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Evidence for the Structure of Brain Triphosphoinositide from Hydrolytic Degradation Studies

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In the preceding paper (Dittmer & Dawson, 1961) we have described the isolation from brain tissue of a new lipid, triphosphoinositide, which contains inositol, phosphate, glycerol and fatty acid in the molar proportions 1 : 3 : 1 : 2. In this paper a study of the chemical hydrolysis products of triphosphoinositide is reported, from which it can be deduced that the phospholipid is a diacylglycerylphosphorylinositol diphosphate. Similar observations have also been made upon a 'diphosphoinositide' fraction obtained from ox brain by the method of Folch (1949), which suggests that it may contain, as well as triphosphoinositide, a lipid that corresponds to a true diphosphoinositide. Preliminary results in this study have already been reported (Dawson & Dittmer, 1960).

EXPERIMENTAL

Methods. Triphosphoinositide and other brain-phospholipid preparations were prepared as described by Dittmer & Dawson (1961). These preparations were usually stored in chloroform at −18°. Since under these conditions preparations of the free acid were found to undergo slow hydrolysis over a period of several weeks, the triphosphoinositide was usually freshly prepared for each experiment and used within a few days or alternatively it was stored as the sodium salt.

The term fraction I is used here to designate the phosphoinositide-rich precipitate obtained by the addition of ethanol to a chloroform solution of brain kephalin as described by Folch (1949). The term Folch 'diphosphoinositide' refers to the insoluble fraction obtained from fraction I after a sequence of precipitations from chloroform with methanol (Folch, 1949). Although no evidence was obtained for decomposition of these fractions when stored in chloroform at −18°, freshly prepared fractions were used for the hydrolysis studies described.

Analysis. The methods used for the estimation of glycerol, inositol, phosphorus and acyl ester were those described by Dittmer & Dawson (1961).

Acid hydrolysis of phospholipids. A sample of a chloroform solution of triphosphoinositide containing 250–500 μg. of P was dried *in vacuo* and hydrolysed for 5–180 min. with 2 ml. of N- or 5N-HCl at 105°. After cooling, chloroform (4 ml.) was added and, after shaking and centrifuging, the upper aqueous layer was withdrawn and dried *in vacuo*. The lower chloroform layer was reserved when necessary for examination of the lipid degradation products. In some experiments these were removed by extracting twice with ether instead of chloroform. Even after short hydrolysis periods the P was almost all in the aqueous layer.

Mild alkaline hydrolysis of phospholipids. Mild alkaline hydrolysis was carried out by the procedure described by Dawson (1960) or alternatively a sample of the lipid, containing approx. 500 μg. of lipid P, was suspended in 8–10 ml. of aqueous 80% (v/v) ethanol, 0·25 ml. of aqueous N-NaOH was added and the mixture incubated for 5–20 min. at 37°. Neutralization of the hydrolysate and subsequent preparation of the phosphate esters for chromatography was carried out as described by Dawson (1960). However, the use of Amberlite IRC-50 was preferable to neutralization with ethyl formate if the hydrolysate was to be examined directly by paper ionophoresis. Excess of sodium formate in hydrolysates that were spotted directly for paper ionophoresis resulted in poor resolution of the phosphate esters. No such effect was observed if a prior chromatographic run was made with a solvent system as in the two-dimensional systems described below.

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Separation of phosphate esters in hydrolysates. Phosphate esters were separated either by paper ionophoresis at pH 3·6, or two-dimensionally by a combination of paper chromatography in phenol–water–acetic acid–ethanol and ionophoresis as described by Dawson (1960). Details of the detection and estimation of phosphate ester spots are also given in that paper.

For isolation of the phosphate esters from single-dimensional ionograms, the hydrolysate was applied as a band and after ionophoresis for 90 min. at 40 v/cm. the volatile buffer was removed by drying. [Pyridine–acetic acid–water (1:10:89, by vol.), pH 3·6, was used as the buffer for acid hydrolysates; increasing the pyridine to 5 vol. gave a better separation for alkaline hydrolysates.] The paper was then dipped in a modified form of the Rorem (1959) reagent (0·025% quinine sulphate dihydrate in ethanol), washed twice with ethanol to remove excess of quinine sulphate, and the separated bands were then marked by their fluorescence in u.v. light. Each band was cut out and the quinine salt of the phosphate ester eluted chromatographically with water. In a few experiments the bands were located by spraying with an indicator (0·04% bromocresol green in ethanol) but this was not as sensitive as the quinine sulphate method.

Examination of the lipid acid-hydrolysis products. The lipids in the ether or chloroform extract of the acid hydrolysates were examined by two methods.

(1) The ether extract was washed with 4% Na_2CO_3 to remove free fatty acids. The ether extract was dried and the mono- and di-glycerides were separated by four transfers in a countercurrent distribution system of equal volumes of light petroleum (b.p. 40–60°) and aqueous 85% (v/v) ethanol (Olley & Lovern, 1954). The mono- and di-glyceride fractions were characterized by measurement of their acyl ester and glycerol content.

(2) The lipids were fractionated on a silicic acid column according to a simplified form of the method of Hirsch & Ahrens (1958). The column (10 cm. long × 2 cm. diam.) was treated with light petroleum (60–80° b.p.) and then successively eluted with 100 ml. of 8% (v/v) of ether in light petroleum, 100 ml. of 25% (v/v) of ether in light petroleum and finally with 50 ml. of ether.

RESULTS

Acid hydrolysis of triphosphoinositide. Fig. 1 (*a*) shows the separation of the phosphorus products obtained after hydrolysis of triphosphoinositide (prepared from chloroform–methanol-extracted brain residue) for 5 min. in 5N-HCl at 100°. After such hydrolysis all the free and combined fatty acids were removed by extraction with organic solvents so none of the phosphate esters examined contained fatty acids. The minor components, inositol monophosphate, glycerophosphate and inorganic phosphate, were identified by using markers. The two major components were identified by isolation and analysis. The large fast-running ionophoretic spot (*A*), identified as inositol triphosphate, contained no glycerol, and contained phosphate and inositol in the molar ratio 3 : 1·01 and, in a second isolation, 3 : 0·98. The spot *B* contained phosphate and inositol in the ratio 2 : 1·08 and no glycerol, which indicates that it is due to an inositol diphosphate. In five experiments the inositol triphosphate yields were 77, 81, 77, 70 and 77% of the water-soluble P after 5 min. hydrolysis; the inositol diphosphate amounted to about 15–20%, whereas the glycerophosphate was 4–5% and inorganic phosphate and inositol monophosphate were each less than 1%. Examination of the hydrolysate for P-free polyols (Dawson, 1958) indicated that it contained free glycerol and a little free inositol.

The ether-soluble lipid degradation products were applied to a silicic acid column and eluted with chloroform. The first fraction obtained contained 40% of the acyl ester in the original triphosphoinositide hydrolysed, the ratio of acyl ester : glycerol was 2·01 : 1·0, and no inositol or P was present. This indicated that at least 40% of

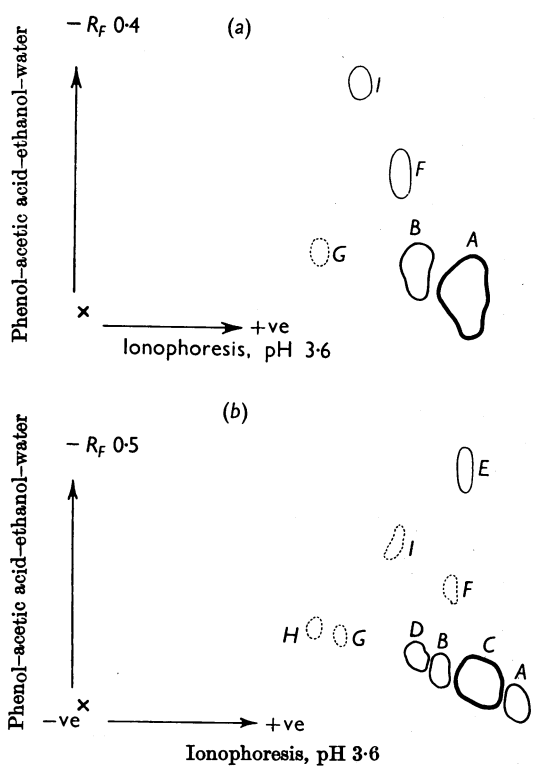

Fig. 1. Separation by paper chromatography and ionophoresis of (*a*) acid hydrolysis P-containing products and (*b*) alkaline hydrolysis P-containing products of triphosphoinositide. The thicknesses of the spots' outlines indicate relative intensities. Identity of spots: (*A*), inositol triphosphate; (*B*), inositol diphosphate; (*C*), glycerylphosphorylinositol diphosphate; (*D*), glycerylphosphorylinositol monophosphate (?); (*E*), cyclic glycerophosphate; (*F*), inorganic phosphate; (*G*), inositol monophosphate; (*H*), glycerylphosphorylinositol; (*I*), glycerophosphate.

the original acyl ester present in the lipid had been converted into diglyceride. In a second experiment the lipids, after being washed with Na_2CO_3 to remove free fatty acids, were run on a Hirsch & Ahrens (1958) column. Diglyceride was isolated in a yield of 30%; its acyl ester : glycerol ratio was 1·98 : 1·0.

When the triphosphoinositide prepared from a solvent-soluble 'diphosphoinositide' fraction (see Dittmer & Dawson, 1961) was examined a very similar pattern was found. Thus hydrolysis for 10 min. at 100° in N-HCl produced 75% of inositol triphosphate (inositol : P ratio, 1 : 3·1), 10% of inositol diphosphate (inositol : P ratio, 1 : 2·08), 6% of glycerophosphate and 6% of inorganic P and traces of inositol monophosphate. The distribution of glycerol in the hydrolysate was as follows, the lipids in this case being separated by the countercurrent distribution method: diglyceride (acyl ester : glycerol ratio, 2·07 : 1), 20%; monoglyceride (acyl ester : glycerol ratio, 0·96 : 1), 38%; glycerophosphate, 4%; free glycerol, 33%.

Experiments were performed to test how the phosphate-ester distribution changed on prolonging the acid hydrolysis. Table 1 shows that the inositol triphosphate disappeared, and at the same time there was an accumulation of inositol diphosphate, inositol monophosphate and a large amount of inorganic P. The glycerophosphate content of the hydrolysate did not change appreciably. If isolated inositol triphosphate was hydrolysed with 5N-HCl at 100° there was a slow accumulation of inositol diphosphate and inorganic phosphate.

Alkaline-hydrolysis products of triphosphoinositide. Mild alkaline hydrolysis of triphosphoinositide prepared from solvent-extracted brain tissue gave a relatively complex phosphate ester picture (Fig. 1*b*). A similar multiple breakdown has been observed to occur with phosphatidylinositol (Dawson, 1960). The products have been identified: (1) by the use of markers; (2) from the known behaviour of hydrolysis products of other lipids, e.g. phosphatidylinositol; (3) by isolation and analysis. The fastest-running spot on ionophoresis (*A*) had a mobility relative to inorganic phosphate which was identical with that of the main P-product of acid hydrolysis, i.e. inositol triphosphate. On analysis it contained no glycerol and a phosphorus : inositol ratio which was 3·0 : 1·04 and 3·0 : 0·98 in two separate isolations; thus it was confirmed that it was inositol triphosphate.

The major spot (*C*) contained phosphate, inositol and glycerol in the molar ratios 3 : 1·07 : 0·99 and 3·0 : 1·16 : 0·98 in two separate experiments. On acid hydrolysis (10 min. in 5N-HCl at 100°) *C* completely decomposed forming inositol triphosphate, glycerophosphoric acid and inositol diphosphate, the latter two compounds containing phosphorus in the concentration ratio of 1 : 2·05 respectively. The major phosphorus-containing product of mild alkaline hydrolysis is therefore probably a glycerylphosphorylinositol diphosphate. Under normal conditions of alkaline hydrolysis (Dawson, 1960) the compound was obtained in a yield of 44, 47, 46% in three experiments, but on using the modified method and avoiding acid conditions the yield was much higher (71, 78%).

Compound *B* (Fig. 1*b*) proved difficult to separate from *D*. On isolation the phosphorus : inositol ratio was 2·0 : 0·98 and 2·0 : 0·87 in two experiments but it also contained a little glycerol. From the relative mobilities and from evidence given later, spot *B* is probably due to inositol diphosphate and spot *D* to glycerylphosphorylinositol monophosphate. The latter may be formed from a true diphosphoinositide contaminating the preparation, as one would expect more inorganic phosphate to be present if it had been formed from triphosphoinositide. A little cyclic glycerophosphate is also present (*E*) and traces of inorganic phosphate (*F*), inositol monophosphate (*G*) and glycerylphosphorylinositol (*H*).

When triphosphoinositide preparations obtained from Folch's fraction I or purified 'diphosphoinositide' were examined by mild alkaline hydrolysis the phosphate esters obtained were very similar to those produced from the triphosphoinositide prepared from brain residue which had been extracted with chloroform–methanol. The major water-soluble phosphate ester on isolation had a molar ratio of glycerol : inositol : phosphate of 1·0 : 1·06 : 3·18, and 1·0 : 0·97 : 3·02 in two experiments. On acid hydrolysis (10 min. in 1N-HCl at 100°) of this compound, inositol triphosphate was formed as the major phosphorus-containing product as well as glycerophosphate and inositol diphosphate.

Examination of Folch's (1949) fraction I and 'diphosphoinositide'. Chromatography of mild alka-

Table 1. *Phosphorus-containing products in acid hydrolysate of triphosphoinositide*

Time of hydrolysis in 5N-HCl (min.)	Temp.	Percentage of total P as: Inositol triphosphate	Inositol diphosphate	Inositol monophosphate	Inorganic P	Glycerophosphate
10	100°	78	15	1	1	5
60	105	61	20	4	5	5
180	105	15	26	12	43	4

line and acid hydrolysates of Folch's (1949) fraction I of brain kephalin and purified 'diphosphoinositide' revealed a rather complex mixture of phosphorus-containing compounds. Both of these fractions produced on mild alkaline hydrolysis glycerophosphorylinositol diphosphate and glycerophosphorylserine which are undoubtedly derived from triphosphoinositide and phosphatidylserine respectively. Inositol triphosphate was also produced and was probably also derived from triphosphoinositide. Three products, glycerylphosphorylinositol, inositol monophosphate and glycerophosphate were found in the hydrolysates of fraction I but not in any appreciable amount in hydrolysates of 'diphosphoinositide'. Although these products may in part be derived from higher phosphoinositides, they probably arise principally from monophosphoinositide. This view is based on the finding (Dittmer & Dawson, 1961) that the monophosphoinositide of brain is separated out into the soluble phase during the precipitation procedure used in preparing 'diphosphoinositide' from fraction I.

Finally, two additional compounds were detected in mild-alkaline hydrolysates from both of these fractions. One of these migrated with inorganic phosphate on paper ionophoresis and the second, although its mobility was slightly less than that of inorganic phosphate, was only poorly separated from it. The combined fraction which migrated with inorganic phosphate, when eluted from the paper strip, was found, after correction for the inorganic phosphate present, to have an inositol : phosphate molar ratio consistently near to 1 : 2, but the glycerol : inositol ratio was of the order of 0·7 : 1. These results suggested that the fraction consisted of a mixture of inositol diphosphate and a compound which contains glycerol, inositol and phosphate in a molar ratio of 1 : 1 : 2. Separation of the mixture by paper chromatography with phenol–water–acetic acid–ethanol followed by elution and analysis of the bands substantiated this. The fractions obtained had glycerol : inositol : phosphate ratios of 0·17 : 1·0 : 1·97 and 1·0 : 0·93 : 2·1 respectively. The latter compound when hydrolysed at 100° with 1 N-HCl for 10 min. produced a mixture which contained the phosphorus distributed as inositol diphosphate (27 %), inositol monophosphate (22 %), glycerophosphate (30 %) and inorganic phosphate (14 %). The nature and yield of the products is consistent with what would be expected from the acid hydrolysis of glycerylphosphorylinositol monophosphate. Other possible structures cannot, however, be excluded.

Acid hydrolysis of fraction I and 'diphosphoinositide' also produced a more complex mixture than that found with triphosphoinositide. The main phosphorus-containing products produced from fraction I were inositol triphosphate, inositol diphosphate, inositol monophosphate and glycerophosphate. On the basis of what is known of the hydrolysis products of triphosphoinositide, the inositol triphosphate and part of the inositol diphosphate, inositol monophosphate and glycerophosphoric acid were formed from triphosphoinositide. It is likely, however, that glycerophosphoric acid is mainly derived from phosphatidylserine and monophosphoinositide, and the inositol monophosphate from monophosphoinositide. The molar ratio of inositol diphosphate to inositol triphosphate in these hydrolysates was much higher than that found for triphosphoinositide.

On mild acid hydrolysis, purified 'diphosphoinositide' gave a mixture of products consisting of inositol triphosphate (37 % of total phosphorus), inositol diphosphate (35 %), inositol monophosphate (7 %) and glycerophosphate (13 %). It seems possible that the additional inositol diphosphate (above that expected from triphosphoinositide hydrolysis) in these acid hydrolysates of fraction I and 'diphosphoinositide', and the compound formed on alkaline hydrolysis (probably glycerylphosphorylinositol monophosphate), are derived from a true diphosphoinositide. If this is so, it is possible from the alkaline hydrolysis products to obtain a picture of the distribution of lipids in Folch fraction I and also in the phosphoinositide after it has been purified by multiple methanol precipitations (Table 2).

Table 2. *Distribution of monophosphoinositide, diphosphoinositide, triphosphoinositide and phosphatidylserine in fraction I and purified 'diphosphoinositide' prepared by the method of Folch* (1949)

Values were derived from an examination of alkaline hydrolysis products.

	Fraction I		'Diphosphoinositide' Purified by 8 methanol precipitations		'Diphosphoinositide' Purified by 10 methanol precipitations	
	(% of total P)	(% of total mol.)	(% of total P)	(% of total mol.)	(% of total P)	(% of total mol.)
Monophosphoinositide	11·4	14·0	—	—	7·0	13·7
Diphosphoinositide	15·1	9·2	34·8	32·2	39·4	30·0
Triphosphoinositide	16·5	6·7	42·6	25·4	37·4	24·4
Phosphatidylserine	57	70·2	22·4	41·5	16·3	32·0

It can be seen that, although such precipitations partially remove monophosphoinositide and phosphatidyl serine, the ratio between triphosphoinositide and diphosphoinositide remains virtually unchanged.

DISCUSSION

Fig. 2 shows a summary of the chemical hydrolysis products of triphosphoinositide identified and studied in this paper. On mild acid hydrolysis the triphosphoinositide fatty acids, which in the parent compound are all linked through acyl ester bonds, are converted into diglyceride, monoglyceride and fatty acids. Since a diglyceride moiety could be deacylated by the acid either after or before it was hydrolysed away from the triphosphoinositide molecule, producing monoglyceride and free fatty acids, it seems reasonable to assume that in the parent compound the fatty acids are both esterified to the glycerol. Similarly, on acid hydrolysis 70–81 % of the phosphorus is liberated in the form of inositol triphosphate, and since triphosphoinositide contains fatty acid : glycerol : inositol : phosphate in the molar ratio 2 : 1 : 1 : 3, this would imply that the vacant hydroxyl group of the diglyceride is esterified to an inositol triphosphate unit. This conclusion is confirmed by the isolation of glycerylphosphorylinositol diphosphate as a major product of the deacylation of triphosphoinositide with alkali.

Acid and alkaline degradation therefore indicate that triphosphoinositide is a diacylglycerylphosphorylinositol diphosphate (phosphatidylinositol diphosphate). Since all naturally occurring phospholipids investigated have so far possessed the α configuration (see, for example, Baer & Maurukas, 1955), it seems likely that in triphosphoinositide the inositol triphosphate is also esterified to an α hydroxyl group in the glycerol. The present studies have shown that the two isomeric inositol triphosphates isolated by Grado & Ballou (1961) from acid hydrolysates of purified 'diphosphoinositide' are almost certainly derived from triphosphoinositide. These authors have reported that the major inositol triphosphate has the structure L-myo-inositol 1:4:5(6)-triphosphate and suggest that the intact lipid is similarly substituted. If this is so, it still remains to be determined to which phosphate grouping the diglyceride moiety is esterified.

Although it is certain that alkali preferentially attacks the acyl ester bonds of triphosphoinositide, the way in which acid attacks the lipid is less clear. Since diglyceride and inositol triphosphate are major hydrolysis products, it can be assumed that the bond between them is one site of attack. It is possible for the inositol diphosphate and glycerophosphate to be formed: (1) through deacylation followed by hydrolysis of the resultant glycerylphosphorylinositol diphosphate or its monoacyl derivative to inositol tri- and di-phosphate and glycerophosphate and possibly monoglyceride; or (2) through formation of phosphatidic acid and inositol diphosphate followed by deacylation of the former with the production of glycerophosphate and free fatty acid. The results obtained do not make it possible to decide between these two possibilities.

Our examination of Folch I fractions and purified 'diphosphoinositide' preparations has shown that the successive methanol precipitations partially eliminate phosphatidylserine and phosphatidylinositol in the supernatant. Nevertheless the results fully confirm the chromatographic conclusions of Hörhammer, Wagner & Hölzl (1960) on the complexity of the purified 'diphosphoinositide' fraction of Folch (1949). Whereas the molecular proportion of phosphatidylserine (32 %) in the preparations after ten methanol precipitations may at first sight appear large, it represents only about 0·5–0·6 % of N in the preparation [Folch (1949)

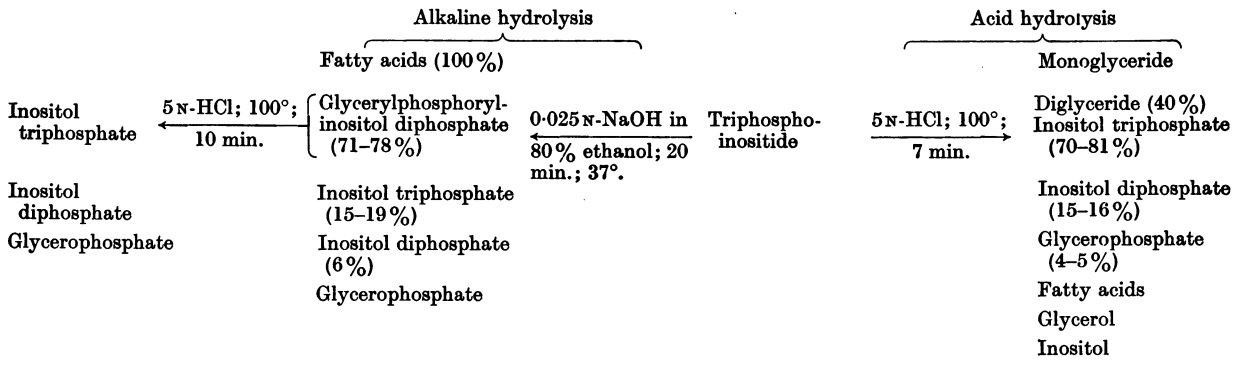

Fig. 2. Summary of the chemical hydrolysis products formed from triphosphoinositide. Phosphate esters formed are expressed as a percentage of the total P hydrolysed. Diglyceride and fatty acids are expressed as a percentage of the yield theoretically possible.

obtained 0·4–0·6% of N, 0·1–0·4% of amino N; and Grado & Ballou (1961) 0·3% of N].

On acid hydrolysis of these fractions (Folch I and purified diphosphoinositide), more inositol diphosphate is formed than can be accounted for by the triphosphoinositide present. Further, on alkaline degradation a considerable proportion of a compound is produced which is probably a glyceryl-phosphorylinositol monophosphate. These results suggest therefore that a true diphosphoinositide may either be present in nervous tissue or be produced from triphosphoinositide. While this compound has not yet been isolated, it is tempting to speculate from the evidence of its deacylation product that it is a phosphatidylinositol monophosphate. This would complete a series in which phosphatidylinositol (monophosphoinositide) and phosphatidylinositol diphosphate (triphosphoinositide) have already been isolated.

SUMMARY

1. Triphosphoinositide preparations isolated both from a 'diphosphoinositide' fraction of brain and from chloroform–methanol-extracted brain tissue have been hydrolytically degraded with alkali and acid.

2. Mild acid hydrolysis of triphosphoinositide produces inositol triphosphate as a major product, as well as inositol diphosphate and glycerophosphate. Diglyceride and monoglyceride have also been isolated from the reaction mixture.

3. Deacylation with alkali produces glyceryl-phosphorylinositol diphosphate as a major product, together with some inositol triphosphate, inositol diphosphate and a little normal and cyclic glycerophosphate. On acid hydrolysis the first compound splits, producing inositol triphosphate, inositol diphosphate and glycerophosphate.

4. From the above results it is deduced that triphosphoinositide is a diacylglycerylphosphoryl-inositol diphosphate (phosphatidylinositol diphosphate).

5. An examination of Folch (1949) fraction I and 'diphosphoinositide' preparations has shown that these probably contain, as well as triphosphoinositide, a true diphosphoinositide. Phosphatidylinositol and phosphatidylserine are also present.

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Interrelationships in Trace-Element Metabolism in *Aspergillus niger*

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The existence of an antagonism between heavy metals, e.g. cobalt, nickel, zinc, cadmium and copper, and essential ones, e.g. iron and magnesium, has been known for some time. The well-documented iron-deficiency 'chlorosis' in plants can be induced by high, extra-physiological concentrations of these heavy metals (Hewitt, 1951). Similar phenomena have not been studied as extensively in micro-organisms. Abelson & Aldous (1950) found that excess of cobalt, nickel, zinc, cadmium and manganese all interfered with magnesium metabolism in *Torulopsis utilis*, *Aerobacter aerogenes*, *Escherichia coli* and *Aspergillus niger*. The toxic effect on growth, with some of these organisms, could be counteracted by supplementing the culture media with magnesium. Healy, Cheng & McElroy (1955), working with *Neurospora crassa*, obtained evidence of deranged iron metabolism as

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