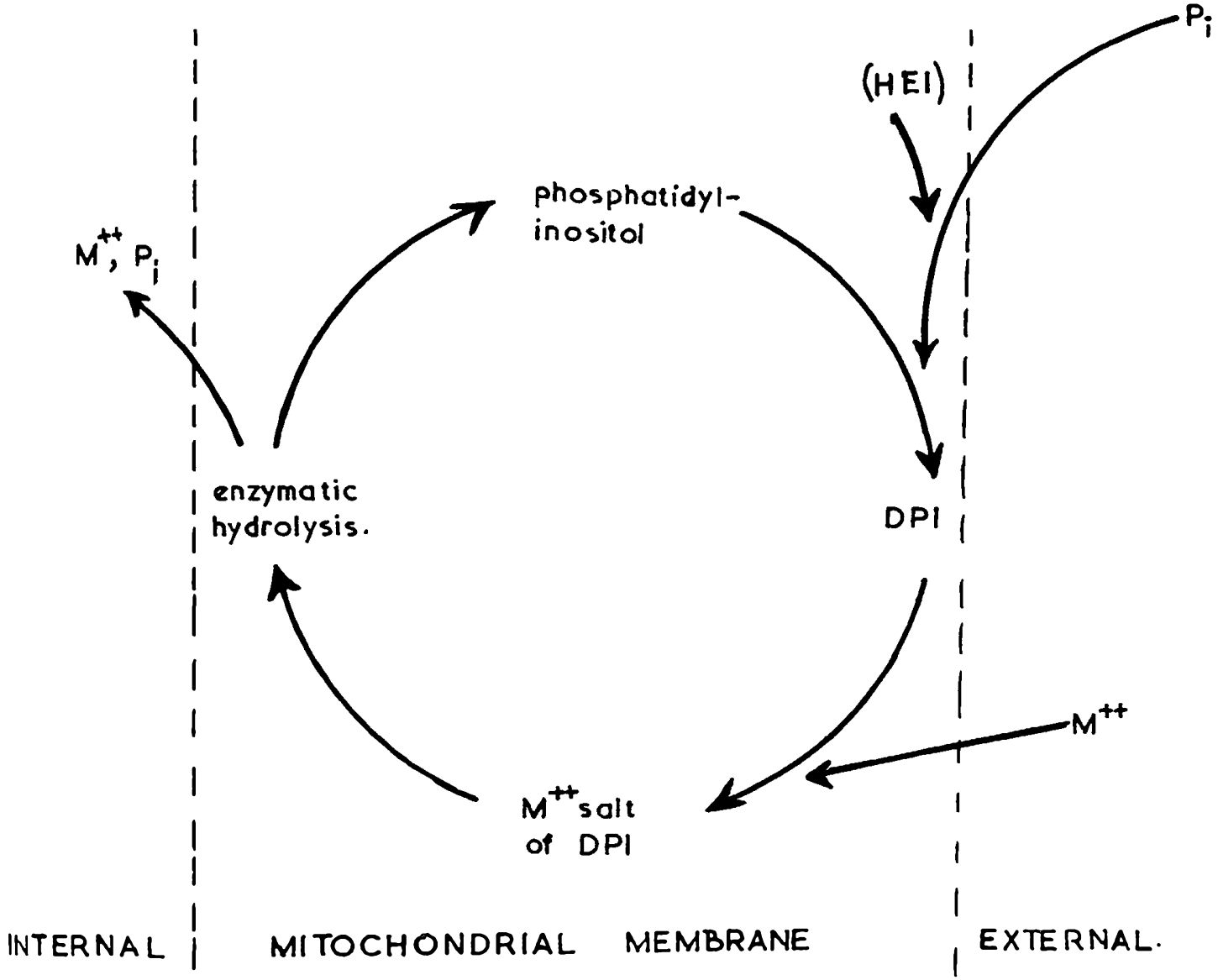


FIG 36. A suggested mechanism for mitochondrial ion accumulation (Hawthorne 1964a)

carry them across the membrane. Enzymic dephosphorylation of the DPI would release Ca^{++} or Mg^{++} phosphate inside the mitochondria, yielding PI. This, in turn, could move to the outside of the membrane, and after rephosphorylation of the PI to DPI the cycle could be repeated. This mechanism of transport bore a close resemblance to the phosphatidic acid pump for Na^{+} transport proposed by the Hokins. However, in their system the transport of phosphate inherent in the mechanism was an unexplained problem, as no phosphate transport was observed. In the mitochondrial system any proposed mechanism would have been inadequate without the inclusion of a specific mechanism for the transport of phosphate as the only major anion.

The weakness of this theory was that it rested upon a single unconfirmed observation that mitochondrial DPI labelling was insensitive to inhibition by oligomycin.

Effects of added adenine nucleotides.

In the early stages of these studies we were forced to resort to indirect methods for testing this hypothesis, as no oligomycin was available. These indirect methods soon cast severe doubts on the correctness of the theory, but were not conclusive.

The effects of added adenine nucleotides were somewhat anomalous (see Table 5). ADP, which was expected to inhibit DPI labelling by competing for the high energy intermediate

postulated to be the energy source for the DPI synthesis, was usually an inhibitor, but occasionally stimulated the system, especially at low concentrations.

ATP, which either had no effect on or stimulated ion-accumulation in most systems deriving their energy from substrate oxidation, was a consistent inhibitor. These results would have been very difficult to explain with the proposed system, but favoured phosphorylation of the lipid from ATP or a derivative of ATP.

The quantity of DPI in liver.

The second major obstacle to this theory was the very low DPI content found both in fresh liver and in liver mitochondria after incubation under the conditions used for the labelling of DPI. For a flux of cations of 0.1 µmole per min. per mg. protein (some systems are capable of supporting transport at a considerably higher rate than this), 0.1 µmole of DPI phosphate should be exchanged per min. per mg. protein. After 5 min. incubations of mitochondria the maximum level of DPI observed was of the order of 0.05 mµmole DPI per mg. protein. This would have to turn over 2,000 times a minute to support the rate of cation transport quoted. However, maximal levels of DPI labelling were observed, not after less than one-tenth of a second, but after 5 min.

These results suggested that the Hawthorne theory was

incorrect, but the final decision had to await the arrival of oligomycin.

Morgan (1964) quoted a figure for the DPI content of pig kidney of 1.84 parts per million, based on dioctanoyl-glycerylphosphorylinositol phosphate. This represents a concentration of approximately 2 mμmoles DPI per g. of tissue. This figure is considerably higher than that found in liver by Galliard and Hawthorne (1963); rabbit liver contained approximately 33 μg. DPI inositol per kilogram of tissues, which corresponds to about 0.2 mμmole DPI per gram of tissue.

Polyphosphoinositides and mitochondrial shrinkage.

Vignais, P.V. et al. (1964) reported that if "aged" mitochondria were incubated with PI and either ^{32}P-ATP or $^{32}P_i$ no labelling of the mitochondrial inositides occurred. The conditions for this experiment were not given.

Figure 13 shows the interrelationships of ATP and high-energy intermediates (HEI's), both phosphorylated and non-phosphorylated, in the energy-transfer pathway involved in mitochondrial shrinkage. It seems that oligomycin blocks the interconversion of the phosphorylated and the non-phosphorylated HEI's (Huijing and Slater, 1961). When ATP is the energy source both ion-accumulation and shrinkage are blocked by oligomycin. Thus it seems that the non-phosphorylated HEI is the energy source for these processes. It was therefore expected that if

the phosphorylation of PI was involved in shrinkage the phosphate group incorporated would not be derived from the ATP which was driving the process, but from some other phosphorylated compound which would be synthesised with the energy derived from the breakdown of the non-phosphorylated HEI. To test this, we allowed "aged" mitochondria to swell in the presence of PI. Shrinkage was then initiated either with labelled ATP or with unlabelled ATP and labelled P_i. In the former no labelling was observed. In the latter two labelled lipids were produced, the deacylation products of which eluted from Dowex-1 columns in the positions expected for GPIP and $GPIP_2$, with most of the radioactivity in the latter. This labelling did not occur either in the absence of ATP or in the presence of oligomycin, when shrinkage was inhibited. These results were in complete accord with the hypothesis. Unfortunately several attempts to repeat them were unsuccessful, leaving the problem unresolved. Several lines of further study present themselves. The concentration of PI which was required for optimal shrinkage of the mitochondria was very low (10^{-5}M). The shrinkage did not respond to PI unless this was added before the initiation of swelling, which suggested that PI was being bound by the particles during the swelling phase of the cycle. A study of this binding, using labelled PI, and a comparison of the quantity bound with the quantity of DPI in mitochondria might

be useful. In the studies of $^{32}P_i$ incorporation reported here the swelling agent used was 5 mM P_i, which naturally diluted the $^{32}P_i$ added with the ATP. More clear-cut results on $^{32}P_i$ incorporation might be obtained if further studies, using oleate, thyroxine or Ca^{++} as the swelling agent, were attempted. Certainly, this is a problem which deserves further study.

Effects of inhibitors of mitochondrial function.

Garbus et al. found that DPI labelling was inhibited by all the inhibitors which they tested, with the exception of oligomycin. The effective inhibitors were azide, antimycin A, dicoumarol, dinitrophenol, cyanide, arsenate and gramicidin S. Galliard and Hawthorne (1963) confirmed the effects of dinitrophenol and cyanide and also found a decrease in labelling under anaerobic conditions.

Effects of oligomycin.

It was found in the present work that oligomycin, far from being ineffective, was a very potent inhibitor of $^{32}P_i$-dependent DPI labelling. The inhibitions were unchanged by the tonicity of the incubation medium and were observed whether the total lipid labelling or that of GPIP was measured. Maximal inhibition occurred at above 2.5 µg. oligomycin/ml. and half-maximal at 0.7 µg. oligomycin/ml. This inhibition was also recently observed by Hajra et al. (1965), concentrations of

0.83 and 1.66 µg. oligomycin/ml. giving inhibitions of DPI labelling of 64% and 76%, respectively.

As the suggested mechanism for transport of ions into mitochondria by DPI was entirely dependent on the DPI synthesis being an oligomycin-insensitive process, the finding of oligomycin-sensitivity made the hypothesis completely untenable. It also lent support to the previous observations which had suggested that ATP, and not a high-energy intermediate, was the agent which phosphorylated PI. This fell into line with the finding that in erythrocyte ghosts (Hokin and Hokin, 1964c) and brain microsomes (Colodzin and Kennedy, 1964; M.Kai, unpublished) DPI was formed by the phosphorylation of PI by ATP.

However, some doubts still remain whether the effect of oligomycin is simply due to an inhibition of energy transfer in oxidative phosphorylation. In the present work the situation seemed fairly clear. In the presence of 5 µg./ml. of oligomycin the inhibitions of DPI labelling and of labelling of acid-labile water-soluble phosphates, presumed to be primarily ATP, were similar. It was concluded that as ATP synthesis was inhibited the labelling of DPI, derived from the ATP, also fell.

The doubts stem from the results of several other groups of workers. Hajra et al. have found that under conditions which caused an 80% inhibition of DPI labelling (the oligomycin concentration was not stated) ATP labelling fell by only 20%.

Peter and Boyer (1963) found that much higher concentrations of oligomycin than those used in the studies of DPI labelling did not inhibit the turnover of endogenous mitochondrial ATP. Garbus et al. found neither inhibition of DPI labelling nor of ATP labelling at 12 µg./ml. oligomycin.

In direct contradiction of these results are our own results and also those of Slater et al. (1964) and Heldt et al. (1964), who both found oligomycin to inhibit the labelling of endogenous mitochondrial nucleotides, although at higher concentrations than those used in the studies of DPI labelling.

It now seems established, by our work and that of Hajra et al., that $^{32}P_i$-dependent DPI labelling in liver mitochondria is an oligomycin-sensitive process. Hajra et al. have also shown, as was expected, that when ^{32}P-ATP is the phosphate donor the process is oligomycin-insensitive.

Mitochondria and $^{32}P_i$-dependent DPI labelling.

As the evidence so far discussed pointed to either ATP or a derivative of it being the phosphate donor for the DPI synthesis it was thought that the synthesis from $^{32}P_i$ would be largely confined to the fractions containing mitochondria. Figure 17 shows the results of an experiment in which mitochondria were prepared and washed several times to free them of contaminants.

The succinate:INT reductase and DPI-synthesising capacities of the fractions rose in a parallel way, as the endoplasmic reticulum fragments (glucose-6-phosphatase) were removed. This suggested a close dependence of the labelling on the presence of mitochondria. This was confirmed in the subsequent experiment (Table 7 , Figure 18), in which it was found that the subcellular distribution of the DPI labelling closely parallelled that of mitochondria (succinate:INT reductase). Thus it was concluded that the mitochondria were essential for the $^{32}P_i$-dependent labelling of DPI, and that ATP was probably the phosphate donor.

Effects of atractyloside.

No results have been presented here on the effects of this inhibitor, but a comment on some of the results of Hajra et al. seems relevant. These authors found that under certain conditions atractyloside would inhibit the $^{32}P_i$-dependent DPI labelling completely, with only a 20% fall in ATP labelling.

Atractyloside had been shown to inhibit a "translocase" responsible for ADP transport into the inner compartment of the mitochondrion (Heldt et al. 1965). This enzyme, functionally similar to the "mesomerase" of Brierley and Green (1965), is said to control the interchange of adenine nucleotides between the freely permeable and the adenine nucleotide-impermeable compartments of the mitochondrion. This interchange may be a

transport of nucleotides (translocase model) or an enzyme-linked transphosphorylation (mesomerase model) between the internal and external compartments of nucleotide. However, the net effect of either system is a control on the entry and exit of adenine nucleotides comprising the tightly-bound bound nucleotide pool of the mitochondrion.

In the system of Hajra et al. it was proposed that although ATP turnover within the mitochondrion was hardly affected by atractyloside, blockage of the exit of labelled ATP from the mitochondrion prevented DPI labelling. Thus, the DPI synthesising system was concluded to be on the exterior of the mitochondrial membrane. This conclusion requires some revision in the light of the results presented here and of those of Klingenberg and Pfaff (1965). The compartment of the mitochondrion rendered adenine nucleotide-impermeable by atractyloside represents only a part of the mitochondrial volume, but atractyloside does control the transport of AMP, ADP and ATP into and out of this compartment (Klingenberg and Pfaff, 1965). Thus Hajra's results must be reinterpreted to mean that DPI labelling occurs outside the atractyloside-controlled compartment of the mitochondrion. They do not exclude the possibility of the reaction still occurring within the mitochondrion. However, as will be discussed later, our results suggest that at least 97% of the DPI labelling in liver,

and probably all of it, occurs at extramitochondrial sites. Thus, Hajra's interpretation would be further modified to one in which DPI labelling is localised, not on the exterior of the mitochondrial membranes, but on other membranes contaminating the mitochondria.

Effect of PI on the P_i-linked phosphorylation.

Morgan (1964), following the work of Garbus at Wisconsin, found that a preparation of soya-bean PI (about 50% pure) or of the Folch inositide fraction, stimulated the labelling of DPI in rat liver mitochondria, using P_i as the phosphate source. I was unable to repeat this observation, using a preparation of PI (70% pure) from brain. However, as will be discussed later, the addition of PI to the isolated cell fractions synthesising DPI with ATP as the phosphate donor showed very clearly the requirement for optimum synthesis.

The reason for this discrepancy is unknown. However, if the preparation of PI used contained as much as 1% of free fatty acid, possibly released by a slow breakdown during storage, this would have been sufficient to uncouple oxidative phosphorylation, inhibit ATP synthesis and thus decrease DPI labelling.

Characteristics of the system labelling DPI from ^{32}P-ATP.

Table 8 gives the results of the first experiment in which the phosphorylation of PI to DPI, using terminally

labelled ATP as a phosphate donor, was demonstrated in the liver system. At that time the only other systems in which this reaction had been reported were the erythrocyte ghost (Hokin and Hokin, 1964 c) and a brain microsomal fraction (Colodzin and Kennedy, 1964). The main points observed in this experiment were that PI, the presumed substrate for the reaction, stimulated the incorporation, and that ADP, one of the presumed products, inhibited it. In the absence of $MgCl_2$ the reaction was not decreased, but this seems to have been an anomalous result, as never after this was the system found to work in the absence of both Mg^{++} and Mn^{++}. Initially studies were made of the conditions for the ATP-linked phosphorylation of PI with a liver mitochondrial fraction as this had been the fraction used in the previous studies on the incorporation of $^{32}P_i$.

Effects of unlabelled nucleotide triphosphates on DPI labelling.

The reaction was inhibited in this fraction by the addition of unlabelled CTP, UTP or GTP, in concentrations slightly less than that of labelled ATP. The observed incorporation was never inhibited by more than 40%. If any of these nucleotides had been the sole phosphate donor for the reaction then nucleotide would have been expected to inhibit the labelling by considerably more than the observed amount. Three possibilities still exist that would explain these nucleotide effects. ATP may be the sole phosphate donor

and the total or specific activity of the ATP may be changed by transphosphorylation between ATP and the other nucleoside 5'-triphosphates. The other nucleotides may be competitive inhibitors of ATP incorporation, though not being utilised themselves. Finally, they may be alternative phosphate donors for the reaction and they are not competing, but substituting for ATP and synthesising unlabelled DPI. This uncertainty will be resolved when studies are made with labelled PI and unlabelled phosphate donors.

Effects of freezing and thawing.

From Table 9 it can be seen that even brief freezing of the mitochondrial fraction caused a reduction in its capacity to label DPI. Table 10 shows the results of freezing the same preparation for 6 days, after which time only 28% of the original activity was retained. However, the fraction of the activity that was still apparent showed the same response to the addition of PI as had the fresh material. No further studies have been made of the effects of storage on the enzyme, but the results of Experiment 40.indicate that the activity of a nuclear fraction which had been frozen for a week was still high. Unfortunately no figures were obtained of the activity of this preparation when fresh, but these results indicate that a further study of the effects of storage on the enzyme should be made. If fractions could be used routinely after storage much time would

of course be saved that is now spent in the preparation of fresh enzyme for each experiment.

The identity of the labelled phospholipid.

The methods used for identification of the labelled phospholipid (Experiments 29 and 30) are based on those used by Ellis et al. (1963) for the characterisation of the components of the brain phosphoinositides. These methods were also used by Galliard and Hawthorne (1963), in their identification of the labelled phospholipid produced when liver mitochondria were incubated with $^{32}P_i$. Little comment is required on the data in this section, as a summary of the results obtained in Experiments 29 and 30 is given after Experiment 30. However, a summary of the evidence accumulated in other laboratories, mainly using $^{32}P_i$ as the phosphate source, seems appropriate.

Morgan (1964), found that the deacylation product of the labelled lipid co-chromatographed with GPIP derived from the brain inositide fraction on Dowex-1-formate, when elution was with an ammonium formate gradient. The compound also corresponded in its mobilities on paper chromatography and ionophoresis with GPIP. On acid hydrolysis a compound similar to inositol diphosphate was released. Hajra et al. also found that the labelled mitochondrial lipid and its breakdown products were indistinguishable from the brain DPI and its degradation

products. It is therefore concluded, on the basis of the accumulated evidence, that the labelled material which has been studied in at least three laboratories is DPI. However, further structural and metabolic studies are required to complete the picture. The liver system has not yet been shown to synthesise DPI from labelled PI and an unlabelled phosphate donor, a reaction which Colodzin and Kennedy have shown in their brain microsomal system. Also, it is not known with any certainty that the labelled lipid has the inositol-1,4-diphosphate structure which has been shown for the brain DPI. Further studies of the inositol diphosphate released from GPIP by periodate and phenylhydrazine treatment could be used to resolve this uncertainty.

Distribution of labelling in the DPI molecule.

Galliard and Hawthorne (1963) reported that in the DPI which was synthesised in liver mitochondria the ^{32}P was almost confined to the monoesterified phosphate group. This has been confirmed, and in Figure 20 b it can be seen that the release of $^{32}P_i$ from the liver lipid by phosphomonoesterase proceeded at the same rate as that from labelled brain DPI. This predominance of labelling in the monoesterified phosphate group has also been reported in DPI labelled in brain slices (Brockerhoff and Ballou, 1962 a) and in both the DPI and the TPI synthesised when erythrocyte ghosts are incubated with ^{32}P-ATP.

The ATP-dependent labelling of DPI in liver subcellular fractions.

In Experiment 34 preliminary results were obtained which indicated that if ATP was used as the phosphate donor the labelling of DPI was by no means confined to the mitochondrial fraction. Most of the activity appeared in the nuclear fraction. The remainder of the results presented here are those which were obtained in a study, first of the conditions for the labelling of DPI from ATP and PI in subcellular fractions from liver, and secondly of the distribution of the activity in these fractions. It was thought that knowledge of the intracellular site of the enzyme activity might give some hint of its functional significance.

Requirement for divalent cations.

As with many phosphokinases (Dixon and Webb, 1958) the enzyme showed a requirement for Mg^{++} ions, these being partially replaceable by Mn^{++}, Ca^{++} being ineffective (see Figure 25). The only other ion so far tested is Fe^{+++}, chosen as a representative trivalent metal. It activated slightly at a low concentration (Figure 25). The optimum concentration of $MgCl_2$ was 15 mM for the mitochondrial fraction and 20 mM or above for each of the other separated fractions. All of the separated particulate fractions (except the microsomal, for which no assays were made) and the homogenate showed a Mn^{++} optimum at 1 mM. In each fraction the maximum activity in the presence of

Mn^{++} was only about half of that obtained in the presence of optimum Mg^{++}. In the supernatant fraction there appeared to be a different response to Mn^{++} ions, with activity still suboptimal at 2.5 mM. However, the appearance of DPI-synthesising activity in this fraction was a rather erratic phenomenon and in two attempts to clarify this Mn^{++} effect no activity at all was detected in the presence or absence of Mn^{++}. This observation may mean that the enzyme being studied is normally bound to a membrane and that a proportion of it can be solubilised during the fractionation procedure. The variation in the amount found in the soluble fraction may be a result of slight variations in the handling techniques used in different experiments.

Requirement for ATP.

The ATP-dependence of the phosphorylation in the subcellular fractions is shown by Figures 23, 26 and 32. The only fraction in which near-optimum synthesis did not appear to be occurring at about 5 mM ATP was the microsomal. When the ATP-dependence of a microsomal fraction was re-tested on another microsomal preparation a sharp optimum was observed at 1 mM. However, in a later experiment (43) the activity at 1 mM ATP was lower than that at 5 mM. The reason for this variation is entirely unexplained and requires further investigation. The effects of the addition of other nucleoside triphosphates to

assays with the mitochondrial fraction has already been discussed. Assessment of the activity of these as alternative phosphate donors is planned.

Requirement for PI.

The requirement for PI as a substrate for DPI synthesis has been shown in erythrocyte ghosts (Hokin and Hokin, 1964 c), a brain microsomal fraction (Colodzin and Kennedy, 1964), in other subcellular fractions from brain (Kai and Hawthorne, unpublished) and in rat-liver mitochondria, Morgan (1964). In the present work no stimulation could be obtained when $^{32}P_i$ was used as a phosphate donor, as discussed earlier. When ATP was the phosphate donor a clear requirement for added lipid could be shown in all cell fractions. However, the degree of stimulation was very variable, particularly in the mitochondrial fraction. This is well illustrated in Table 10 in which two fresh preparations of enzyme are quoted. One was stimulated by 170% in the presence of 0.6 mM PI, whereas the other only showed an increase in activity of 17% with the same addition. Even at optimum PI concentration the activity of this fraction was sometimes barely more than double that in its absence.

The nuclear, lysosomal and microsomal fractions always showed a marked dependence on added lipid, as did the supernatant when it was active at all. Stimulations of up to 30-fold in the activity of the nuclear fraction have been observed (Figure 31).

The net result of this was that as optimal conditions for the system were approached the proportion of the activity in the mitochondrial fraction declined and that in other fractions, particularly the nuclear and microsomal, increased sharply (compare Tables 19, 20, 21 and 22).

The earlier experiments described here were made with brain PI and the later ones with PI from soya-bean "Inosithin". No direct comparison of these lipids as substrates has yet been made, but there appears to be no clear difference in their behaviour. With every preparation from both sources the optimum PI concentrations for this reaction were 5 mM and above. Above 5 mM there is still a slight rise in activity (see Figure 33 and Experiment 43). This, though, is very low compared with the very steep rise found at lower concentrations. In the interests of economy, the preparation of PI in large quantities being a rather time-consuming and expensive business, 5 mM PI, rather than a concentration well on to the plateau of the concentration curve, was used as the standard concentration for assays. In several experiments, including some not included here, the increase in activity observed with a rise in PI concentration from 5 mM to 7.5 mM or higher, has always been less than 15% of the activity at 5 mM.

The effect of the addition of other phospholipids to the system has only so far been investigated with the mitochondrial

fraction (Table 18). Three lipids were tested, egg phosphatidylcholine, phosphatidic acid derived from this and brain phosphatidylethanolamine (about 80% pure). All three inhibited the system to some extent.

The PI optimum for this system is higher than for other systems catalysing the same reaction. In the erythrocyte ghost it is only about 0.5 mM (Hokin and Hokin, 1964 c) and in brain 1 - 2 mM (Kai and Hawthorne, unpublished). In liver the concentration of PI is about 3 - 4 mM (based on a content of approximately 1.2 mg. total phospholipid P per gram of tissue, of which about 8% is PI (Table 2)). As most of the lipid is concentrated in the membranes, this can only give a general idea of the amount of substrate available to the enzyme <u>in vivo</u>. It may, though, be significant that the proportions of the total lipid as PI in these systems rise in the order erythrocyte ghost < brain < liver, as do the requirements of the DPI-synthesising system.

<u>Time-course of labelling</u>.

This is shown in Figure 34. After an initial linear phase the rate of increase in the activity of DPI decreased sharply. Extension of the period of incubation beyond the 10 min. shown in the Figure showed that there was very little change in the subsequent 20 min. There are several possible explanations for this phenomenon. The DPI synthesised may be remaining attached

to an enzyme site, preventing the access of fresh substrate. A loss of an intermediate or a deactivation of the synthetic enzyme may be occurring. Alternatively, there may be a continuous cycle of phosphorylation and dephosphorylation of DPI occurring, and the attainment of a steady-state activity may simply reflect the DPI monoester phosphate group coming into isotopic equilibrium with the ATP in the system. This problem can be resolved by the addition of unlabelled ATP to the system after a constant activity has been attained. If the latter mechanism operates this should cause, in 5 to 10 min., a fall in the activity of the DPI in the system. In one experiment not included here, preliminary evidence has been obtained which suggested that this may indeed be the situation, but further studies are still required on this problem. Such an explanation requires the existence in liver of a DPI phosphomonoesterase, an enzyme only so far found in brain. The liver inositidase preparations of Kemp et al. contained no inositide phosphomonoesterase activity, but this may have reflected selective removal during the purification procedure. This partially purified diesterase was mainly localised in the supernatant fraction. If indeed a cyclic phosphorylation and dephosphorylation is occurring and accounting for the short linear phase of the time-course, it is dependent on the presence of an inositide phosphomonoesterase, or of phosphoinositidase

activity of an unspecific phosphatase. Studies of the time-course in different cell fractions might indicate the location of this catabolic enzyme.

Subcellular distribution.

A subcellular distribution study of the DPI-synthesising activity, with 2 min. incubations and using the near-optimal conditions established above, gave the results in Tables 21 and 22 and Figure 35. A set of marker enzymes was assayed on each set of fractions, as was DNA as a marker for nuclei. From Figure 35, it can be seen that reasonable separation of the subcellular components was achieved. The distribution of 5'-nucleotidase was clearly different from that of any other marker enzyme; activity was mainly in the nuclear and microsomal fractions, as reported by El-Aaser and Reid (1965). These authors (Reid et al., 1964; El-Aaser and Reid, 1965) find that in the nuclear fraction the 5'-nucleotidase activity is not a component of the nuclei and in the microsomal fraction it does not occur in the fragments of endoplasmic reticulum. They have concluded that the 5'-nucleotidase activity of liver cell fractions reflects the presence of the fragmented plasma membrane. This is supported by the finding that isolated liver plasma membranes contain high levels of this enzyme (Emmelot et al., 1964) and that histochemically 5'-nucleotidase is localised around the boundaries of the liver cell (Novikoff et al., 1962),

particularly in the microvilli bordering on the bile canaliculi.

The distribution of DPI-synthesising activity closely parallelled that of 5'-nucleotidase and this is interpreted as indicating the localisation of this lipid synthesis in the plasma membrane. If further work confirms this hypothesis this will be the first description of a biosynthetic enzyme in the plasma membrane of the liver cell.

Inositide synthesis and the plasma membrane.

A number of interesting further lines of attack present themselves, based on the theory that this system is localised in the plasma membrane.

Naturally the first point to be resolved is whether this is a correct interpretation of the results obtained so far and the ways of attacking this problem are discussed in the following section. Before this, a review of the present state of knowledge on the distribution and biosynthesis of polyphosphoinositides, seen in the light of the plasma membrane hypothesis, will be made.

The first and most obvious point is that DPI and TPI are synthesised in at least one other plasma membrane system, the erythrocyte ghost. This shows that whether or not these lipids are primarily localised in plasma membranes, they are at least present in them. Secondly, there appears to be a close correlation in the nervous system between the presence of myelin

and the presence of the higher inositides. This relationship was to some extent discussed in the Introduction. Since the time of writing new data have been published (Eichberg and Dawson, 1965). In purified myelin, which by electron microscopy appeared to consist of a homogenous preparation, the proportion of the total phospholipid in the form of DPI and TPI was 2 - 3 times that of the whole brain. The formation of myelin by the enfolding of the nerve axon by the plasma membrane of the Schwann cell is now a well-documented phenomenon. Thus, in brain the higher inositides are very closely associated with the most concentrated accumulation of plasma membrane material in the body. In the experiments of Kai and Hawthorne, however, it has not been found that the phosphorylation of PI to DPI and of DPI to TPI in brain occurs at the same site in the cell. That of PI to DPI is not concentrated in the myelin-containing fractions, but is distributed mainly through the nuclear, nerve-ending and microsomal fractions. This distribution is similar to that observed by Hosie (1965) for Na^+/K^+-activated ATPase in brain cell fractions prepared by the same technique. Thus it may be suggested that DPI is a component of the plasma membranes and synaptic membranes of the neurone. On the other hand, preliminary studies, also by Kai and Hawthorne, indicate that the phosphorylation of DPI to TPI is about three times more active in a myelin fraction than in the other fractions,

and it may be postulated that TPI is characteristically present in "high" concentrations in myelin. This is certainly supported by some of the observations quoted in the Introduction.

Further studies.

Resolution of the site of DPI labelling will be attacked initially by the use of centrifugal subfractionation of the nuclear and microsomal fractions which contain the bulk of the activity. Initial studies in this direction, using the density-gradient centrifugation systems of Emmelot et al. (1964) and of Dallner (1963) have already been made, with limited success. All that can be said at this stage is that if these two fractions are subfractionated by density gradient centrifugation they can each be separated into two further fractions and 5'-nucleotidase and the DPI-synthesising activity fractionate in parallel. However, as yet the fractionations have been very poor and few reliable results have been obtained. One of the problems, particularly with enzyme preparations derived from the nuclear fraction, is that the protein concentration becomes critical. As the enzyme is partially purified its activity becomes very dependent on there being only a low concentration of protein in the incubation medium.

Purification of plasma membranes by the procedure of Neville (1960), as modified by Emmelot et al. (1964) will be tested as a method of preparing enzyme fractions high in synthetic

activity. However, in view of the very variable activity of the supernatant fraction from liver fractionations, possibly caused by removal of the enzyme from its membrane site, some concern is felt about the rather severely hypotonic conditions used in this preparation.

The production of labelled DPI also occurs in the kidney (Garbus et al., 1963), a tissue with a very large plasma membrane surface area, mainly in the microvillous structure bordering the secretory tubules. A method for the preparation of a membrane fraction very rich in Na^+/K^+-stimulated ATPase has already been described for this tissue (Wheeler and Whittam, 1964) and the application of this technique to kidney and assay of DPI in that tissue may be attempted. Also, an attempt may be made to apply the procedure to the liver. TPI and DPI can be labelled in kidney slices in the presence of $^{32}P_i$ (Santiago-Calvo et al., 1964) and a study of the subcellular distribution of the inositides after this type of incubation should also be helpful.

Possible methods for the study of the distribution of labelled DPI in the membranes by micro-autoradiography, after incubation, are being considered.

Another angle from which to approach a solution to this problem may be by gathering data on the inositide contents and inositide-synthesising activities of a variety of tissues

containing a profusion of plasma membranes modified for some specific function. Possible candidates would be the retina, with the lamellar structure of the rods, the salt glands of birds, and electric organs.

It is hoped that this sort of study may give some sort of clue to the significance of these lipids which are present in such small quantities and which are metabolically so active, especially in the brain where they form the main metabolically active fraction of the lipids of myelin (Eichberg and Dawson, 1965). The studies of cation content and binding with purified Folch fractions and of the equivalence between the divalent cation content and the polyphosphoinositide content of purified myelin are suggestive of some involvement with divalent cations. Ca^{++} appears to be somehow concerned with membrane permeability, and is also involved in the control of inositide turnover in brain slices. These observations point a possible way, but are very small lights in the darkness. It may be that continuation of the present work may add to the light. We hope so, but feel that we have a very long way to go before we see things clearly.

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