Phosphoinositides

4. INOSITOL LIPIDS OF SCYLLITOL-CONTAINING TISSUES*

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*myo*Inositol (I) is the only isomer of inositol yet found in phospholipids. *scyllo*Inositol (*scyllitol*, II) has long been known to occur free in the tissues of elasmobranch fishes (Staedler & Frerichs, 1858), in the acorn (Vincent & Delachanal, 1887) and in various other plants (Müller, 1907, 1912; Goodson, 1920; Hann & Sando, 1926). A search has been made, therefore, in two of these tissues, the acorn and the liver of *Raia clavata* (the common ray), for lipids containing *scyllo*inositol.

MATERIALS AND METHODS

Materials. Liver from *Raia clavata* was obtained from the Marine Biology Laboratory, Plymouth. Batches of 500 g. were packed in ice for transport to Birmingham. Two batches of acorns from the common oak (*Quercus robur*) were extracted. The first batch was picked in early September from local trees. The acorns were not fully ripe and were green. The second batch was collected from the ground in December. They were dark brown and some had begun to germinate. The two batches behaved similarly in the experiments reported below.

Pure *scyllo*inositol, prepared by the reduction of *myo*-inosose-2 according to Raymond (1957), was a gift from Dr R. B. Ellis.

Ionophoresis. Separations of *scyllo*- and *myo*-inositol by paper chromatography in methanol-water mixtures have been described by Fleury, Courtois & Malangeau (1953). Angyal, McHugh & Gilham (1957) suggested phenol-water (4:1, w/w). In the present work, authentic samples of the two isomers failed to separate in these solvents, but ionophoresis in borate solutions (Foster & Stacey, 1953) gave good separations. Strips of Whatman no. 1 paper (50 cm. × 7 cm.) held in position by glass plates were used. The electrolyte was 0.012 M-sodium tetraborate and a potential of 950 v was applied for 3 hr. Under these conditions the relative mobilities towards the cathode were: *scyllo*inositol 15 cm., *myo*inositol 4 cm. The best results were obtained by applying small volumes (approx.

0.01 ml.) of the inositol solutions to the paper strips, so that spots of liquid no bigger than 5 mm. in diameter were produced.

The silver nitrate method of Trevelyan, Procter & Harrison (1950) was used for detection of the spots. In the presence of borate, development of the spots is slower than usual. For this reason, the use of borate solutions stronger than 0.012 M was avoided.

Other analytical methods. Methods for the determination of phosphate and for the chromatography of phospholipid constituents have been described by Hawthorne & Hübscher (1959).

EXPERIMENTAL

Preparation of non-lipid inositols from acorns

Since the method used involves a hydrolysis, the products will include free inositols and inositols liberated from any water-soluble compounds such as phosphate esters. Two methods of preliminary treatment were used. In the first, the minced acorns (880 g.) were homogenized for 1 min. in a high-speed mixer with 1.2 l. of acetone. After 30 min. at room temperature the solvent was filtered off and distilled *in vacuo* until only a watery extract remained. Conc. HCl (2 vol.) was then added and the mixture refluxed for 18 hr.

In the second method, 500 g. of minced acorns was homogenized with 1 l. of 0.6 N-HClO₄. After 18 hr. at room temperature, the mixture was centrifuged and the solid re-extracted in the same way. The combined supernatants were filtered by gravity and neutralized with 5 N-KOH. The precipitated KClO₄ was filtered off and the solution refluxed with 2 vol. of conc. HCl as above.

After refluxing, the mixture in each case was cooled and filtered to remove the copious black precipitate. The clear brown filtrate was concentrated *in vacuo* to a syrup, to remove most of the HCl, then redissolved in 50 ml. of water and decolorized with charcoal. The filtrate after charcoal treatment was mixed with excess of Dowex 50 (100–200 mesh; H⁺ form) and Dowex 2 (100–200 mesh; OH⁻ form). The resins were removed by filtration and the colourless ion-free liquid was tested for the inositols as described below.

Characterization of the non-lipid inositols of acorn

The colourless liquid obtained by the method described above was evaporated on a water bath to a volume of 5 ml., and while it was still hot an equal volume of hot absolute ethanol was added. After an hour at 4°, the mixture deposited white crystals. These melted at a temperature above 300° and so could have contained little

* Part 3: Kemp, Hübscher & Hawthorne (1961).

*myo*inositol (m.p. 225°). *scyllo*Inositol melts with decomposition at 352° (Raymond, 1957).

Ionophoresis of the concentrated aqueous solution (0.01 ml./spot) from another batch of acorns showed a strong *scyllo*inositol spot and a much fainter *myo*inositol spot. To judge from the size and intensity of the spots there was roughly ten times as much *scyllo*inositol as *myo*inositol.

Preparation of free inositols from Raia clavata liver

In this case no hydrolysis step was included, so that the products represent free inositols only.

Again, two preliminary methods were used. In the first, the liver (255 g.) was homogenized with 500 ml. of water in a high-speed mixer. The homogenate was filtered overnight by gravity and the material on the filter paper re-extracted twice with 500 ml. of acetone each time. Acetone and water extracts were combined and the acetone was removed by vacuum distillation.

In the second method, the liver (580 g.) was homogenized with 1 l. of 0.6N-HClO₄. On standing overnight the liver tissue, owing to its high content of oil, rose to the surface and most of the extract could be removed by aspiration. Extraction with a second 1 l. of the acid was performed in the same way. The combined extracts were neutralized with 5N-KOH and filtered.

Extracts obtained by either of these methods were treated as follows. Excess of basic lead acetate was added and the white precipitate allowed to settle for several hours. The clear supernatant was then poured off and the precipitate collected by centrifuging. After three water washes in the centrifuge, the precipitate was suspended in 300 ml. of water and decomposed with excess of H₂S. The PbS was removed by filtration and the clear filtrate concentrated *in vacuo* to about 50 ml. After deionizing with Dowex 2 and Dowex 50 the filtrate was tested for *scyllo*- and *myo*-inositol.

Free inositols of Raia clavata liver

The aqueous extract prepared as described in the section above was treated in the same way as the acorn extract to induce crystallization. An extract prepared from 750 g. of liver gave 230 mg. of a white crystalline solid. A sharp melting point could not be obtained, but the gradual decomposition between 250° and 300° suggested that *scyllo*inositol was the main component. For confirmation the product was acetylated.

Acetylation. The crystals (222 mg.) were dried at 98° *in vacuo* over P₂O₅ for 1 hr. They were then warmed with 10 ml. of acetic anhydride containing 0.3% (v/v) of 72% (w/w) HClO₄ until all had dissolved. After 20 min. the mixture was poured into 500 ml. of ice-water. An oil was produced, which solidified after 2 hr. in the cold with occasional stirring. The solid was filtered off at the pump, washed well with cold water and dried over P₂O₅ *in vacuo* at room temperature for 18 hr. On treatment with boiling absolute ethanol, a little of the product dissolved, leaving 265 mg. of white needles, m.p. (uncorr.) 285–286°. Hann & Sando (1926) give the melting point of *scyllo*inositol hexaacetate as 291° when recrystallized from acetic acid.

Ionophoresis. A solution obtained from a second sample of liver gave two spots on electrophoresis in borate solution. The stronger one corresponded with *scyllo*inositol and the

weaker (roughly one-fifth the amount) with *myo*inositol. Since no attempt at recrystallization had been made, which would concentrate the less soluble *scyllo*inositol, these relative amounts apply to the free inositol isomers in the original liver tissue. The same is true of the acorn electrophoresis results.

Preparation of acorn phospholipids

The residue from the extraction of the acorns (880 g.) with acetone (2 × 1.2 l.) was extracted twice with 1.5 l. of chloroform-methanol (2:1, v/v). The combined chloroform-methanol extracts were concentrated to a volume of about 30 ml. *in vacuo*. Acetone (500 ml.) was then added to precipitate crude phospholipid and remove pigment. After 2 days at 4°, the precipitate was separated by centrifuging and shaken well with 50 ml. of chloroform. After filtering to remove non-lipid material, the chloroform solution was concentrated *in vacuo* to 5 ml. and treated with 100 ml. of acetone. In one case no precipitate could be obtained until a few drops of aqueous 20% CaCl₂ solution were added. The phospholipid precipitate was spun down and stored in chloroform solution. The average yield of phospholipid (as phosphorus) was 2 mg./kg. of fresh acorns.

Hydrolysis of acorn phospholipids

From 880 g. of acorns a chloroform solution containing only about 2 mg. of phospholipid phosphorus was finally obtained. A sample equivalent to 740 μg. of phosphorus was dried and hydrolysed with 0.4 ml. of 2N-HCl in a sealed tube at 110° overnight. The cooled hydrolysate was treated with charcoal (previously shown not to absorb the inositols) and then with Dowex 2 (OH⁻ form). The hydrolysate was evaporated to a small volume and ionophoresis carried out on samples corresponding to about 200 μg. of inorganic phosphorus in the original hydrolysate. A strong spot in the *myo*inositol position was seen, but no *scyllo*inositol.

Washing procedure. Since phospholipids are noted for their ability to carry non-lipid contaminants into solution in organic solvents, a second sample of the chloroform solution of acorn phospholipids was washed before hydrolysis.

A 3 ml. portion of the chloroform solution (360 μg. of phosphorus) was shaken well with 1 ml. of 0.1M-KCl and 0.5 ml. of 2N-HCl. The mixture was centrifuged and the upper aqueous layer removed. To the lower layer and interfacial material 0.5 ml. of 2N-HCl, 1 ml. of 0.1M-KCl and 0.5 ml. of methanol were added. The mixture was again shaken and centrifuged. The aqueous layer was removed. The interfacial lipid and lower chloroform layer were hydrolysed as described above. Again, electrophoresis showed only *myo*inositol, in amounts that showed that very little could have been removed by the washing. The *myo*inositol was therefore a lipid component.

Preparation of Raia clavata liver phospholipid

The fish liver (500 g.) was homogenized with 1 l. of acetone. The solvent, which extracted much oil from the tissue, was filtered off at the pump and the residue re-extracted with 1 l. of acetone. After this second portion of acetone had been filtered, the solid was extracted twice with 1 l. of chloroform-methanol (2:1, v/v). These two

extracts were combined and evaporated *in vacuo* to a volume of about 30 ml. Excess of acetone was then added and the mixture kept at 4° overnight. The precipitate was centrifuged down and suspended in 30 ml. of chloroform. Insoluble matter was removed by centrifuging and 200 ml. of absolute ethanol added to the lipid solution, precipitating a 'kephalin' fraction. The supernatant was concentrated *in vacuo* to approximately 10 ml. and a 'lecithin' fraction precipitated with 200 ml. of acetone. This lecithin was stored in chloroform solution. The kephalin fraction was redissolved in 10 ml. of chloroform, precipitated once more with 200 ml. of absolute ethanol and also stored in chloroform solution. The approximate yield of phospholipid (as phosphorus)/kg. of fresh liver was lecithin 10 mg., kephalin 6 mg.

Hydrolysis of Raia clavata liver phospholipids

Samples of the lecithin and kephalin fractions were washed and hydrolysed as described for the acorn lipid. Chromatography of the hydrolysate in propan-2-ol-acetic acid-water (3:1:1, by vol.) showed glycerol in both hydrolysates. Choline was seen in the lecithin hydrolysate. Three ninhydrin-reactive spots, the strongest of which resembled serine in position, were seen in the kephalin hydrolysate.

After treatment with charcoal and Dowex 2, samples of the hydrolysate were subjected to borate ionophoresis. A strong *myo*inositol spot was given by the kephalin hydrolysate, a weak one by the lecithin. No *scyllo*inositol could be detected.

DISCUSSION

The present work shows that the amount of free *myo*inositol in the acorn and in ray liver is small compared with that of free *scyllo*inositol. Müller (1912) failed to find any of the *myo*-compound in the tissues of the spur dogfish, though *scyllo*inositol was isolated without difficulty.

In spite of the predominance of *scyllo*inositol in the tissues studied, only the *myo*-isomer is selected for phospholipid synthesis. It is difficult to exclude completely the presence of 'phosphoscyllitides', but the evidence suggests that they cannot be present in more than minute amounts.

The chemical structures of *myo*inositol (I) and *scyllo*inositol (II) differ very little. The only difference lies in the presence of an axial 2-hydroxyl in *myo*inositol, giving (if the ring is regarded as planar) a trio of *cis*-hydroxyl groups instead of the all-*trans* *scyllo*inositol structure. Since *scyllo*inositol is not incorporated into the lipids it would appear that the enzyme incorporating inositol into the phospholipid is specific for the *myo*-structure. If the pathway (Paulus & Kennedy,

1960) applies to the tissues studied here, this enzyme catalyses the reaction:

The actual linkage of *myo*inositol to phosphate in phosphatidylinositol, the only phosphoinositide completely characterized so far, is through the 1-hydroxyl (Pizer & Ballou, 1959), but it is likely that the 2-hydroxyl and other neighbouring groups affect the enzyme-substrate interaction. Paulus & Kennedy showed that D-inositol (III), L-inositol and pinitol (5-O-methyl-D-inositol) failed to react. Taking these compounds and *scyllo*inositol into consideration, the minimum requirements for enzymic reaction appear to be a trio of adjacent *cis*-hydroxyl groups (i.e. equatorial hydroxyl group on C-1 and C-3, axial on C-2).

SUMMARY

1. Free *scyllo*inositol and *myo*inositol have been found in the liver of the common ray (*Raia clavata*) and in the acorn, the latter compound in relatively small amounts.

2. In the phospholipids of these tissues *myo*inositol, but no *scyllo*inositol, could be detected.

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REFERENCES

- Angyal, S. J., McHugh, D. J. & Gilham, P. T. (1957). *J. chem. Soc.* p. 1432.
 Fleury, P., Courtois, J. & Malangeau, P. (1953). *Bull. Soc. Chim. biol., Paris*, **35**, 537.
 Foster, A. B. & Stacey, M. (1953). *Chem. & Ind.* p. 279.
 Goodson, J. A. (1920). *J. chem. Soc.* **117**, 140.
 Hann, R. M. & Sando, C. E. (1926). *J. biol. Chem.* **68**, 399.
 Hawthorne, J. N. & Hübscher, G. (1959). *Biochem. J.* **71**, 195.
 Kemp, P., Hübscher, G. & Hawthorne, J. N. (1961). *Biochem. J.* **79**, 193.
 Müller, H. (1907). *J. chem. Soc.* **91**, 1767.
 Müller, H. (1912). *J. chem. Soc.* **101**, 2383.
 Paulus, H. & Kennedy, E. P. (1960). *J. biol. Chem.* **235**, 1303.
 Pizer, F. L. & Ballou, C. E. (1959). *J. Amer. chem. Soc.* **81**, 915.
 Raymond, D. (1957). *Helv. chim. acta*, **40**, 492.
 Staedler, G. & Frerichs, T. F. (1858). *J. prakt. Chem.* (I), **73**, 48.
 Trevelyan, W. E., Procter, D. P. & Harrison, J. S. (1950). *Nature, Lond.*, **166**, 444.
 Vincent, C. & Delachanal (1887). *C.R. Acad. Sci., Paris*, **104**, 1855.