A FURTHER STUDY OF INOSITOL-CONTAINING LIPIDS

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Several lipids containing inositol have been shown to occur in plant or animal tissues, but the chemical structure of none is completely known. The present study of the inositol phosphates liberated by mild hydrolysis of these inositides was undertaken to obtain further information on this subject.

At least three different types of inositol phosphatide have been described. The first, about which most is known, is represented by brain diphosphoinositide[1]. According to FOLCH, who isolated it, this compound contains inositol diphosphate, glycerol and fatty acid in equimolecular proportions. The second type also occurs in animal tissues but contains inositol mono-phosphate, fatty acids and probably glycerol[2,3,4]. The third type, found so far only in plant and bacterial lipids[5–8] again contains inositol mono-phosphate, but this time linked to sugars, giving a more complex structure.

The phosphorus-containing hydrolysis products of these phosphatides have been conveniently separated by gradient elution with dilute HCl from strong-base anion exchange resin columns. The alcohol-insoluble phosphates from soy bean and groundnut inositides were compared by this method. When graphs of phosphate content against eluant volume were plotted, two identical curves were obtained, suggesting that the same lipids occur in each plant. This confirms previous chemical studies, inositol mono-phosphate, galactose and arabinose being obtained from both sources[7,8]. It seems unlikely that inositol diphosphate occurs in the molecule, as MALKIN AND POOLE suggested[7].

Mild alkaline hydrolysis of FOLCH's diphosphoinositide removes the fatty acids and gives a compound which is only eluted with difficulty from the resin columns mentioned above. It takes up 4 moles of periodic acid (2 per phosphate), which is twice as much as inositol *meta* diphosphate[1]. A similar compound was isolated in poorer yield by HAWTHORNE AND CHARGAFF after acid hydrolysis[8]. FOLCH's analyses indicate that inositol diphosphate, glycerol and fatty acid (1:1:1) are the only constituents of diphosphoinositide, and also that it has one titrateable acid group per phosphorus atom. In addition, glycerophosphate cannot be isolated from this compound. The unusual ring structure indicated in Fig. 1 would explain all these observations, but in the absence of more direct evidence it is only tentatively suggested.

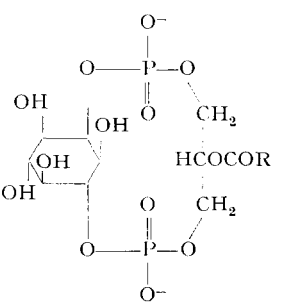

Fig. 1. Possible structure of diphosphoinositide.

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A previous study of the oxidation of this lipid with free periodic acid[8] showed an uptake of 3 moles per phosphate group. More careful studies using sodium periodate at pH 7.5 have shown the fairly rapid uptake of 1 mole HIO_4 per P. The pH by then had fallen to 5.5, presumably because of the liberation of formic acid, and a much slower uptake of a second mole of HIO_4 then took place. Under these conditions no more than 2 moles HIO_4 per P were taken up even after seven days. If the suggested structure is correct, the two stages of the oxidation would correspond with (1) a fairly rapid attack on the inositol ring (*cf.* FOLCH[1]) and (2) a slow hydrolysis with the liberation of either free glycerol or glycerol diphosphate and the aldehyde formed when the inositol ring was broken. The course of the hydrolysis would depend on whether cyclisation took place. Presumably either the β OH of the glycerol or the remaining OH of the inositol could be involved in such a cyclisation. In any case one product would be susceptible to further oxidation by periodate.

In Fig. 1 the positioning of the phosphates on the inositol ring is arbitrary, though if one of them is on the axial OH, bridging with glycerol is easier, to judge from models. The facts could also be accounted for by assuming a poly-inositol structure, with glycerol bridges between molecules of inositol diphosphate.

For comparative purposes an inositol monophosphate has been prepared from phytic acid by the action of wheat bran phytase[9]. Though bran phytase is not specific for this compound, the product obtained was optically inactive. FLEURY, DESJOBERT AND LECOCQ[10] recently produced a similar optically inactive monophosphate by enzymic hydrolysis of phytate, and showed it to be the 2-phosphate. The compound isolated in the present work is likely to be the same. Its optical inactivity suggests the 2 or 5 compounds or a racemic mixture.

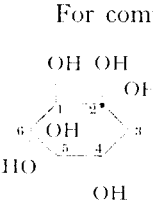

Fig. 2. *Meso*-inositol (• = axial OH).

Meso-inositol, of which phytic acid is the hexaphosphate, is generally agreed to exist in the chair form (see [11]), a model of which shows that the 2-OH group has an axial position, the other five lying in an equatorial plane. This may account for the apparent specificity of the phytase, hydrolysis of the 2-phosphate being sterically hindered by the adjoining groups.

The strain of *Acetobacter suboxydans* which oxidises polar hydroxyl groups in the free inositols[11] was without effect on this enzymically produced monophosphate. The apparent oxidation of the monophosphate from soy bean inositide observed by HAWTHORNE AND CHARGAFF[8] has now been shown to be due to an impurity in the phosphate, so that the position of the phosphate group remains an open question. It could be 2- or 5-. Pure samples of inositol monophosphate from soy bean and ground-nut inositides were both resistant to oxidation by *A. suboxydans**. A sample of inositol monophosphate prepared by the alkaline hydrolysis of phytic acid is also resistant to oxidation**. This compound may possibly be a mixture of isomers, but it contains little of the 2-compound[10,13], therefore it appears that even when the axial OH group is free, inositol monophosphate is not attacked by *A. suboxydans*. A phosphate group anywhere in the molecule exerts a general inhibitory effect on the oxidation.

* I should like to thank Prof. E. CHARGAFF for suggesting a re-investigation of the action of *A. suboxydans* on the inositol phosphates.

** Kindly supplied by Prof. P. FLEURY, Faculté de Pharmacie de Paris.

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METHODS

Most of these have been described previously[8].

EXPERIMENTAL

Preparation and hydrolysis of soy bean and ground-nut inositides

The soy bean phosphoinositide was prepared by the method used before[8]. The ground-nut lipid was obtained from the crude acetone-insoluble fraction of Nigerian ground-nut oil*. A product rich in inositide was prepared by dissolving 200 g crude lipid in 600 ml chloroform and precipitating with 1200 ml methanol. The precipitate was redissolved in 400 ml chloroform and filtered at the pump through a 2 cm layer of cellulose powder to remove water-soluble impurities. It was then reprecipitated with 1.5 volumes of methanol. After a third methanol precipitation similar to the second the phosphatide was emulsified with 500 ml water. 250 ml concentrated HCl were added and the mixture was boiled for 10 minutes in nitrogen. A similar method of hydrolysis was used for the soy bean lipid.

Isolation of soy and ground-nut inositol derivatives

The method consisted essentially of removal of HCl by vacuum distillation, precipitation of phosphates as their lead salts, decomposition with H_2S and trituration of the dried residue with alcohol, to remove phosphoric and glycerophosphoric acids. Details are given in a previous paper[8]. The alcohol-insoluble material was a white powder giving a strong positive Molisch test. A typical preparation from the ground-nut lipid had 8.9% P after two precipitations with alcohol, and was strongly dextrorotatory: $[\alpha]_D^{20} = +85°$ (water solution containing 0.35 mg P per ml).

A compound prepared by more vigorous acid hydrolysis (3.5 hours with boiling 5 *N* HCl) gave after one alcohol precipitation P = 9.38%, $[\alpha]_D^{20} = 4.43°$ (water solution, 2.49 mg P per ml). The Molisch test was negative, and by analogy with the soy bean lipid this substance was largely inositol monophosphate. On hydrolysis of this phosphate (6 *N* HCl, refluxed 18 hours) free inositol was liberated. It was detected by chromatography using the method of MEREDITH AND SAMMONS[14]. The developing reagent was 0.5% aqueous uranyl acetate (MEREDITH AND SAMMONS, unpublished)**, with which, while the paper is still damp, the inositol shows up in ultra-violet light as a yellow fluorescent spot. On heating at 90° for a few minutes the spot appears dark blue against a yellow background, in U.V. light.

Resin column treatment of vegetable inositol phosphates

The column, 28 cm long and 2.4 cm in diameter, was prepared from the strong-base anion exchanger De-Acidite FF (Permutit Co., London; Dowex-1 is an equivalent resin). Before use it was washed with *N* HCl and then distilled water until the eluant was neutral. The flow rate was about 5 ml per minute. 10 ml of a solution of the soy bean or ground-nut alcohol-insoluble phosphates, containing in all 5–15 mg phosphorus, were applied to the column and eluted with 0.01 *N* HCl. The eluate was collected in 25 ml fractions. After the first peak had been eluted no more phosphate could be found in the eluate, so the 0.01 *N* HCl was replaced by 0.01 *N* HCl containing 0.01 *N* NaCl. A second peak was then eluted. By increasing the NaCl concentration to 0.02 *N* a small amount of inorganic phosphate was sometimes eluted. Peak 1 evidently contained two components 1A and 1B, which were incompletely separated (see Fig. 3). It is possible that one is an arabinoside, the other a galactoside of inositol monophosphate, but further work will be needed before their structures are clear. Substances eluted from the pooled fractions had the following properties: (1A) 4.12% P, $[\alpha]_D^{20} = +84°$ in water; (1B) P = 4.65%, $[\alpha]_D^{20} = +109°$. After hydrolysis in an evacuated sealed tube with *N* H_2SO_4 at 110° C for 13

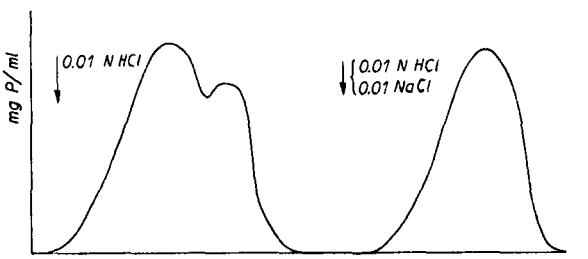

Fig. 3. Inositol compounds from soy bean and ground-nut inositides.

* I am indebted to Mr. H. H. HUTT of Messrs. J. J. Bibby & Sons, Liverpool, England, for a generous supply of this material.

** I am grateful to the authors, my colleagues in the Dept. of Pharmacology, for permission to use their unpublished data.

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hours, substance 1A showed one reducing group per phosphate present. Both substances gave a positive orcinol test for pentose, but this may have been due to incomplete separation of the peaks. The reason for the low phosphorus contents is unknown. Peak 2 contained no sugar and was optically inactive. Inositol monophosphate was eluted at the same concentration.

Hydrolysis of brain diphosphoinositide

The sample of diphosphoinositide used was prepared by FOLCH's method[1] but with fewer methanol reprecipitations. It contained 5.37% P and considerable amounts of serine phosphatide were present. 200 mg lipid was dissolved in 60 ml aqueous *N* KOH and put at 37° for 2 days. It was then brought to pH 6 with acetic acid and treated with 3 ml 25% lead acetate. Ammonia was added to bring the pH to 8.5 and the white precipitate was centrifuged down and washed twice with water. The lead was removed by H_2S and the filtrate concentrated in vacuum. The final solution, in 5.0 ml water, contained 3.03 mg P altogether.

2 ml of the above solution and 20 ml 0.14% sodium periodate were made up to 25 ml with water in a graduated flask. After 46 hours 4 ml aliquots were titrated, in the presence of excess sodium bicarbonate and potassium iodide, with 0.01 *N* arsenite. A blank was treated similarly. The periodate consumption was 1.9 moles per P-atom. The slightly low figure is presumably due to the presence of serine phosphatide in the original lipid.

The same solution gave two peaks when fractionated on the resin column. The first, representing about a third of the phosphate present, was eluted by 0.01 *N* HCl containing 0.02 *N* NaCl, the second only by 0.01 *N* HCl with 0.25 *N* NaCl. The latter arises from diphosphoinositide.

Periodate oxidation of intact diphosphoinositide

The sample used had 6.14% P and 0.51% N. It was emulsified in water and treated with Zeo-Karb 215 (Dowex-30 is similar) to remove cations. The solution was then brought to pH 7 with sodium bicarbonate: it contained 0.256 mg P per ml. 5 ml of it, with 20 ml 0.14% sodium periodate, were made up to exactly 50 ml. 2 ml aliquots were titrated at intervals in the manner outlined above. The results were corrected, on the basis of nitrogen content (diphosphoinositide is assumed nitrogen-free), for the presence of serine phosphatide. In 24 hours at room temperature 0.9 moles of HIO_4 per P were used. The uptake was then much slower, 1.8 moles per P being used in 7 days.

Inositol monophosphate from phytic acid

This was prepared by POSTERNAK's method[9]. The product was a white, optically inactive powder P = 10.18%. A crystalline cyclohexylamine salt was prepared by adding the theoretical amount of cyclohexylamine sulphate to the barium salt in a little water. The barium sulphate was centrifuged down and 10 volumes of *n*-butanol added to the supernatant. After shaking and standing at 0° for an hour the solution was filtered and an equal volume of ether added. Fine needles came down and were recrystallised by similar butanol-ether treatment. The m.p. was 192° (dec.) after some darkening at 185° (P = 6.47%, theory 6.76% for anhydrous salt).

Action of A. suboxydans *on inositol phosphates*

The experiments with *Acetobacter suboxydans* (A.T.C.C. No. 621) were performed in the Warburg apparatus using the conditions described by MAGASANIK AND CHARGAFF[11]. If the phosphates had been washed with ether or prepared by alkaline decomposition of the cyclohexylamine salt followed by ether extraction to remove cyclohexylamine, a large apparent oxygen uptake was noticed. The use of ether was therefore avoided. A slight oxidation of the inositol phosphate glycosides from soy bean was observed, also of arabinose and galactose, but this involved only about 0.2 atoms of oxygen per molecule. The inositol monophosphates prepared from phytic acid using phytase or alkaline hydrolysis[13] and those isolated from soy bean or ground-nut lipids were unattacked.

ACKNOWLEDGEMENTS

The author is indebted to Professor A. C. FRAZER for facilities used in this work and to Mr. JOHN WINROW for expert technical assistance.

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SUMMARY

The inositol phosphates released on hydrolysis of soy bean, ground-nut and ox brain inositides have been separated on an anion exchange resin column.

The inositol phosphatides of soy bean and ground-nut appear to be identical. Inositol monophosphate, linked glycosidically to galactose or arabinose, appears among the hydrolysis products. Inositol diphosphate could not be isolated.

A possible structure of brain diphosphoinositide is suggested, in which glycerol esterifies both phosphates of inositol diphosphate.

Acetobacter suboxydans did not attack any of the inositol monophosphates studied, at least one of which had a free axial hydroxyl group.

RÉSUMÉ

Les phosphates d'inositol libérés par hydrolyse des inositides du soja, de l'arachide et du cerveau de boeuf ont été séparés sur une colonne d'une résine échangeuse d'anions.

Les inositol phosphatides du soja et de l'arachide semblent identiques. Le monophosphate d'inositol, lié par une liaison glycosidique au galactose ou à l'arabinose, se trouve parmi leurs produits d'hydrolyse. Il n'a pas été possible d'isoler du diphosphate d'inositol.

Une structure possible du diphosphoinositide du cerveau est proposée; selon cette structure, le glycérol estérifierait les deux fonctions phosphate du diphosphate d'inositol.

Acetobacter suboxydans n'attaque aucun des monophosphates d'inositol étudiés, dont l'un au moins possède un groupe hydroxyle axial libre.

ZUSAMMENFASSUNG

Durch Hydrolyse von Inositiden aus Sojabohnen, Erdnüssen und Ochsengehirn erhaltene Inositolphosphate wurden an Hand einer Anionenaustauschsäule aus Kunstharz getrennt.

Inositolphosphatide aus Sojabohnen und Erdnüssen scheinen identisch zu sein. Inositolmonophosphat, durch Glykosidbindung an Galaktose oder Arabinose gebunden, erscheint unter den Hydrolyseprodukten. Inositoldiphosphat konnte nicht isoliert werden.

Es wird eine Strukturmöglichkeit der Gehirndiphosphoinositide vorgeschlagen, laut welcher beide Phosphate des Inositidphosphates durch Glyzerin esterifizert werden.

Acetobacter suboxydans griff keines der untersuchten Inositolmonophosphate an, deren mindestens eines eine freie axiale OH-Gruppe besass.

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Received May 4th, 1955