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Review

The inositol phospholipids

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"The dried crystals melted with decomposition at about 215°. The substance is probably an organic acid but we have not yet had time to examine it thoroughly." (1) In this modest way inositol seems to have made its first appearance as a lipid constituent. Later in 1930, Anderson and Roberts (2) established the identity of their crystals, obtained from a phospholipid present in avian tubercle bacilli, as *myo*-inositol. The occurrence of inositol in a plant lipid, soybean oil, was first reported in 1939 by Klenk and Sakai (3). Three years later Folch and Woolley (4) described an inositol lipid in brain. The subsequent work of Folch, who coined the name phosphoinositide, stimulated the much wider recent interest in such lipids. Their chemistry has been reviewed by Folch (5) and by Folch and LeBaron (6).

NOMENCLATURE

Several systems for the classification and naming of phosphatides have been put forward (7 to 10). The inositol phosphatides prove difficult to accommodate in some of these. Folch and Sperry (7), for instance, divide the phosphatides into three groups: (1) phosphosphingosides, (2) phosphoglycerides, and (3) phosphoinositides. Most of the inositol phosphatides so far described also contain glycerol and could therefore appear in two of these groups. It is perhaps simpler to make only two subgroups: (*a*) glycerophosphatides (containing glycerol but no sphingosine), and (*b*) phosphosphingolipids (containing sphingosine but no glycerol). Derivatives of sphingosine and the closely related phytosphingosine (11) would be classed together. Inositol lipids also containing glycerol would form a subdivision of group (*a*) called "phosphoinositides," as Folch suggested. The inositol lipid described by Carter *et al.* (11) would belong to group (*b*) since it contains phytosphingosine but no glycerol. If this compound is derived from a more complex lipid containing a "phosphatidyl" residue, the latter's classification would be more difficult according to any scheme.

Like the name phosphoinositide, the names monophosphoinositide and diphosphoinositide for specific compounds are also in general use, but have less to recommend them. The substitution of the name phosphatidylinositol for monophosphoinositide has several advantages. First, it brings the compound into line with the better-known phosphatidylcholine, phosphatidylethanolamine, and phosphatidylserine, which have related structures. Phosphatidyl then has a specific chemical meaning, derived from phosphatidic acid (di-acyl glycerophosphoric acid), according to Channon and Chibnall (12). Second, use of the name phosphatidylinositol allows more complex lipids, such as Vilkas and Lederer (13) described (phosphatidylinosito-di-D mannoside), to be given a related name indicating structure. Third, it avoids confusion of phosphatidylinositol with other plant monophosphoinositides, which also release inositol monophosphate on hydrolysis but are actually quite different. The name glyceroinositophosphatidic acid for phosphatidylinositol (14) could also lead to confusion.

The name lipositol, used by Woolley (15) for an inositol lipid from soya beans, is now rarely used. Its reintroduction would be undesirable since the product was probably a mixture, and the term could be applied to any lipid containing inositol. As Folch and LeBaron point out (6), insufficient structural evidence is available for the naming of other phosphoinositides. When such evidence is produced, it may be possible to replace diphosphoinositide by a more systematic name.

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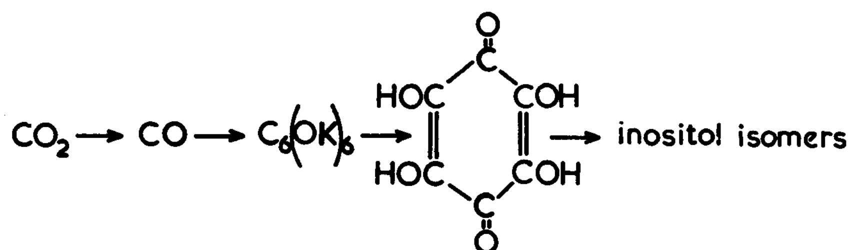
CHEMISTRY OF INOSITOL

Since this subject has been well reviewed by Angyal (16) and Angyal and Anderson (17), only a few points will be mentioned here. There are eight possible isomers of hexahydroxycyclohexane, one of which is racemic, but so far only one has been reported as a lipid constituent. Now called *myo*-inositol (I*a* and I*b*), it was known for many years as *meso- or i*-inositol.

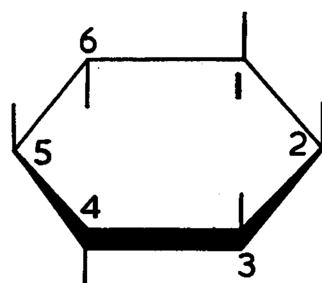
For polyhydroxy compounds, the inositols are surprisingly stable. *Myo*-inositol has been isolated from a rat carcass which had been digested with 6N sulfuric acid overnight in an autoclave at 17 pounds pressure (18). After neutralization of the digest, treatment with charcoal, and passage through a mixed-bed ion-exchange resin, the inositol could be crystallized directly by adding alcohol. Charalampous and Abrahams (19, 20) used a similarly vigorous procedure for the isolation of C^{14}-labeled inositol from yeast grown on C^{14}-labeled glucose. The washed yeast was refluxed for 12 hours with 22 per cent hydrochloric acid. C^{14}-inositol may also be prepared from plants grown in an atmosphere containing $C^{14}O_2$ (21).

Other methods are available for the production of isotopically labeled *myo*-inositol. Weygand and Schulze (22) obtained it in 2.2 per cent yield from $BaC^{14}O_3$ by the following sequence of reactions:

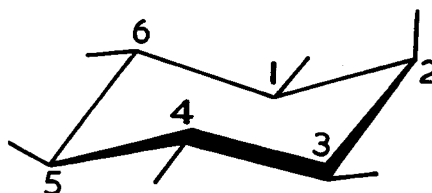


The inositol isomers produced by the catalytic hydrogenation of tetrahydroxybenzoquinone were separated by chromatography on cellulose powder columns in an acetone-water mixture (16). Posternak *et al.* (23) synthesized *myo*-inositol (2-C^{14}) from $C^{14}H_3NO_2$ and aldo-5-0-isopropylidene, 1,2 D xylo-pentofuranose in a seven-stage synthesis. The radiochemical yield was 4.2 per cent. From suitably labeled glucose, inositol labeled in other positions could be synthesized using this method. Wilzbach (24) has described the production of uniformly tritium-labeled inositol by equilibration with tritium gas. Posternak *et al.* (25) produced inositol labeled with deuterium in the 2-position, by the reduction of *scyllo*-inosose. Agranoff *et al.* (26) used a similar method to prepare inositol-2-H^3.

Though *myo*-inositol is often represented for convenience in structural formulas with a planar carbon ring (I*a*), the preferred conformation is actually the "chair" form of the ring giving one axial and five equatorial hydroxyls (I*b*). The photographs (II and III) of a model in this conformation will give a better



(I*a*)
Myo-inositol,
planar carbon ring.



(I*b*)
Myo-inositol,
preferred "chair"
form.

idea of the spatial relationships. Use of this conformation makes certain reactions easier to understand. For instance, the relatively nonspecific enzyme phytase is able to remove all the phosphate from phytic acid (*myo*-inositol hexaphosphoric acid), but if the reaction is stopped when 70 per cent of this phosphate has been released, inositol 2-phosphate may be isolated from the digest. The phosphate on the axial 2-hydroxyl is less readily hydrolyzed than the other five which have the equatorial configuration. Studies by Ellis (27) have confirmed this. Inositol 2-phosphate was hydrolyzed less readily by prostate phosphomonoesterase than an equatorially substituted phosphate. It is of interest that the natural phosphatidylinositols so far studied have the inositol 1-phosphate structure, avoiding the less reactive 2-position.

The fascinating studies of Magasanik and coworkers (28, 29) on the oxidation of inositol isomers by *Ace-*

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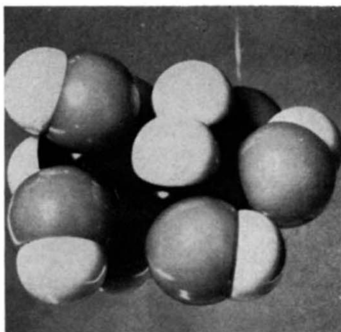
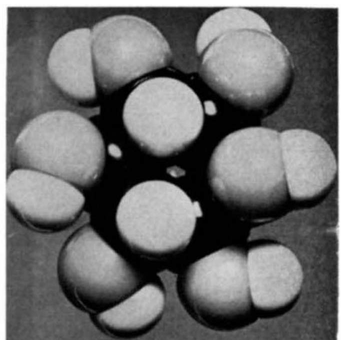


(II)

Model of *myo*-inositol in preferred "chair" form, axial 2-hydroxyl uppermost.



(III)

Same model from above, axial 2-hydroxyl on right.

tobacter suboxydans show that only axial hydroxyls are oxidized to keto-groups. Platinum-catalyzed dehydrogenation (30) gives similar results. This may at first seem to contradict what has been said about the reactivity of axial OH groups. In this case, however, the attack is at the equatorial hydrogen; e.g., *myo*-inositol has only one such hydrogen, in the 2-position, and so the 2-inosose (*scyllo*-inosose) is produced. The conformation of *myo*-inositol is also important in connection with the hydrolysis of diesters based on inositol phosphates. This subject is discussed below.

CHEMISTRY OF THE INOSITOL PHOSPHATES

Courtois (31) has comprehensively reviewed the earlier work on the chemistry and biochemistry of these compounds. Of the different inositol isomers, only *myo*-inositol has been found naturally in combination with phosphate (17). Phytic acid, the best-known phosphoric acid ester of inositol, is dealt with in detail by Courtois and so will be mentioned here only as starting material for the preparation of inositol monophosphate. Posternak and Posternak (32) first carried out this preparation using the phytase of bran, although Anderson (33) had previously obtained the monophosphate by acid hydrolysis of phytic acid. At present the method used most often is the modification of the enzymic method due to McCormick and Carter (34). Fleury *et al.* (35) compared the inositol monophosphates produced by enzymic and alkaline hydrolyses of phytate. Only the former was believed to be identical with synthetic inositol 2-phosphate. More recent work (36, 37) has shown that all three compounds are identical, as would be expected on theoretical grounds (38). This 2-phosphate has less activity as a growth factor for cells in tissue culture than the phosphate obtained by hydrolysis of phosphatidylinositol, which is now known to be a mixture of inositol 1- and 2-phosphates (39).

The esters obtained by hydrolysis of phytic acid have been partly separated on ion-exchange columns (40), and also by paper ionophoresis (41). The behavior on Nalcite SAR columns of inositol monophosphate isolated from liver has been described by Hübscher and Hawthorne (42). Desjobert and Petek (43) were able to separate inositol mono-, di-, tri-, tetra-, penta-, and hexaphosphates on paper chromatograms, though the penta- and hexaphosphates ran to almost the same positions. The developing reagents of Wade and Morgan (44) were used but the alkali-silver nitrate method of Anet and Reynolds (45) is rather more sensitive.

An inositol monophosphate was synthesized in 1931 by Horiuchl (46), who phosphorylated free *myo*-inositol and thus probably obtained a mixture of isomers containing little of the 2-phosphate. Iselin (47) published the first synthesis of an inositol phosphate of known configuration, taking advantage of the enzymic oxidation of the 2-hydroxyl of *myo*-inositol mentioned above. *Scyllo*-inosose thus produced was acetylated and the product reduced in the presence of a platinum catalyst to 1,3,4,5,6-penta-O-acetyl inositol and a small amount of penta-O-acetyl scyllitol. The mixture was phosphorylated with diphenyl phosphochloridate and the scyllitol derivative separated by fractional crystallization. After removal of the protecting acetyl and phenyl groups, inositol 2-phosphate and scyllitol phosphate were obtained. 1,3,4,5,6-penta-O-acetyl-inositol can be prepared in another way, which avoids the enzymic oxidation. Angyal *et al.* (48) acetylated the acetone derivative of inositol, thus obtaining (±)-3,4,5,6-tetra-O-acetyl-1:2-O-isopropylidene-*myo*-inositol. Mild acid treatment converted this to the (±)-3,4,5,6-tetra-O-acetyl-*myo*-inositol. Davies and Malkin (49) acetylated this compound with 1.25 moles of acetyl chloride and obtained 1,3,4,5,6-penta-O-acetyl-*myo*-inositol. This provides another example of the greater reactivity of equatorial than axial hydroxyls in the inositols.

Kilgour and Ballou (50) have synthesized levo-inositol 3-phosphate. The phosphorylation of the tetra-acetate of *myo*-inositol mentioned above appears to

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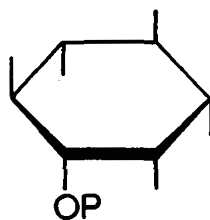
give a cyclic 1:2 phosphate and from this Pizer and Ballou (51) prepared a (±) 1-phosphate by alkaline hydrolysis. Some 2-phosphate is also formed but may be removed since it has a more soluble cyclohexylamine salt.

The *myo*-inositol 2-phosphate obtained from phytic acid may be converted to a 1:2 cyclic phosphate by reaction with dicyclohexyl-carbodi-imide (51, 52). Hydrolysis of the cyclic phosphate in acid or alkali gave a mixture of (±) 1-phosphate (70-80 per cent) and 2-phosphate. These compounds may be distinguished by chromatography on paper in isopropanol: ammonia:water (7:1:2, v/v) and so the above authors have been able to demonstrate phosphate migration for the first time in the inositol series (51, 52, 53). Acid catalyzed the formation of (±) 1-phosphate from 2-phosphate, the final mixture being similar to that produced by hydrolysis of the cyclic *myo*-inositol 1:2-phosphate (53). These results are important in deciding the structure of the naturally occurring phosphoinositides, as will be seen later.

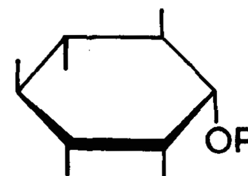
Until recently the inositol monophosphates obtained by hydrolysis of phosphoinositides were believed to be optically inactive, but Pizer and Ballou (51) have now prepared an active 1-phosphate ($[\alpha]^{25}_{589}$ + 3.4° for the cyclohexylamine salt at pH 9) from an alkaline hydrolysate of soybean lipids. Their results have been confirmed by Hawthorne *et al.* (54), who also prepared a 1-phosphate from liver phosphatidylinositol by enzymic hydrolysis. This latter compound appears to be optically active[1] and may be identical with the inositol phosphate which occurs free in liver and other tissues (42).

Brown *et al.* (55) have oxidized glycerylphosphorylinositol prepared from phosphatidylinositol to a glycolaldehyde derivative which broke down to inositol 1-phosphate on treatment with phenylhydrazine at pH 6. There was no evidence of phosphate migration.

Starting from the naturally occurring inositol galactoside "galactinol," first isolated from sugar beet by Brown and Serro (56), Ballou and Pizer (57) have synthesized an optically active *myo*-inositol 1-phosphate. Kabat *et al.* (58) had previously shown that galactinol was 1-O-α D galactopyranosyl *myo*-inositol and that inversion at the inositol-galactose linkage led to (-)-inositol. In the synthesis galactinol is first completely benzylated. After methanolysis to break the galactosidic linkage, the penta-O-benzyl inositol was phosphorylated with diphenyl phosphochloridate. The usual hydrogenation gave an inositol phosphate whose cyclohexylamine salt had $[\alpha]_D$ − 3.2° at pH 9. The soybean inositol phosphate has $[\alpha]_D$ + 3.4°. Ballou and Pizer concluded that the respective structures were:



(IV) Synthetic



(V) Soybean

They proposed to call the soybean compound L-*myo*-inositol 1-phosphate.

Myo-inositol 5-phosphate has also been synthesized (17) but the details are not yet in print.

CHEMICAL SYNTHESIS OF PHOSPHATIDYLINOSITOL AND RELATED COMPOUNDS

Davies and Malkin (59) have published the first chemical synthesis of an inositol phospholipid, 2,3-distearyl-glyceryl 2-*myo*-inosityl phosphate. They reacted glycerol 1-iodide 2,3-distearate with silver 2-(1,3,4,5,6-penta-O-acetyl) *myo*-inosityl phenyl phosphate. Protecting groups were removed by hydrogenation followed by reflux with ethanolic hydrazine. This excellent synthesis should stimulate progress in the field of inositol phosphatide biochemistry, where work is being done at present on poorly characterized compounds. Combining this method with that of Ballou and Pizer (57), it should be possible also to synthesize a lipid having the inositol 1-phosphate structure.

Work in two laboratories has led to the synthesis of glycerol 1-(inositol 2-phosphate) by different routes and the work has been published simultaneously (49, 60). In the first method 1,3,4,5,6-penta-O-acetyl *myo*-inositol was treated with 1.1 moles phenyl phosphochloridate in lutidine, followed by the addition of 5 moles (DL)1,2-isopropylidene-glycerol. A crystalline product was isolated which gave glycerol 1-(inositol 2-phosphate) after removal of protecting groups. It is interesting that when D-1,2-isopropylidene glycerol was used, the final product was optically inactive, even though the intermediates were active. In the second method (60) 1,3,4,5,6-penta-O-acetyl *myo*-inositol 2-phosphoric acid was reacted with DL-1,2-isopropylidene glycerol in the presence of dicyclohexylcarbodiimide and pyridine. Ellis and Hawthorne[2] have also prepared

[1] P. Kemp, unpublished observations.

[2] Unpublished results.

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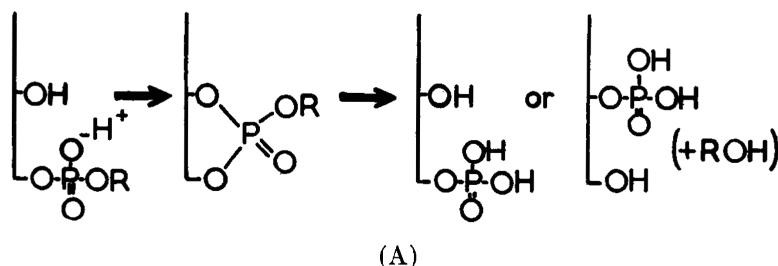

(A)

the corresponding scyllitol diester by this second route. Brown *et al.* (55) have synthesized glycerol 1-(inositol 2-phosphate) and glycerol 1-(inositol 1-phosphate) by letting glycidol react with the corresponding inositol phosphates. Their products contained small amounts of the glycerol 2-derivatives.

Glycerylphosphorylinositol has also been prepared by mild alkaline hydolysis of liver phosphatidylinositol (61). The pure diester was isolated by chromatography on Dowex 1 in the presence of borate. It is now known that this compound has the glycerol 1-(inositol 1-phosphate) structure (51, 55, 62).

HYDROLYSIS OF GLYCERYLPHOSPHORYLINOSITOL AND RELATED COMPOUNDS

Though phosphate migration occurs less readily in the inositol phosphates than in glycerophosphate derivates, it can easily invalidate structural conclusions based on the properties of phospholipid hydrolysis products. This was not realized by the earlier workers in the field, but the studies of Brown and his colleagues in Cambridge, using model compounds (37, 55, 63), have considerably improved our understanding of the factors involved. Modern views are based on the theory that an intermediate cyclic triester is formed during the hydrolysis of phosphoric acid diesters having a free hydroxyl vicinal to the phosphate group (Scheme A). The cyclic triester then rapidly breaks down to release the group ROH and a cyclic diester which hydrolyzes to a mixture of isomeric α and β phosphates. The case of phosphatidylinositol[3] is more complicated than this, since once the fatty acids are removed, cyclization is possible with both an inositol and a glycerol hydroxyl. Alkaline hydrolysis could take place according to either Scheme B or Scheme C.

[3] The convention in which the inositol ring is considered planar will be used in this discussion, hydroxyls being described as being in *cis* or *trans* relationship. Use of the nonplanar convention does not affect the argument (16), though the presentation becomes more complicated.

Brown *et al.* (63) studied the corresponding derivatives of *cis*- and *trans*-cyclohexanediol rather than inositol. Alkaline hydrolysis of glycerol 1-(*cis*-2-hydroxycyclohexyl phosphate) gave only 14 per cent glycerophosphate. The remaining phosphate was in the form of 2-hydroxycyclohexyl phosphate, no inorganic phosphate being produced. The *trans* diester gave 77 per cent glycerophosphate. It might be expected that inositol 1(3)- or 2-derivatives would resemble the *cis* compounds, and inositol 4(6)- or 5-derivatives the *trans* (see I*a*). In fact, the situation is complicated by the presence of the other inositol hydroxyl groups. Table 1 summarizes the results of hydrolysis studies

TABLE 1. HYDROLYSIS OF THE GLYCERYLPHOSPHORYLINOSITOLS

Compound	Inositol Phosphate*	Reference
Glycerol 1-(inositol 2-phosphate) *synthetic*	31, 35	(48)
Glycerol 1-(inositol 2-phosphate) *synthetic*	31	(59)
Glycerol 1-(inositol 2-phosphate) *synthetic*	39, 40	(54)
Glycerol 1-(inositol 1-phosphate) *synthetic*	34, 35	(54)
Glycerylphosphorylinositol *from liver*	35	(64)
Glycerylphosphorylinositol *from liver*	39, 34	(65)

* As per cent of total P in the hydrolysate, all of which was organic.

on the inositol diesters. Though Brown *et al.* (55) were able to detect a small difference between the inositol 1- and 2-phosphate derivatives, this appears comparable with the experimental error in the analyses of other workers. It would be unwise to make structural decisions on the basis of this difference.

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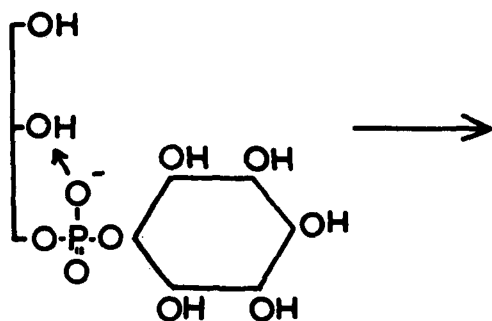
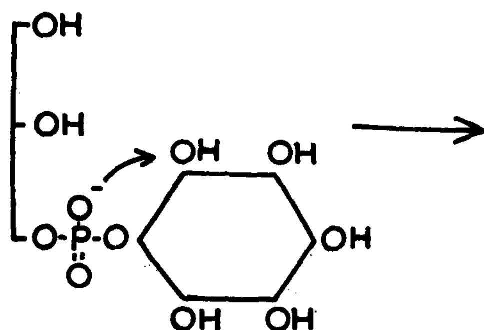


inositol + glycerol

1- and 2- phosphates

(B)

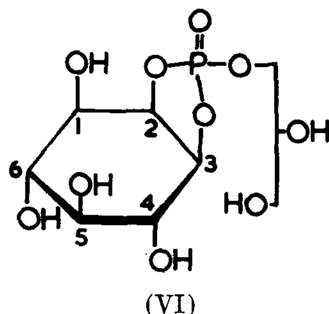


glycerol + isomeric

inositol phosphates

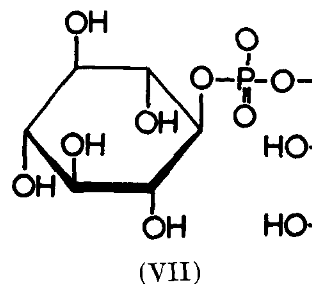
(C)

In all of the synthetic compounds (Table 1) the phosphate has neighboring *cis* hydroxyls on the inositol ring, yet the yield of inositol phosphate is much smaller than in *cis*-cyclohexanediol derivatives. Cyclization is apparently hindered by the presence of a third *cis* hydroxyl in the *myo*-inositol (VI). Evidence from related compounds supports this idea (65).



(VI)

Diesters having the inositol 1(3)- or 2-phosphate structure would be expected to give similar amounts of inositol phosphate on hydrolysis, but inositol 4(6)- or 5-phosphate derivatives would give much less. Glycerol 1-(scyllitol phosphate) (VII), in which the phosphate is flanked by two scyllitol *trans* hydroxyls,



(VII)

should be very similar in its behavior to the inositol 4(6)- or 5-compounds. For this reason it has been synthesized and its hydrolysis studied.[4] Preliminary results indicate that on alkaline hydrolysis only about 3 per cent scyllitol phosphate is produced. This provides further evidence that the natural inositol diester and therefore liver phosphatidylinositol also have the 1(3)- or 2-structure.

[4] R. B. Ellis and J. N. Hawthorne, unpublished observations.

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ISOLATION OF PHOSPHOINOSITIDES FROM TISSUES

One of the great obstacles in the study of phospholipids is the lack of easy and reliable methods for the isolation of pure compounds from tissue extracts. Apart from contamination of one phospholipid with another and with nonlipid impurities, the range of fatty acids present in any particular phospholipid makes further difficulties. It is almost impossible to be certain, for example, that phosphatidylinositol isolated from heart muscle has a fatty acid composition identical with that in the original tissue. Suppose, as an illustration, that heart contains only a distearyl and a dioleyl phosphatidylinositol. They will have very different solubility properties. During purification it is most unlikely that identical proportions of each will be lost. If silicic acid chromatography is employed in the isolation, the unsaturated phosphoinositide will appear in the early fractions and the saturated compound may overlap with a phospholipid eluted later from the column. If precipitation with organic solvents is used, there is a possibility that more unsaturated phosphoinositides will be lost in the supernatant. A comparison of the fatty acid composition of phosphatidylinositols isolated by various methods is given in a later section.

Considerable purification of phosphoinositides can be obtained by solvent treatment and reprecipitation. Folch has perfected methods of this type (5, 6), dissolving the lipid in chloroform and using carefully controlled volumes of methanol or ethanol for precipitation. The purity of the product must be checked by analysis, e.g., the nitrogen content will indicate contamination of many phosphoinositides, which are nitrogen-free when pure. The inositol to P molar ratio, which should be a simple one, is also a useful guide.

McKibbin and Taylor (66) developed a method for the fractionation of phospholipids by absorption on silicic acid columns and elution with increasing concentrations of methanol in chloroform. The procedure of Hanahan *et al.* (67) seems to this writer to be the best modification so far described for the preparation of phosphoinositides. These authors used 20 per cent (v/v) methanol in chloroform as their first eluting agent. This removed phosphatidylserine and phosphatidylethanolamine as one peak. The second solvent, 40 per cent methanol in chloroform, removed phosphatidylinositol and then lecithin. The method gave good results with liver phospholipids but has not been applied to those from brain.

Dils (68) has shown that ox brain diphosphoinositide is not separable from phosphatidylserine on silicic acid columns. Vilkas and Lederer (13) have successfully applied silicic acid chromatography to the separation of tubercle bacillus phosphoinositides. Dils and Hawthorne (69) used cellulose acetate columns for the purification of liver phosphatidylinositol. Using countercurrent extraction, Hörhammer *et al.* (70) have separated phosphatidylinositol and diphosphoinositide from an ox brain phospholipid fraction ("Folch fraction I"). These workers also obtained three inositol lipids from soybean fat by the same method.

Although the pursuit of purity is essential for work on the chemistry of phosphoinositides, it takes us a long way from conditions inside the cell, where phospholipids are certainly impure. For biochemical studies it is necessary to isolate larger units as well. Most of the lipoproteins are appalling mixtures from the chemist's point of view, but they are more significant metabolically than their "purified" lipid components. Though different criteria of purity have to be applied, these large units may also be obtained in a "pure" state.

ANALYSIS OF PHOSPHOLIPID MIXTURES

As yet there is no satisfactory method of separating all the individual phospholipids of a tissue from one another. Some fractionation is possible on silicic acid columns or silica-impregnated paper, but neither method can be applied directly to the quantitative analysis of phospholipid mixtures. It seems doubtful if physical methods of this sort will provide a complete separation, since each phospholipid is really a family of molecules with differing fatty acid composition. As already mentioned, the fatty acids may profoundly alter the physical properties of a phospholipid.

Much analytical work on tissue phospholipids has therefore been done in other ways. One method involves a vigorous hydrolysis of the lipid mixture and estimation of individual components such as choline or inositol in the hydrolysate (71, 72, 73). It has the disadvantage that the same component may occur in more than one phospholipid. Another method is based on mild alkaline hydrolysis, which removes only the fatty acids, followed by separation of the resulting phosphate esters (e.g., glycerylphosphorylcholine) by two-dimensional paper chromatography (74) or on ion-exchange columns (61).

These methods, however, provide no help in studies involving the fatty acids of phospholipids, so several workers have turned to paper chromatography of the intact compounds. Lea *et al.* (75) described the separation of certain phospholipids on silica-impregnated

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filter paper, but phosphoinositides were not included. Rouser *et al.* (76) used a variety of polar or ionic solvents on untreated filter paper, detecting the phospholipid spots with Rhodamine B or G. Unfortunately, phosphatidylinositol does not move in these solvents (77). Marinetti and Stotz (78) also separated a series of phospholipids on silica-impregnated papers using both one- and two-dimensional chromatography. The method has been applied to phospholipid mixtures from several different tissues (79, 80). Phosphatidylinositol has an R_f of 0.20 in one of the solvents used (78), a mixture of diisobutylketone, acetic acid, and water (40:30:7, v/v). In the same solvent, diphosphoinositide does not appear to move (81). Agranoff *et al.* (26), using this solvent with silica-impregnated glass fiber papers, found that phosphatidylinositol "tailed" from R_f 0.55 back to the origin. They obtained better results without the silica impregnation and in a solvent consisting of diisobutlylketone and acetic acid (30:5, v/v). It will be apparent that phosphoinositides do not behave well on paper chromatograms.

Hörhammer *et al.* (82) obtained what seem to be better separations on formaldehyde-treated paper with an *n*-butanol-acetic acid-water solvent (4:1:5). The R_f value of phosphatidylinositol was 0.43. Diphosphoinositide separated into three components (R_f values 0.19, 0.24, and 0.28).

An unusual method for separating phospholipids is paper electrophoresis. This has been used by Zipper and Glantz (83), who separated lecithin, phosphatidylethanolamine, phosphatidylserine, and diphosphoinositide in several different solvents. Relative rates of migration in a solvent consisting of methanol, pyridine, and acetic acid (8:1:1, v/v) made to 0.05 N with sodium acetate, were as follows: Lecithin + (i.e., toward anode) 0.43; phosphatidylethanolamine + 4.12; phosphatidylserine + 150; diphosphoinositide − 27.8. It is surprising that at both acid and alkaline pH, diphosphoinositide moved toward the cathode, while the less acidic phosphatidylserine moved in the opposite direction. Dimyristyl and dioleyl lecithins were not separable. There do not seem to be any other reports of work based on electrophoresis, which, theoretically at least, seems well suited to the separation of phosphoinositides.

ESTIMATION OF LIPID-BOUND INOSITOL

The lack of a simple specific chemical method for the estimation of *myo*-inositol has long been a difficulty in work on the inositol lipids. Microbiological assay with an inositol-requiring yeast has been widely used since Woolley first proposed it in 1941 (84). A reliable and sensitive modern procedure using a strain of *Schizosaccharomyces pombe* is described by Norris and Darbre (85).

Several enzymic methods have recently been proposed. Two of these are based on the oxidation of *myo*-inositol to glucuronic acid by a kidney enzyme (86). Another enzymic assay makes use of an inositol dehydrogenase from *Aerobacter aerogenes* (87). The enzymic and microbiological methods are much more specific than the chemical ones described below, but they involve specialized techniques such as culture of microorganisms and enzyme purification which may not always be available. In addition, inhibitors produced by hydrolysis of a lipid sample may cause difficulty.

The chemical methods that seem most useful are both based on oxidation of *myo*-inositol by periodic acid. Here the chief problem is the removal of interfering compounds such as glycerol, serine, ethanolamine, and the sugars. One version uses paper chromatography to accomplish this and was designed specifically for the analysis of lipid inositol (88). Sealed-tube hydrolysis with a small volume of HCl is employed and the hydrolysate applied directly to paper chromatograms. The chromatographic solvent is isopropanol-acetic acid-water (3:1:1, v/v). Position of the inositol spots is determined by spraying a "marker" strip to which pure inositol is applied. The spots are then cut out and extracted. The extracts are oxidized with periodate and the excess oxidizing agent determined iodometrically. Standards of inositol are put through the whole procedure. The method requires at least 50 to 100 μg. inositol. Hübscher and Hawthorne (42) obtained an accuracy of ± 3 per cent with it.

A more sensitive method depends on the spectrophotometric estimation in U.V. light of periodate (89). Inositol is oxidized at 65°C under controlled conditions. LeBaron *et al.* (90), as well as Agranoff *et al.* (26), describe this method.

Using the yeast bioassay, Taylor and McKibbin (73) reported the values in Table 2 for the lipid inositol content of various animal tissues. Inositol was released by hydrolysis with 4N HCl under reflux for 40 hours.

PHOSPHOINOSITIDES OF ANIMAL TISSUES

Though Thudichum, the great pioneer of phospholipid chemistry, recognized inositol as a constituent of brain (91), it was not until 1942 that Folch and Woolley (4) found it combined in a brain lipid.

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TABLE 2. Lipid-Bound Inositol of Animal Tissues*

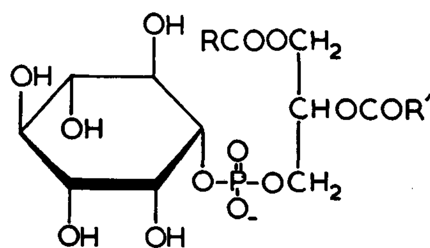
Tissue	Total Lipid Inositol† (μM)	Molar Ratio-Lipid Inositol:P
Dog liver (2)	13.0	.0730
Dog kidney (2)	13.2	.0653
Dog heart (2)	7.39	.0438
Beef heart (1)	8.53	.0546
Dog brain (2)	22.4	.0338
Dog intestine (2)	9.64	.0713
Dog skeletal muscle (1)	3.60	.0480
Dog pancreas (1)	19.3	.0860
Dog lung (1)	10.4	.0500
Dog plasma (2)	6.38	.0253
Rabbit plasma (1)	3.70	.0327
Human plasma (1)	5.05	.0204
Human plasma (10 patients)	7.45	.0255
Human plasma (10 patients) range	5.23-10.7	.0230-.0277

* Reproduced by kind permission of the authors, Taylor and McKibbin (73), and the editors of the *Journal of Biological Chemistry.*

† μM inositol per g. dry lipid-free tissue residue and per 100 ml. plasma. Number of individuals in parenthesis.

Burmaster (92) also showed the presence of inositol phospholipids in brain cephalin. Macpherson and Lucas (93, 94) first showed that liver phospholipids contained inositol.

At present two well-defined phosphoinositides are known in animal tissues, phosphatidylinositol (VIII), whose structure is almost certainly the one shown here, and "diphosphoinositide," isolated by Folch from brain (95, 96).



(VIII)

Hörhammer *et al.* (70) have provided some evidence of a lysophosphatidylinositol in ox brain. Hokin and Hokin (97) obtained two phosphoinositide spots on autoradiograms of pigeon pancreas phospholipids. One appears to be due to phosphatidylinositol, and both give inositol monophosphate and glycerophosphate on hydrolysis. The evidence is not contrary to the idea that the second lipid is a lyso-compound, though the two differ considerably in their turnover of glycerol, inositol, and phosphate.

Phosphatidylinositol was first isolated by Faure and Morelec-Coulon from wheat germ (98) and heart muscle (99). McKibbin (100) and Hawthorne (101) obtained evidence of a similar compound in liver. Subsequent work in several laboratories has provided a sound basis for structure (VIII). Faure and Morelec-Coulon (102), after a series of carefully controlled solvent extractions and precipitations, were able to obtain from heart muscle a crystalline sodium salt of phosphatidylinositol. This lipid, therefore, can be added to the very short list of phospholipids which have been obtained from natural sources in crystalline form. The same authors (103, 104, 105), by analysis of the phosphoinositide and its hydrolysis products, showed that it contained fatty acids, glycerol, *myo*-inositol, and phosphoric acid in molar proportions of 2:1:1:1. Analysis of the barium salt of the original lipid showed that the phosphate residue had only one free acid group. Pure preparations were free from nitrogen. The compound from ox heart contained equimolar amounts of stearic and unsaturated fatty acids, the latter having an iodine number of 222 and an average molecular weight of 291. On hydrolysis, inositol monophosphate and glycerophosphate were liberated (106), suggesting that the inositol and glycerol were both linked to phosphoric acid. Confirmation of this was obtained by the liberation of the diester glycerylphosphorylinositol after mild alkaline hydrolysis (107). This diester was isolated and analyzed with ion-exchange columns in the presence of borate (61).

McKibbin's phosphatidylinositol (106), obtained from horse and dog liver by chromatography on silicic acid and countercurrent extraction, contained fatty acids of a much lower iodine number than those of the heart compound (102). The difference may reflect different methods of preparation (108), as has been mentioned before, though a process depending on precipitation, like that of Faure and Morelec-Coulon, would be expected to give more saturated compounds, since these are less soluble. However, these authors point out that their mother liquors did not contain any less saturated phospholipids (108). In addition, silicic acid columns appear to release unsaturated phospholipids quite readily (68, 109), the early fractions of any peak always being highly unsaturated. It is more likely that the difference is due to McKibbin's use of countercurrent extraction, since Hanahan and Olley (64) obtained figures very similar to those of the

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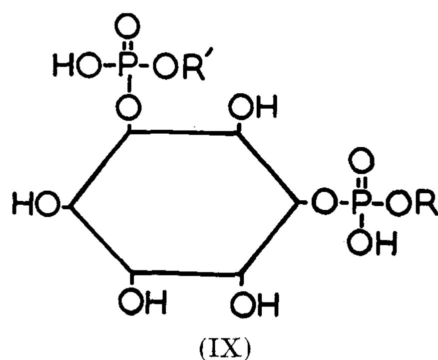
French workers. Table 3 summarizes the results on fatty acid composition.

Hanahan and Olley (64) showed that a short HCl hydrolysis preferentially released inositol monophosphate and diglyceride from phosphatidylinositol. Le Cocq and co-workers (110, 111), using acetic acid, also studied this type of hydrolysis. They found that the first product was an optically active cyclic inositol phosphate which then broke down to a mixture of inositol 1- and 2-phosphates. The optical activity and the fact that a cyclic ester could form strongly suggest that the inositol 1-structure was present in the original phosphatide. This inositol 1-structure was first suggested by Pizer and Ballou (36) for soybean phosphatidylinositol, though these authors mention similar findings with the liver compound. Studies of the hydrolysis of glycerylphosphorylinositol from the liver lipid confirm this (64, 112). Brockerhoff and Hanahan (62) also provide evidence that the inositol is esterified in the 1- (or 4-) position. Periodate oxidation of glycerylphosphorylinositol from the liver lipid gave an optically active glycolaldehydeinositol phosphate, which also showed that the glycerol was α-linked. Brown *et al.* (55) used periodate in the same way, as mentioned above, finally obtaining the free inositol 1-phosphate. Their reaction conditions provide an elegant chemical method for the preparation of the natural inositol 1-phosphate.

Diphosphoinositide was isolated from ox brain by Folch (96), who suggested structure (IX) for this lipid.

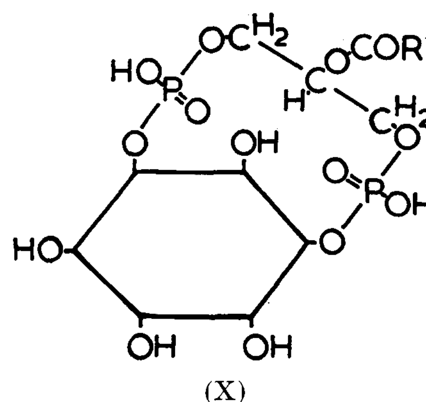


(IX)

(Configuration of ring hydroxyls unknown.)

After many reprecipitations, Folch obtained a compound which appeared to be almost pure. Analysis of its recognized hydrolysis products suggested that all of the compound was accounted for. Inositol, fatty acid, glycerol, and phosphoric acid were found in molar ratios of 1:1:1:2, respectively. When cations were removed, diphosphoinositide had two free acid groups.

On the basis of these analyses and information from periodate oxidation, it was suggested that diphosphoinositide may have the structure (X) (38) or be a polymer in which inositol diphosphate residues were linked by monoglyceride. The latter idea was also put forward independently by Folch and LeBaron (6).



(X)

Recent studies in this laboratory[5] seem to exclude structure (X) and make it even more difficult to envisage the true structure. Carboxylic ester determinations on purified diphosphoinositide from ox brain gave a molar ratio of unity for P:ester. In the original work of Folch (96), the value for this ratio was obtained by weighing the fatty acids from a known amount of lipid and using the equivalent weight of 400 obtained by titration. This figure is unusually high for the equivalent weight of a phospholipid fatty acid. If the more usual figure of 270 is used, Folch's weight data give a molar ratio of 0.87:1 for fatty acid:P. Hawthorne and Chargaff (113) obtained a fatty acid to P ratio of 1.1:1, the equivalent being 330. It seems, therefore, that diphosphoinositide has two fatty acids for each inositol. In the same study phosphatidylinositol was found in brain, confirming the observation of Hörhammer *et al.* (114).

Dittmer and Dawson (115) provide evidence that two triphosphoinositides occur in ox brain. On hydrolysis with acid, inositol triphosphate is released from these complex phospholipids. The simplest composition of one of them is given as $(\text{fatty acid})_6$ $(\text{phosphate})_6$ $(\text{glycerol})_3$ $(\text{inositol})_2$. They are tightly bound to brain protein. Their relation to diphosphoinositide is not yet clear.

The phosphatido-peptides, first described in brain tissue by Folch and LeBaron (116, 117), have attracted the attention of several workers, partly because

[5] J. N. Hawthorne, P. Kemp, and R. B. Ellis, unpublished experiments.

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TABLE 3. Fatty Acids of Different Phosphatidylinositol Preparations

Tissue	Iodine Number	Δ/P*	Equivalent Wt. of Fatty Acids	Nature of Fatty Acids	Reference
Liver	44-56 (fatty acids)	0.42-0.59	236-274	Polyunsaturated + 70% saturated	(106)
Liver	75 (phospholipid)	1.69	287	50% unsaturated 50% stearic	(108)
Heart	78-84 (phospholipid) 222 (unsaturated fatty acids isolated)	1.83	291	50% unsaturated 50% stearic	(103)
Ox liver	——	1.77	250	——	(64)
Rat liver	——	1.90	254	——	(64)

* Double bonds /atom P in the phospholipid.

they contribute phosphate to the RNA fraction in the tissue-P analysis of Schmidt and Thannhauser (118). These substances occur in many tissues and have a high phosphate turnover (119). Phosphatido-peptides seem to be inositol phosphatides linked to peptide or protein by saltlike linkages (117). It is not yet clear whether they occur in this state in the tissues, since the procedures used to isolate them are quite likely to produce artifacts by degradation of the original tissue component. In the fractionation scheme of Schneider (120) phosphatido-peptides appear in the same fraction as phosphoproteins, which also have a high phosphate turnover (121). Huggins and Cohn (119) describe a method for the separation of these two types of compound. A phosphatido-peptide fraction from liver was studied by Hutchison *et al.* (122) and shown to release inositol monophosphate on mild alkaline hydrolysis. A similar fraction from brain contained the diphosphate as well. Glycerophosphate was not observed. Hawthorne (123) obtained evidence that liver phosphatido-peptide contained phosphatidylinositol. These interesting compounds deserve further study from both the chemical and metabolic points of view.

Freinkel (124) showed that sheep thyroid contained a phospholipid releasing inositol and glycerol on hydrolysis.

Spiro and McKibbin (125) have determined the lipid inositol content of various subcellular fractions of liver. Mitochondria, microsomes, nuclei, and supernatant contained 0.07, 0.11, 0.09, and 0.11 moles of lipid inositol, respectively, per atom of phospholipid P. The animals used were rats. A choline-deficient diet did not affect these ratios.

Basford (126) showed that the "co-enzyme Q lipoprotein" from heart mitochondria contained 42 per cent of its phospholipid in the form of lecithin, 48 per cent as "cephalin" (phosphatidylserine plus phosphatidylethanolamine), and 4 per cent as phosphoinositide. This phospholipid pattern differed from that of whole mitochondria as observed by Hanahan—lecithin, 57 per cent; cephalin (as defined above), 28 per cent; phosphoinositide, 9 per cent; sphingomyelin, 1 per cent (see Basford and Green, 127). These authors suggest that other mitochondrial lipoproteins may be richer in inositol lipids and sphingomyelin, each having a specific pattern of components. The role of lipids in electron-transfer systems has been reviewed by Green and Lester (128).

In 1949 Celmer (8) was able to find inositol in the phospholipids of egg yolk. Malangeau (129) studied a phosphoinositide from the same source which had the unusual inositol to P molar ratio of 2:1. Rhodes and Lea (130) and Dils and Hawthorne (131) also reported the occurrence of small amounts of inositol phospholipids in egg yolk.

Lovern and Olley (132) found two types of phosphoinositide in haddock flesh. They concentrated on countercurrent distribution between petroleum ether (b.p. 40°-60°C) and 85 per cent ethanol at the extremities of the system. Garcia *et al.* (133) found a different phosphoinositide pattern when cod flesh lipids were distributed in the same solvent system. The ethanol-soluble compound was the predominant inositol lipid, but one of intermediate solubility was also found. The petroleum ether-soluble component of haddock was absent in cod. Garcia *et al.* also observed that 84 per cent of the cod lipid inositol was retained on a cellulose powder column used for removing water-soluble contaminants. The solvent was chloroform-methanol-water (80:20:2.5, v/v). This retained lipid was eluted by methanol. With haddock, only 19.4 per

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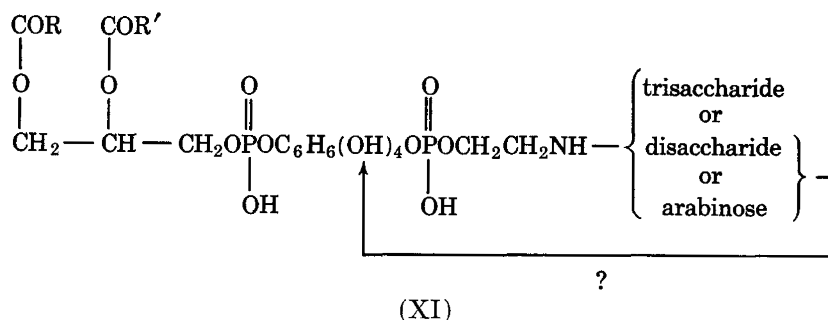
cent lipid inositol remained on the column under similar conditions.

An inositol phospholipid occurs in the tapeworm *Taenia saginata* (134). On hydrolysis it released inositol monophosphate. Phospholipid accounted for only 4.3 per cent of the total lipid in this organism. Phosphatidylethanolamine was not found, but lecithin was present. Saha and Igarashi (135) have studied the inositol lipids of certain aquatic animals.

VEGETABLE PHOSPHOINOSITIDES

Klenk and Sakai (3) made the first study of a plant inositol lipid by the isolation of inositol monophosphate from soybean phospholipid. They also reported, but did not examine closely, a dextrorotatory phosphate associated with the inositol phosphate. This substance gave a positive Molisch test. Woolley (15) obtained an inositol-containing fraction from the same source, and named it lipositol. On hydrolysis, lipositol released galactose, ethanolamine, fatty acids, inositol monophosphate, and tartaric acid. Folch and LeBaron (6) consider that the tartaric acid was an artefact, possibly due to the vigorous hydrolysis conditions used by Woolley, since Folch (5) was unable to detect this acid in soybean inositol lipid. In the latter study Folch fractionated dialyzed soybean phospholipids by chloroform-ethanol partition until the composition of his product remained constant. At this stage the phosphoinositide contained "hexose" [2], fatty acid [3], primary amine [1], glycerol [1], inositol [2], and phosphoric acid [2]. The figures in brackets represent approximate molar ratios; 97.2 per cent of the weight of material was accounted for by these components. Nomura[6] (136) obtained a similar lipid from soybean "cephalin." On hydrolysis it gave inositol monophosphate, glycerophosphate, galactose, and arabinose.

Using peanut (groundnut) lipids as starting material, Hutt *et al.* (138) also found a phospholipid that contained inositol and sugars. Further study of this material led Malkin and Poole (139) to suggest tentatively the following structure (XI). It is probable that Malkin and Poole were dealing wth a mixture



(XI)

of inositol lipids. They found a P:N molar ratio of 1.85:1. Ethanolamine appeared to be the only base. Inositol monophosphate, galactose, arabinose, and a possible disaccharide of galactose and arabinose were found among the hydrolysis products. Glycerophosphate was also found, but no inositol diphosphate.

Scholfield *et al.* (140, 141) fractionated soybean phospholipids by countercurrent distribution in a hexane-95 per cent methanol system. They found two quite different phosphoinositide fractions. Surprisingly, the one containing arabinose, galactose, and mannose in addition to inositol was more soluble in hexane than the other, which contained no sugars. This second compound was more soluble in methanol and nitrogen-free, though contaminated at first with phosphatidylethanolamine (142). It is now known to be phosphatidylinositol.

The most impressive study of seed oil phosphoinositides is that of Carter *et al.* (143). They found phosphatidylinositol and two new types of inositol lipid in most of the oils. The first of these, "lipophytin," contained inositol polyphosphate, fatty acids, some glycerol, and amino acids. Analysis showed 11 to 12 per cent P. The second, "phytoglycolipid," was a complex alkali-stable lipid. In 1953 Van Handel (144) had obtained evidence that soybean contained a lipid of this type. Resistance to dilute alkali provided the key to its purification. Phytoglycolipid was found in corn, flaxseed, soybean, peanut, cottonseed, and sunflower seed. Suitable hydrolyses released the following components: phytosphingosine (145), fatty acids, inositol, glucosamine, hexuronic acid, galactose, arabinose, mannose, and phosphoric acid. The following tentative structure (XII) was put forward (11).

The glucuronic acid and glucosamine were linked as hyalobiuronic acid (146). It was considered that the original inositol lipid might have been a phosphatidyl

[6] Imai (137) has also studied the phosphoinositides of soybean, but I have been unable to obtain the original publication.

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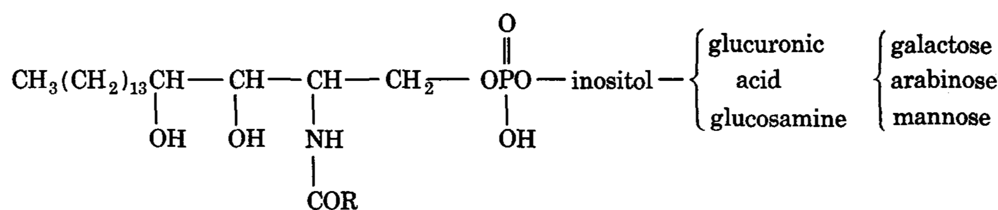
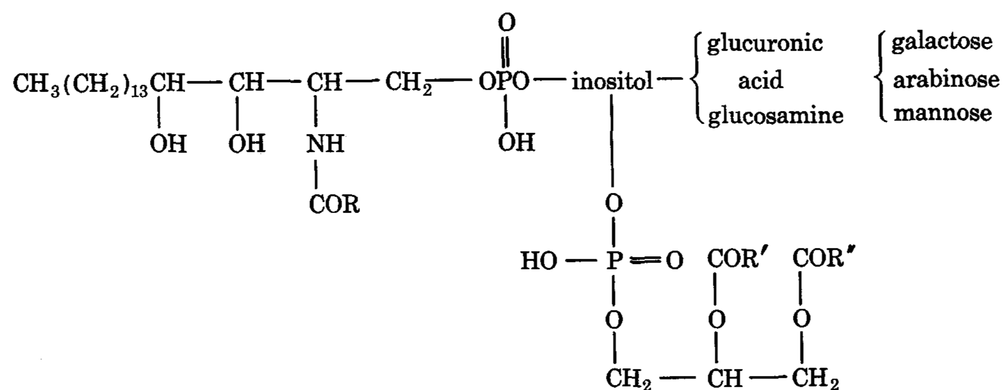


(XII)

derivative of (XII), which gave (XII) on treatment with alkali, since the solubility of phytoglycolipid and the original compound were quite different. Mild alkaline hydrolysis of (XIII), however, would be more likely to remove fatty acids and leave an inositol diphosphate derivative.

phosphatidylinositol was present. This same compound was isolated from wheat germ by Faure and Morelec-Coulon (98, 148). It is also present in peas, where it makes up 5 per cent of the total lipids (149). Peas provide a good starting material for the preparation of pure phosphatidylinositol by a relatively simple



(XIII)

process (150). Hörhammer *et al.* (70), using countercurrent extraction, found three inositol lipids in the alcohol-insoluble fraction of soybean phospholipids. They were phosphatidylinositol, lysophosphatidylinositol, and a more complex sugar-containing phosphoinositide.

McGuire and Earle (151) found 5.7 per cent inositol in crude linseed phospholipids. Countercurrent distribution by the method already mentioned (140, 141) showed that two phosphoinositides were present. The one more soluble in alcohol had a molar ratio of approximately 2:1:1 for P:N:inositol. The corresponding ratio for the hexane-soluble compound was approximately 4:1:1. The authors point out that the compounds were not necessarily pure.

In cottonseed oil, Olcott (152) found a phospholipid resembling lipositol (15).

In rubber latex, Smith (153) found an inositol phospholipid that released reducing sugar on hydrolysis.

Hawthorne and Chargaff (113), hydrolyzing what was probably a relatively impure soybean phosphoinositide, obtained strongly dextrorotatory compounds (cf. 3) which appeared to be glycosides of inositol monophosphate. They could be separated from inositol monophosphate itself by paper chromatography, or by ion-exchange columns (38). Arabinose and galactose derivatives were present and analysis indicated that each had inositol, sugar, and phosphate in equimolar proportions. The presence of other constituents was not excluded. It is not yet clear whether these substances are breakdown products of phytoglycolipid or another still unknown plant phosphoinositide. Nor is it clear whether Folch's soybean phosphoinositide (5) is a mixture of phytoglycolipid and phosphatidylinositol or whether it contains a third inositol lipid different from both.

Okuhara and Nakayama (147) methylated and degraded a soybean phosphoinositide, concluding that

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The constituents analyzed occurred in the following molar proportions: inositol:sugar:phosphoric acid: fatty acid, 1:1:1:2. The latex lipid contained 10.5 per cent of this substance.

Fuller and Tatum (154) found that most of the inositol in *Neurospora crassa* was bound in phospholipid. The relation between morphology and phosphoinositide content was studied. A colonial form resulting from inositol deficiency contained only 20 per cent as much phosphoinositide as the normal strain.

PHOSPHOINOSITIDES OF MICROORGANISMS

The classical work in this field was done by Anderson and his colleagues at Yale. Without the aid of modern techniques such as chromatography, they made great advances in phospholipid chemistry. In 1939 Anderson (155) reviewed the subject.

After the first discovery of inositol in a phospholipid, which has already been referred to (1), Anderson and Roberts (2) went on to study the inositol lipids of several strains of *Mycobacterium tuberculosis*. From human, avian, and bovine strains, a phospholipid containing inositol and mannose was obtained. Glucose and fructose were also found in the lipid from the human strain. Hydrolysis of the wax from this strain, which differs considerably in solubility from the phospholipid fraction, gave fatty acids and a complex phosphorylated polysaccharide (156). Hydrolysis released arabinose, mannose, and galactose from the latter, but only traces of inositol.

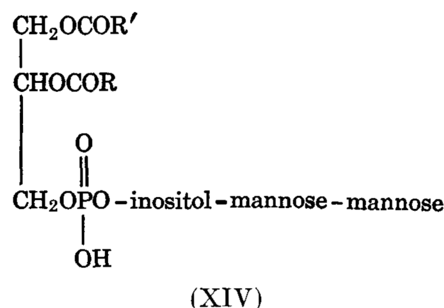
Chargaff *et al.* (157) showed that the nonpathogenic "Timothy grass bacillus" *(Mycobacterium phlei)* contained much less lipid than *M. tuberculosis*. The phospholipid of *M. phlei,* like that of *M. tuberculosis,* had little nitrogen (0.22 per cent). Hydrolysis released 2 per cent inositol, 9 per cent mannose, 10 per cent glycerophosphoric acid, and 60 per cent fatty acids; 19 per cent of the water-insoluble material could not be accounted for (158).

Since all the acid-fast bacteria investigated by Anderson and his collaborators appeared to contain little nitrogenous phospholipid and significant amounts of unusual phosphoinositides, another acid-fast organism was studied, the so-called *Bacillus leprae*[7] (159). This also proved to have a phospholipid which hydrolyzed to a mixture of palmitic acid, oleic acid, high molecular weight liquid fatty acids, glycerophosphoric acid, inositol, mannose, and another sugar. The alcohol-insoluble material obtained by alkaline hydrolysis seemed to be a mixture of glycerophosphate and a phosphorylated polysaccharide (4.7 per cent P). On further hydrolysis with boiling 3.5 per cent sulfuric acid, the polysaccharide was broken down to mannose (2 moles), inositol (1 mole), glucose and fructose (taken together, 1 mole).

In a more detailed study of the human *M. tuberculosis* phospholipid, Anderson *et al.* (160) obtained a polysaccharide by mild alkaline hydrolysis. This, when hydrolyzed with ammonia, lost its phosphate and gave a nonreducing polysaccharide which the authors called "manninositose." It contained 2 moles of mannose for every mole of inositol. Its molecular weight and analysis strongly suggested that it was a dimannoside of inositol. A compound of glycerol, mannose, and phosphoric acid was also thought to be present in the original lipid hydrolysate.

From the same source, de Sütö-Nagy and Anderson (161) isolated several different organic phosphates. The nature of these compounds varied considerably from one culture to the next when synthetic media were used. One of the phosphates was called inositol glycerol diphosphoric acid. It contained no sugar and liberated inositol phosphate and glycerophosphate on hydrolysis. The yields of these compounds are not given, and it is possible that the phosphate was a mixture containing glycerylphosphorylinositol.

Using silicic acid columns to fractionate their lipid mixtures, Vilkas and Lederer (13) have shown the presence of two distinct phospholipids in a streptomycin-resistant mutant of *M. tuberculosis* (H 37 Rv). One of these phospholipids was the magnesium salt of phosphatidic acid and the other was a complex phosphoinositide, phosphatidylinosito-di D-mannoside (XIV), also in the form of its magnesium salt. The fatty acids of these phospholipids were saturated, in



(XIV)

contrast to those found by Anderson. They consisted of palmitic and stearic acids. Vilkas (162) has shown that the mannose residues are linked 1:4 or 1:6 as a mannobiose, which is then linked through its free 1-

[7] Now called *Mycobacterium leprae.*

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position to inositol. The point of attachment on the inositol ring is not yet known. In the strain BCG, Vilkas also found evidence of phosphatidylinositol itself, as well as phosphatidylinosito-mono-mannoside, phosphatidylinosito-di-mannoside, and phosphatidylinosito-penta-glucoside.

Cason and Anderson (163) showed that the wax of bovine *M. tuberculosis* contained inositol lipids. The same was true of the human strain wax (164). Nojima *et al.* (165) found 3 per cent inositol in the Wax D fraction of BCG, and in further work Nojima (166) showed that the inositol compounds were phosphatidylinosito-di-, tetra-, and penta-mannosides.

Pangborn (167) has recently isolated serologically active lipopolysaccharides (similar to the compounds above) from pyridine extracts of acetone-dried *M. tuberculosis*. One of the compounds contained 1.73 per cent P, 10.54 per cent inositol, and 50 per cent mannose. The fatty acids were saturated.

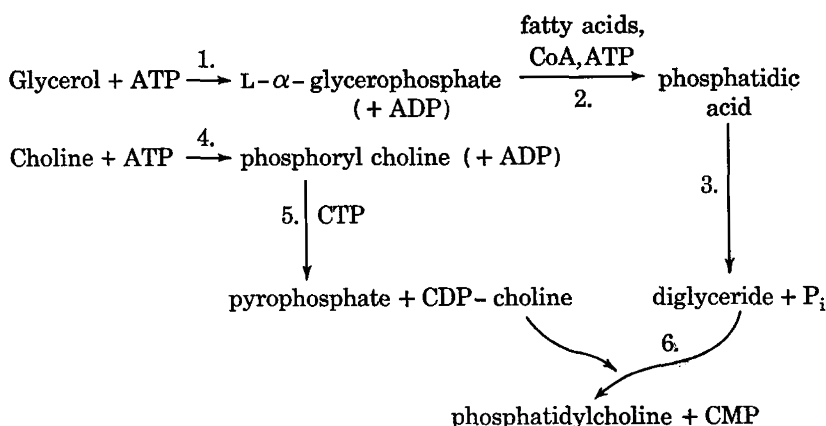
A phosphoinositide fraction has been obtained from yeast by Hanahan and Olley (64). Analytical data and behavior on silicic acid columns suggested that it was phosphatidylinositol. Yarbrough and Clark (168) showed than an inositol-requiring yeast, *Schizosaccharomyces pombe,* incorporated most of its inositol into an alcohol-soluble lipid. No "cephalin-like" phospholipids were detected.

Penicillium chrysogenum contains an inositol lipid which liberates the monophosphate on hydrolysis (169).

BIOSYNTHESIS

In 1939 Chargaff (170) suggested a pathway for phospholipid biosynthesis in which "amino-ethyl alcohol or choline is phosphorylated and the resulting phosphoric acid esters combine with a diglyceride." Recent studies have led Kennedy and his colleagues to the same conclusion. In addition, they have shown for the first time the importance of cytidine nucleotides in phospholipid biosynthesis (171). Kennedy's suggested scheme for phosphatidylcholine or phosphatidylethanolamine is summarized in Scheme D.

Though a full discussion of this scheme is not possible here, one point needs to be made because it bears on the biosynthesis of inositides. The evidence for reaction 4 in animal tissues is not overwhelming. The enzyme catalyzing it, "choline phosphokinase," was isolated from brewer's yeast by Wittenberg and Kornberg (172), but these authors found that liver, brain, and kidney had only one-tenth to one-fortieth the activity of yeast. In addition, Kennedy failed to show the incorporation of free choline into the lipids of isolated liver mitochondria (171). His successful synthetic studies use phosphoryl choline, as do those of several other workers (171, p. 338). However, Berry *et al.* (173) found choline phosphokinase activity in nervous tissue. Dils and Hübscher (174) showed the incorporation of labeled choline into the lecithin of rat liver mitochondria. Their system differed from Kennedy's in that CMP proved a more effective co-



SCHEME D

Abbreviations: ATP, adenosine triphosphate; ADP, adenosine diphosphate; P_i, inorganic phosphate; CTP, cytidine triphosphate; CDP-choline, cytidine diphosphate choline; CMP, cytidylic acid; CoA, coenzyme A.

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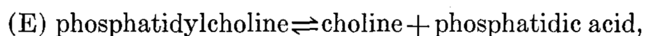
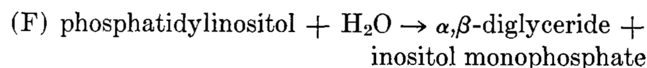
factor than CTP. Preliminary experiments, furthermore, showed that the incorporation of choline or serine was stimulated by calcium ions, alone or in the presence of other cofactors (175). Though it is tempting to think that the calcium-activated incorporation of choline and serine is due to phospholipase action,


(E) phosphatidylcholine ⇌ choline + phosphatidic acid,

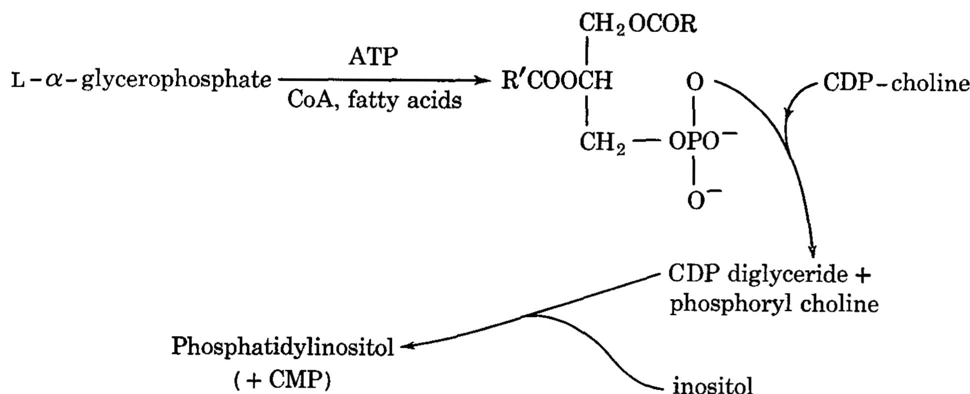
the exact mechanism has not been elucidated.

Work on the biosynthesis of inositol lipids began before the chemical structure of any was established. For these compounds and for phosphatidylserine a pathway similar to Scheme D was sought (176, p. 137). Such a pathway would require inositol monophosphate as an intermediate in the synthesis of phosphatidylinositol. This compound does in fact occur in several tissues (42), but attempts to show the phosphorylation of inositol by tissue extracts in the presence of ATP have been unsuccessful. In a thorough investigation, Hübscher (177) failed to find enzymic activity in any of the following tissues: rat liver, heart, kidney, brain, and intestine; dog liver, kidney, and heart; pig intestine; baker's and brewer's yeast. Two separate assay systems were used. One depended on removal of nucleotides by charcoal and estimation of the increase in non-nucleotide organic phosphate. In the other, ADP formation was measured by addition of phospho-enol-pyruvate and lactic dehydrogenase to give a diphosphopyridine nucleotide-linked system.

Hoffman-Ostenhof *et al.* (178) reported that partially purified preparations of yeast hexokinase were able to phosphorylate inositol in the presence of ATP and Mg^{++} ions. Brain hexokinase also phosphorylates inositol, but at a much slower rate than glucose (179). Hübscher (177) confirmed the yeast hexokinase phosphorylation, but considered it of little significance, since the rate was less than one-sixtieth of that with glucose. It seems more likely, therefore, that the inositol phosphate found free in tissues is a breakdown product of phosphatidylinositol. An enzyme hydrolyzing this lipid, according to reaction (F) below, has been isolated from rat liver (180). It appears to be widely distributed.


(F) phosphatidylinositol + H_2O → α,β-diglyceride + inositol monophosphate

Agranoff *et al.* (26) studied the incorporation of tritium-labeled inositol into the lipids of guinea pig kidney and other tissues. Kidney mitochondria, in the presence of Mg^{++} and CMP or CDP-choline, were able to incorporate inositol into a lipid. Chromatography of the lipid and its water-soluble hydrolysis product suggested that it was phosphatidylinositol. Incorporation of labeled inositol was stimulated by phosphatidic acid, but not by D-α,β-diglyceride. In the absence of inositol, kidney preparations were able to catalyze the synthesis of a substance soluble in chloroform-methanol mixtures and believed to contain cytidylic acid. The substance was thought to be CDP-diglyceride. Agranoff *et al.* suggested the following scheme of biosynthesis:



Scheme G

The scheme differs from that for phosphatidylcholine in that the phospholipid P comes from glycerophosphate. Inositol is incorporated directly into the lipid, so that inositol phosphate does not appear as an intermediate. This would explain the failure of earlier workers to find an "inositol kinase."

The same workers make an interesting suggestion

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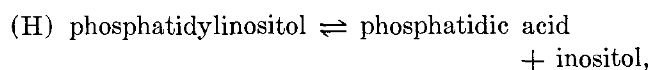
about the relatively high P^{32}-labeling *in vitro* of phosphatidic acid and inositide in certain tissues (74, 181). Evidence for the presence of phosphatidic acid in animal tissues has been doubtful until the recent isolation of the pure compound from ox liver by Hübscher and Clark (182, 183). Phosphatidic acid phosphatase (184), which hydrolyzes phosphatidic acid to diglyceride and inorganic phosphate, is inhibited by Mg^{++}. If the *in vitro* studies use a higher Mg^{++} concentration than is found *in vivo*, phosphatidic acid will be available for inositide synthesis instead of being hydrolyzed to diglyceride. This cannot be the complete explanation for brain, however, because the high P^{32} incorporation is also observed *in vivo* (185). The great difference in phosphate turnover between phosphatidylcholine and the phosphoinositides in brain does suggest that they are synthesized by different routes. In liver, though, this difference is not found (186).

Paulus and Kennedy (187, 188) consider that there are two possible mechanisms by which inositol may enter lipid molecules. The first is an exchange reaction catalyzed by guinea pig liver microsomes in the presence of Mn^{++} and tris (*tris* [hydroxymethyl] amino-methane), but not phosphate, buffer. Using phosphate buffer and low concentrations of Mn^{++}, the reaction is stimulated by the cytidine derivatives which Agranoff *et al.* quote (26). Otherwise the nucleotides have no effect.

The second mechanism is a true synthesis, stimulated by CTP. The evidence of Paulus and Kennedy (188) supports Agranoff's pathway (G) except that the former workers consider that CTP, rather than CDP-choline, is involved in the formation of CDP-diglyceride. Using a homogenate of whole guinea pig liver, they showed that inositol reduced the yields of an ether-soluble nucleotide, thought to be CDP-diglyceride. This would be expected if CDP-diglyceride reacts with inositol to give phosphatidylinositol and (ether-insoluble) cytidylic acid. Paulus and Kennedy also synthesized CDP-dipalmitin chemically, using dicyclohexylcarbodiimide. Addition of this compound greatly increased the incorporation of tritium-labeled inositol into the lipids of washed, dialyzed chicken liver microsomes. The phosphate of phosphatidylinositol was believed to come from glycerophosphate, in contrast to the results of McMurray *et al.* (189) on brain tissue.

This work illustrates one difficulty in working with radioactive intermediates in subcellular particulate systems. The tracer method is in a way too sensitive, and may detect reactions that have little significance *in vivo*. A false picture can result, for example, when these reactions are the reverse of those taking place in the tissues. The exchange reaction above may be due to an enzyme catalyzing the reaction:


(H) phosphatidylinositol $\rightleftharpoons$ phosphatidic acid + inositol,

though there is no other evidence for such a phospholipase. At equilibrium there may be 99 per cent hydrolysis of phosphatidylinositol, yet labeled inositol would appear to show synthesis of inositide because the reaction is reversible. Dils and Hübscher make the same point in their work on Ca^{++}-activated incorporation of choline into lecithin (174). In tracer studies, supporting chemical evidence of net synthesis is desirable.

Some work has been done on the incorporation of labeled compounds into the inositides of brain tissue, but, as McMurray *et al.* (189) point out, speculation about biosynthetic pathways is unprofitable until we know the structure of diphosphoinositide. Rossiter and his colleagues have studied uptake of P^{32} into rat brain phospholipids, using two systems, a hypotonic anaerobic system supporting labeling under optimal conditions for glycolysis and an aerobic system requiring conditions for oxidative phosphorylation. Thompson *et al.* (190) found CTP and CDP more effective than CMP or CDP-choline in stimulating incorporation of tritium-labeled inositol into the lipids of such systems. Phosphatidic acid increased labeling in the glycolyzing homogenate only. McMurray *et al.* (189) found that CTP stimulated the incorporation of P_i^{32}, but not P^{32}-labeled glycerophosphate into brain inositide. The biosynthetic problem in nervous tissue is further complicated by the recent demonstrations that phosphatidylinositol occurs in brain tissue as well as diphosphoinositide (81, 70). In addition, Hokin and Hokin showed that the former lipid had a much higher turnover of phosphate and inositol than diphosphoinositide. These authors also showed that acetylcholine stimulates incorporation of labeled phosphate and inositol equally into the phosphatidylinositol of brain slices. Inositol phosphate appeared to be incorporated as a unit into the inositide, while C^{14}-labeled glycerol incorporation was not stimulated by acetylcholine. These observations are not easy to reconcile with the pathway suggested by Agranoff.

ENZYMES HYDROLYZING PHOSPHOINOSITIDES

The phospholipases, of which the best known are the lecithinases, may be divided into four groups,

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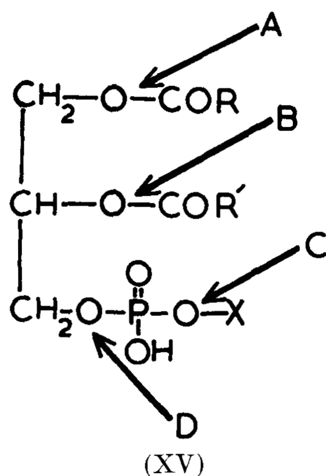
A, B, C, and D, each group attacking a different bond in a phospholipid of the structure shown (see review on lecithinases by Hanahan [191]).



(XV)

The B enzyme is more properly a lysophospholipase B.

The pathway by which phospholipids such as lecithin are broken down in animal tissues is far from clear (72), but there is less information still about the phosphoinositides.

Long and Penny (192) found that moccasin venom phospholipase A had no action on diphosphoinositide or phosphatidylinositol. Sloane-Stanley (193) first reported the enzymic hydrolysis of an inositol lipid. He used diphosphoinositide as substrate and worked with brain homogenates. Rodnight (194) showed that the enzyme occurred in other tissues such as liver, kidney, and spleen, and that it was activated by Ca^{++}. Inorganic phosphate and an organic ester believed to be inositol monophosphate were released from diphosphoinositide. Kemp *et al.* (195, 196) obtained the same type of enzyme from liver in a purer state. Phosphatidylinositol was also hydrolyzed by the enzyme. None of the other phospholipids studied, which included lecithin and cardiolipin, were attacked. Inorganic phosphate was not produced, and the authors considered that its presence in their less pure enzyme preparations indicated contamination with a phosphomonoesterase. Pileggi (197) has shown the presence of "phytase" activity in several animal tissues. Liver extracts were also able to hydrolyze inositol monophosphate.[8] The "phosphoinositidase" appeared to break down phosphatidylinositol to diglyceride and inositol 1-phosphate, but the products of its action on diphosphoinositide remain obscure.

Phosphatidylinositol has the ability to activate certain phospholipases. Dawson (198) showed that in concentrations as low as 30 μM it had this effect on a phospholipase B from *Penicillium notatum*. Lecithin was used as substrate. Liver "polyglycerophosphatide" (199) had similar activating properties. The *P. notatum* enzyme preparation was able to hydrolyze phosphatidylinositol itself, giving a mixture of glycerylphosphorylinositol, free inositol, inorganic phosphate, and fatty acids (200). This breakdown was inhibited by lecithin or lysolecithin. In contrast, the liver enzyme was not affected by these lipids (195, 196). Pancreatic phospholipase was also able to hydrolyze phosphatidylinositol. In this case diglyceride and inositol monophosphate may have been formed, but the evidence was not conclusive.

The mechanism of attack on lecithin by the *P. notatum* enzyme has been studied by Bangham and Dawson (201, 202). For enzymic attack to take place, they showed that the lecithin micelles had to have a net negative charge. This could be imparted by a variety of substances including dodecyl sulfate and dicetyl phosphoric acid, as well as the lipids cited above. Positive ions, such as Ca^{++}, inactivated the system. The nature of the net charge was shown by electrophoresis. An elegant and novel method of assaying phospholipase activity was used in some of these experiments. A film of P^{32}-labeled lecithin was spread on a Langmuir trough and a Geiger counter arranged above it. Hydrolysis of the lecithin released phosphate into the underlying aqueous phase, so reducing the rate of counting.

The lecithinase C of cabbage[9], which released choline from the lipid, is also activated by phosphatidylinositol (203). Maximum activity was obtained in the presence of Ca^{++}. In the case of the *P. notatum* enzyme, Ca^{++} appeared to inhibit activation by phosphatidylinositol.

PHOSPHOINOSITIDE TURNOVER STUDIES

Dawson (74, 204) showed that the incorporation of P_i^{32} into brain phosphoinositide and phosphatidic acid was greater *in vitro* than that into the other phospholipids. Brain dispersions were incubated with P_i^{32} in the presence of suitable cofactors. The hydrolysis products of the labeled phospholipids were separated by two-dimensional paper chromatography. It is doubtful whether the phosphate esters from phosphatidylinositol and diphosphoinositide separate well under these conditions. When these experiments and the

[8] P. Kemp, unpublished observations.

[9] Unfortunately, sometimes known as lecithinase D.

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similar ones of Hokin and Hokin (181) were performed, only diphosphoinositide was thought to occur in brain. Hokin and Hokin (81), in a more recent paper, state that in brain slices similarly incubated, phosphatidylinositol rather than diphosphoinositide was the heavily labeled compound. Results were based on chromatography of the intact phospholipids and electrophoresis of their hydrolysis products.

McMurray *et al.* (189) confirmed Dawson's results in two systems which are described in the section on biosynthesis. Under conditions for anaerobic glycolysis, as well as those for oxidative phosphorylation, inositol phospholipid and phosphatidic acid were much more heavily labeled than the other phospholipids. Identification was again based on paper chromatography.

Similar high turnover of P^{32} in rat brain phosphoinositide was found *in vivo* 3 hours after injection of the isotope into young animals (185). The specific activity of phosphoinositide was about ten times that of phosphatidylcholine. Certain psychotropic drugs affect phospholipid metabolism, though this is not necessarily specific or related to their therapeutic effects. Using brain slices, Magee *et al.* (205) found that Azacyclonol (10^{-3}M) stimulated synthesis of phosphoinositide, phosphatidylserine, and phosphatidic acid, but depressed that of phosphatidylcholine and phosphatidylethanolamine. Higher drug concentrations (5×10^{-3}M) depressed biosynthesis in all cases. Ansell and Morgan (206) found this same depression *in vivo* with rat brain, after a dose of 200 mg. per kg. body weight. Reserpine® (5 to 10 mg. per kg.) had no effect.

In liver, on the other hand, no great difference between the P^{32}-labeling of phosphatidylinositol and the other phospholipids was observed *in vivo* with rats (186). Six hours after injection of the radioisotope, phosphatidylcholine showed the highest P^{32} uptake, whereas phosphatidylethanolamine and phosphatidylinositol had about half the amount of P^{32}. Marinetti *et al.* (79), using rat liver homogenates, also failed to find a high turnover of inositol lipid. High P^{32} uptake was observed only in phospholipids resembling phosphatidic acid, but even this was not seen *in vivo* (76, 78).

Hokin and Hokin found relatively high incorporation of P_i^{32} into pancreas slices (207). Acetylcholine raised the rate of incorporation still farther. The results with pancreas and other tissues are summarized in Table 4.

Freinkel (124) incubated sheep thyroid slices with P_i^{32} or C^{14}-labeled glycerol. After 4 hours the phosphoinositide had almost five times the specific activity of the lecithin, when P^{32} was used. With labeled glycerol there was, if anything, less incorporation into the phosphoinositide than into lecithin, but the rates were low in both cases. This is probably because the glycerol derivatives needed for phospholipid biosynthesis (e.g., diglyceride, glycerophosphate) are available in the tissue slices. Thyrotropic hormone stimulated phosphoinositide-P turnover (see Table 4).

The Hokins have made the general suggestion that phosphoinositides are concerned with the active transport of proteins and other substances out of the cell. Higher turnover of phosphate in phosphoinositide was observed under conditions which caused secretion of amylase, epinephrine, ACTH, mucin, and thyroxin from suitable tissue slices (references in Table 4). Under the same conditions phosphatidic acid turnover was usually stimulated too, and a theory has been put forward that this lipid plays a part in the transport of cations across membranes (213, 214). According to this theory, phosphatidic acid is synthesized from diglyceride and ATP at the inner surface of a membrane consisting essentially of lipid material. This phosphatidic acid diffuses through the membrane, taking with it the cations. At the outer surface of the membrane phosphatidic acid phosphatase hydrolyzes phosphatidic acid, releasing inorganic phosphate and the cations, while the diglyceride diffuses back to the inner surface to be used again. In the case of the albatross salt gland, where sodium chloride rather than sodium phosphate is secreted, an additional mechanism for the removal of phosphate ions would be required. While phosphoinositide may be functioning in some similar way, only further work will lead to an understanding of the increased turnover at the molecular level.

Several interesting points arise from the work of the Hokins, however. When slices were incubated with C^{14}-glycerol instead of P_i^{32}, no increased turnover with acetylcholine was seen. The authors have suggested (81) that the phosphate and inositol of phosphatidylinositol turn over independently of the diglyceride, and that in brain tissue at least, inositol phosphate is incorporated into the phospholipid as a unit. The argument is based on the fact that acetylcholine produces similar percentage increases in the turnover of both phosphate and inositol, but, of course, the initial rates could be quite different. It does not seem possible to calculate these rates from the data presented by Hokin and Hokin.

Two other observations are relevant. The enzyme hydrolyzing phosphatidylinositol (195, 196), which is

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widely distributed, removes inositol phosphate as a unit. On the other hand, in the currently accepted biosynthetic pathway for phosphatidylinositol, the phosphate comes from phosphatidic acid, while the inositol is incorporated independently.

The stimulation of phosphate turnover in phosphoinositides has been shown only in preparations such as tissue slices, where the cell membranes are intact. This bears out the suggestion of the Hokins that these lipids are connected with transport of substances out of the cell. In subcellular particles from guinea pig brain, acetylcholine increased only the incorporation of P^{32} into phosphatidic acid (215).

A high phosphate turnover has also been observed in the "phosphatido-peptide" fraction from various animal tissues (119). This fraction often contains phosphatidylinositol. Huggins (216) found that acetylcholine increased still more the incorporation of P^{32} into the phosphatido-peptide of chicken pancreas slices. Turnover of the total phospholipid fraction was increased about threefold, while that of the phosphatido-peptide increased sixfold. The acetylcholine concen-

TABLE 4. STIMULATION OF P^{32} TURNOVER IN PHOSPHOINOSITIDE AND PHOSPHATIDIC ACID OF TISSUE SLICES

Tissue	Stimulating Agent	Effect	P^{32} Ratio: Stimulated/Control		Reference
			Phosphoinositide	Phosphatidic acid	
Rabbit submaxillary gland	Acetylcholine 10^{-5}M	Secretion of mucin	5 (whole phospholipid fraction)		Hokin & Hokin (207)
Rabbit parotid gland	Acetylcholine 10^{-5}M	Secretion of amylase	4 (whole phospholipids)		Hokin & Sherwin (208)
Pigeon pancreas	Acetylcholine 10^{-3}M	Secretion of amylase	17	1.6; 3	Hokin & Hokin (181)
	Acetylcholine 10^{-4}M	Secretion of amylase	(a)* 9-18; (b) 2-18	2	Hokin & Hokin (97)
Pigeon pancreas	Pancreozymin 100 μg./ml.	Secretion of amylase	5 (whole phospholipids)		Hokin & Hokin (209)
	Secretin (0.005-50 units/ml.)	Secretion of H_2O, HCO_3-	No effect	No effect	
Sheep thyroid	Thyrotropic hormone (0.1 units/ml.)	Secretion of thyrotoxin ?	5.3	—	Freinkel (210)
Guinea pig brain	Acetylcholine 10^{-2}M	?	2.3	2.1	Hokin & Hokin (181, 81)
Guinea pig adrenal medulla	Acetylcholine 10^{-5}M	Secretion of epinephrine	2.5	2.0	Hokin *et al.* (211)
Rat adenohypophysis	CRF† 0.3 or 0.9 μg./ml.	Secretion of ACTH‡	1.4	1.4	Hokin *et al.* (212)
Rat adrenal	ACTH‡ 3.3 milliunits/ml.	Cortico-steroid synthesis and secretion	No effect	No effect	
Albatross salt gland	Acetylcholine 10^{-4}M	Secretion of NaCl	3.5	16	Hokin & Hokin (213)

* (a) and (b) are the two phosphoinositides separable on paper chromatograms.
† CRF, posterior pituitary corticotropin-releasing factor.
‡ ACTH, adrenocorticotropin.

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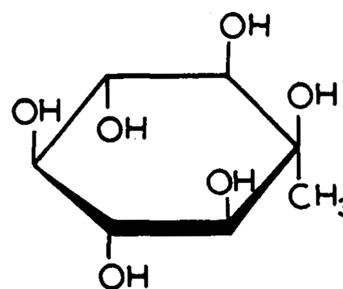
tration was 10^{-4}M. Acid-soluble phosphate turnover was unaffected. Huggins *et al.* (217) studied the P^{32}-labeled lipids of the phosphatido-peptide in greater detail. Chromatography on silica-impregnated paper or silicic acid columns revealed three components. One resembled phosphatidic acid and the other two were similar to phosphoinositides "a" and "b" of Hokin and Hokin (97).

It is possible that phosphatido-peptides are artifacts (123), due to nonspecific binding of acidic phospholipids by protein or peptide during the extraction procedures. These procedures are usually quite drastic, high speed homogenization of the tissue with 10 per cent trichloroacetic acid often being the initial step. It would be interesting to see whether the turnover of phosphoinositide in the phosphatido-peptide fraction is significantly different from the total phosphoinositide turnover in a given tissue.

Vladimirov *et al.* (218) equilibrated the acidified chloroform-methanol extract of P^{32}-labeled brain phosphatido-peptide with water. The phosphate compounds going into the aqueous phase had the highest specific activities, yet the inositol compounds remained in the chloroform. The exact nature of the various labeled phosphates has not been elucidated.

Moscatelli and Larner (219) injected C^{14}-inositol into rats and showed that after several hours the radioactivity appeared in exhaled CO_2, in liver glycogen, and in the tissue lipids. In starved rats, inositol incorporation into lipids was reduced by 90 per cent. Glucose and C^{14}-inositol were injected into a rat fed on a high protein and high carbohydrate diet. After 10 hours the animal was killed and the total lipids of brain, liver, kidney, and heart were isolated and counted. It was not proved that inositol was the only labeled lipid constituent, but it is at least likely to have been the major one. Moscatelli and Larner quote figures based on disintegrations per minute per mg. lipid. From data in the literature on phospholipid and inositol lipid contents of these tissues, the approximate relative specific activities of the tissue phosphoinositides can be calculated. Assuming that phosphatidylinositol is the only inositol lipid present, the relative specific activities would be: brain 17, liver 12, kidney 14, heart 85. The high turnover of heart phosphoinositides is noteworthy.

Posternak and Schopfer (220) studied the phospholipids of an inositol-requiring strain of *N. crassa.* Acid hydrolysis of the lipids released two different inositol phosphates separable by paper chromatography. Isomytilitol (XVI), which inhibited growth of the *N. crassa,* was found to be incorporated into the phospholipid in place of inositol. This interesting result may lead to an understanding of the mechanism of growth inhibition by isomytilitol.



(XVI)

OTHER ASPECTS OF METABOLISM AND FUNCTION

Under certain conditions (e.g., in fatty livers caused by biotin feeding) *myo*-inositol can act as a lipotropic agent. There is no definite evidence, however, that this lipotropic action is connected with the formation or metabolism of inositol phospholipids. It does not seem possible to produce fatty livers in rats by feeding diets deficient in inositol. With choline, which is a much more powerful lipotropic agent, this can easily be done. Deuel (221) has reviewed this subject and concludes that the mechanisms of action of choline and inositol as lipotropic agents are quite distinct.

McKibbin and Brewer (222) found that inositol feeding affected human plasma lipids very little. There were minor increases in total phospholipid, total cholesterol, and free cholesterol. Neutral fat apparently decreased.

Needless to say, the effect of oral phosphoinositides on experimental atherosclerosis has been studied (223). The rats used had been fed on diets rich in cholesterol. The phosphoinositide had no effect. Inositol itself was similarly inactive (224).

Challinor and Daniels (225) studied the growth of *Saccharomyces cerevisiae* in media containing reduced levels of growth factors. When the inositol content of the medium was reduced from 10 μg. per ml. to 0.6 μg. per ml., the appearance of the yeast changed considerably and it produced much more fat (24.5 per cent as compared with 1.6 per cent based on dry weight). The nature of the fat produced and the mechanism of this dramatic effect are still unknown.

It was once thought that pancreatic amylase contained inositol which was essential for enzymic activity. Use of purer preparations has shown this to be untrue (references in 226), but Sarma and his col-

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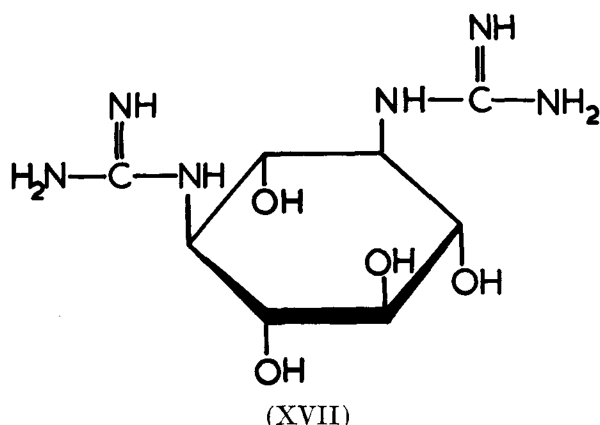
leagues (226, 227) have found an interesting relationship between inositol metabolism and amylase synthesis. In pigeon pancreas slices (227) ω-methyl pantothenic acid inhibited amylase activity, turnover of phospholipids, and synthesis of phosphoinositide. A relation between amylase synthesis and formation of inositol lipids was suggested. In several other biological systems inositol stimulated amylase synthesis (226). In the germinating cereal *ragi,* the pantothenic acid antagonist mentioned above reduced the level of amylase and of lipid inositol. Addition of pantothenic acid restored both to their original values.

Streptomycin is a derivative of 1,3-diguanido-*scyllo*-inositol (XVII). Its usefulness against *M. tuberculosis*



(XVII)

and the fact that this organism is rich in phosphoinositides have prompted the suggestion that the antibiotic may act by interfering with the metabolism of these lipids. Rhymer *et al.* (228) obtained some evidence that lipositol and a brain lipid fraction could suppress the activity of streptomycin against *S. aureus.*[10] Ata *et al.* (230) showed that *myo*-inositol was inactive against dihydrostreptomycin when the growth of *M. tuberculosis avium* was studied. However, after eight transfers in a medium containing 0.5-1 per cent *myo*-inositol, a streptomycin-resistant strain was obtained.

The remainder of this review must be largely speculative since so little is known of the function of phosphoinositides. Eagle *et al.* (39), who showed that *myo*-inositol is an essential growth factor for many human cells in tissue culture, suggest that its primary function may be in phosphoinositide metabolism. The concentrations of inositol required for optimum growth were higher than those expected of a typical vitamin or cofactor and point to a metabolic need for inositol.

The work of the Hokins, which has been summarized before, provides the soundest evidence so far with regard to function. It seems likely that the inositol lipids are concerned with the transport of materials across the cell membrane. Like all phospholipids, they may form part of many membranes within the cell, too, and it is unlikely that this role is merely structural (if indeed anything in a cell can be called merely structural).

After years of neglect, the phospholipids are beginning to be slightly fashionable in biochemical circles. One of the most promising fields in which they appear is that of the subcellular particles such as the electron transfer systems (128). The so-called "microsomal" fraction is also rich in phospholipid, and there are already (so far, doubtful) claims that phospholipids play a role in amino-acid activation for protein synthesis.

Murray *et al.* (231) have shown that the metaphase arrest caused in rat fibroblast culture by the mitotic poison colchicine could be reversed by *myo*-inositol. It is tempting to deduce from this that inositol derivatives play a part in the cell division process. However, tropolone, which has a seven-carbon ring structure, foreign (as far as we know) to animal tissues, is an even more powerful inhibitor of the colchicine effect (232). The possible role of inositol or its derivatives must therefore remain an open question. Soybean phosphoinositide (232) and liver phosphatidylinositol[11] were not effective in this system, but this may partly be due to the difficulty of getting such molecules inside cells.

Levin *et al.* (233) have studied phospholipid metabolism in rat liver following partial hepatectomy. As measured by P^{32}, the turnover of phospholipid increased with mitotic activity. This was due to a higher turnover of lecithin in all parts of the cell and a higher turnover of nuclear cephalin. Since cephalin from liver nuclei contains phosphoinositide, the latter may well take part in this stimulated activity.

From a chemical point of view it is paradoxical that phospholipids containing an unreactive cyclitol should be metabolically active. It is the inositol phosphate moiety in the phosphoinositides that holds most interest for the future. There is no doubt that this part of the molecule can be removed enzymatically, but we know little about the reasons for its removal or its further reactions with other cellular components. Chargaff (234) has speculated that it may act as a phosphate

[10] Later work failed to confirm this. (H. E. Carter, personal communication; also Reference 229.)

[11] H. H. Benitez, E. Chargaff, and J. N. Hawthorne, unpublished observations.

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donor, combining, for instance, with nucleosides to give nucleotides and free inositol. Reactions of this sort would depend on the ability of hydroxy-compounds to form cyclic phosphate intermediates, as has been indicated already. Phosphate cyclization could be of great importance in the metabolism of inositol phospholipids.

Though an attempt has been made to make this review comprehensive, I must apologize for overlooking significant papers, particularly in languages other than English. My thanks are due to Professor Erwin Chargaff, in whose stimulating company I first met the phosphoinositides, and to Dr. Georg Hübscher for helpful discussion of the metabolic aspects. I am also grateful to Mrs. R. Williams for all her work in preparing the manuscript.

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