crude product at this point. The combined solutions were chromatographed on Alorco F-20 alumina (3.3 cm. diam., 15 cm. long), then developed and eluted with 250 cc. of benzene, 1000 cc. of 9:1 and 2500 cc. of 4:1 benzene–chloroform. The ether traveled through the column as a deep-red band, and was saved in a separate eluate. Unetherified 7-hydroxyphenothiazone-3 and some dark impurities remained strongly adsorbed at the top of the column. Evaporation of the eluate left the ethers as a partially crystalline solid. Vacuum treatment at 78° removed some volatile material, and recrystallization from hexane–benzene and from acetone provided garnet-red lath-like crystals (1) m. p. 124.5–126°. The substitution of amyl bromide for the iodide resulted in only 7% yield of the desired ether.

Repetition of this preparation with a larger amount of crude material (31.5 g.), eliminating the vacuum heating step, gave material (2) melting at 131–132°.

Acknowledgment.—The writers are indebted to Dr. E. J. Eastmond, Mrs. Bernice Williams and Mr. G. L. Bailey for the measurements of spectral absorption, to Mr. L. M. White and Miss Geraldine Secor for microchemical analyses, and to Dr. F. T. Jones for microscopical investigation of numerous products.

Summary

Thionol (7-hydroxyphenothiazone-3) has been prepared by condensation of *p,p'*-dihydroxydiphenylamine with sulfur, and by sulfuric acid oxidation of phenothiazine followed by isolation as the lithium salt. The yield from the previously reported latter process has been increased by shortening the reaction time.

The ethyl, amyl, octyl, dodecyl and hexadecyl ethers of 7-hydroxyphenothiazone-3 have been prepared by treating the silver salt with the appropriate alkyl iodides.

3-Octoxyphenothiazine-5-oxide has been made by alkaline peroxide treatment of 3-octoxyphenothiazine.

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[CONTRIBUTION FROM THE BANTING AND BEST DEPARTMENT OF MEDICAL RESEARCH, UNIVERSITY OF TORONTO]

Synthesis of Inositol-5-monophosphoric Acid and Scyllitol Monophosphoric Acid[1]

BY BEAT M. ISELIN[2]

The widespread occurrence of inositol phosphoric acids in plants has long been recognized.[3] For many years inositol hexaphosphoric acid, or phytic acid was the only inositol phosphate known until Anderson,[4] in 1914, succeeded in isolating inositol monophosphoric acid from wheat bran. The same substance has been obtained by the action of the enzyme phytase on phytic acid[5,6] or by partial hydrolysis of phytic acid with dilute sulfuric acid.[7] More recent investigations have shown that inositol monophosphoric acid is a constituent of many phosphatides. Anderson has found this substance as a polysaccharide in the phosphatide fraction of tubercle bacilli.[8,9] Klenk and Sakai have described a preparation of inositol monophosphoric acid from soy bean lipositol[10] in which, as has been demonstrated by Woolley,[11] it is combined with galactose in glycosidic linkage.

The inositol monophosphoric acids isolated from natural sources are optically inactive. Their structure has not been investigated as yet. However, in the light of the present knowledge of the configuration of meso-inositol it is evident that only those mono-substituted derivatives are optically inactive in which the substituent is in position 2 or 5[12]; substitution in other positions is expected to yield products with optical activity. Assuming that the natural inositol monophosphates are not resolvable it may be concluded that the phosphoric acid residue is attached to carbon atom 2 or 5.

The synthesis of an inositol monophosphoric acid carrying the substituent on carbon atom 5 was effected by taking advantage of the known fact that *Acetobacter suboxydans* oxidizes meso-inositol specifically in position 5 yielding scyllo-meso-inosose. This substance, by acetylation and subsequent catalytic hydrogenation of the keto group with platinum oxide in glacial acetic acid, is converted to 1,2,3,4,6-pentaacetyl-meso-inositol as has been described by Posternak.[13] When the directions for the hydrogenation of scyllo-meso-inosose pentaacetate given by this author were closely followed, a product was obtained that had the recorded melting point (161–162°); acetylation to the hexaacetate and the bioassay of the hydrolyzed material with *Saccharomyces cerevisiae* revealed, however, that the product contained approximately 25% of the scyllitol isomer. It was found impossible to achieve a satisfactory separation of the two isomers by fractional crystallization. If the hydrogenation of scyllo-meso-inosose pentaacetate was carried out using methanol in-

(1) Presented at the 115th meeting of the American Chemical Society, San Francisco, March, 1949.

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(3) *E. g.*, E. Winterstein, *Ber.*, **30**, 2299 (1897).

(4) R. J. Anderson, *J. Biol. Chem.*, **18**, 441 (1914).

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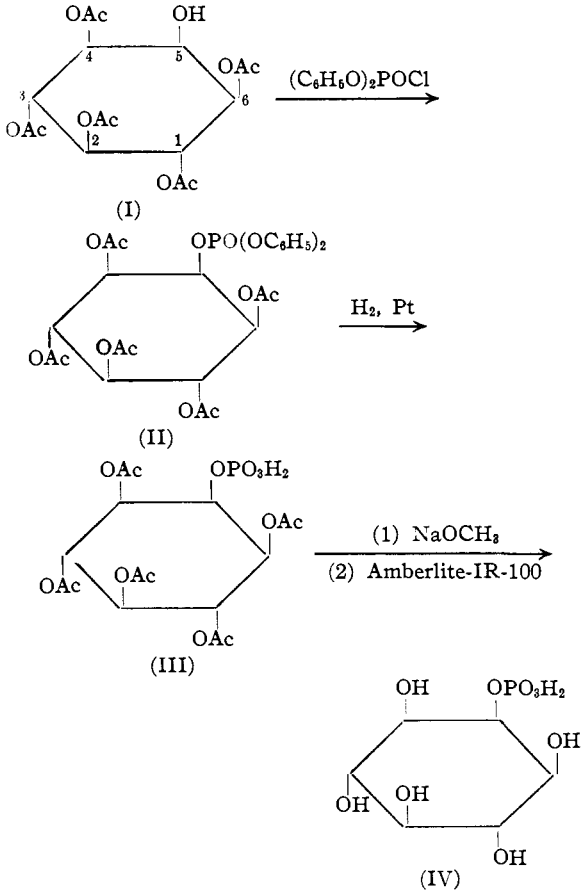
stead of acetic acid as a solvent, 1,2,3,4,6-pentaacetyl-meso-inositol (I) melting at 177–179° was obtained that contained no detectable amount of the scyllitol isomer.

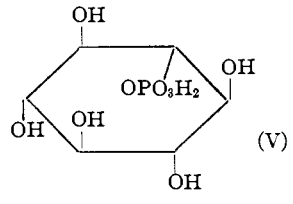


Attempts to phosphorylate this substance by treatment with phosphorus oxychloride were unsuccessful. Quite unexpectedly the substance also proved to be rather resistant to phosphorylation with diphenylchlorophosphonate at low temperatures though this phosphorylating agent has been shown to react very rapidly with primary or secondary hydroxyl groups of carbohydrates.[14,15,16] By increasing the reaction temperature to 80° and by the use of an excess of diphenylchlorophosphonate it was possible to obtain 1,2,3,4,6-pentaacetyl-inositol-5-diphenylphosphate (II) in 64% yield. Removal of the phenyl groups by reductive cleavage with hydrogen in the presence of platinum oxide yielded 1,2,3,4,6-pentaacetyl-inositol-5-phosphoric acid (III) (80%).

The hydrolysis of the acetyl groups could be effected by various methods. Best results were obtained by catalytic saponification with sodium methylate in methanol. The sodium salt of inositol-5-phosphoric acid formed in the course of this reaction in quantitative yield is of sufficient purity for biological purposes. Removal of the cation by means of ion exchange gave inositol-5-phosphoric acid (IV) that crystallized from aqueous ethanol in hexagonal plates melting at 198–200° with decomposition. Both the free acid and the sodium salt were optically inactive. All the salts of inositol-5-phosphoric acid, with the exception of the lead salt, were easily soluble in water. The barium salt was precipitated as a white amorphous powder from an aqueous solution by addition of ethanol. Hydrolysis of inositol-5-phosphoric acid with hydrochloric acid gave meso-inositol which, after acetylation, proved to be identical with the hexaacetate of natural meso-inositol.

Inositol-5-phosphate was not attacked by *Acetobacter suboxydans* as was to be expected since the point of attack by the bacterial enzyme, the hydroxyl group on carbon atom 5, is blocked by a substituent. In bioassays with *Saccharomyces cerevisiae* the substance had approximately 4% of the activity shown by free meso-inositol whereas in assays with *Neurospora crassa* it was about 10% as effective as inositol. This is in agreement with the finding of Woolley[17] who demonstrated that inositol monophosphate isolated from soy bean lipositol had an activity of the same order. On hydrolysis of inositol-5-phosphoric acid the calculated amount of meso-inositol was liberated as could be shown in tests with *Neurospora crassa*.

The preparation of scyllitol monophosphoric acid[18] started from the mixture of inositol- and scyllitol-pentaacetates as obtained by catalytic reduction of scyllo-meso-inosose pentaacetate in glacial acetic acid.[13] Phosphorylation with diphenylchlorophosphonate gave a mixture of pentaacetylinositol-5-diphenylphosphate and of pentaacetylscyllitol-diphenylphosphate. The two components could not be separated at this stage, but after reductive cleavage of the phenyl groups the pentaacetyl-scyllitol-phosphoric acid could be separated easily from the corresponding derivative of meso-inositol by fractional crystallization. Catalytic saponification of the acetyl groups with sodium methylate yielded the sodium salt of scyllitol phosphoric acid. Removal of the cation by means of ion exchange gave scyllitol monophosphoric acid (V) that melted at 212–214°. The acid and its salts have characteristics similar to those of inositol-5-phosphoric acid. Hydroly-



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(18) Not numbered since all mono-substituted derivatives of scyllitol are identical due to the symmetric structure of the molecule.

sis with hydrochloric acid gave scyllitol in nearly quantitative yield.

Scyllitol monophosphoric acid was completely inactive in the bioassay with *Neurospora crassa* before and after hydrolysis of the phosphoric acid residue.

Experimental[19]

1,2,3,4,6-Pentaacetyl-inositol (I).—A suspension of 4 g. of scyllo-meso-inosose pentaacetate,[13] m. p. 147°, in 80 ml. of dry methanol was shaken with hydrogen at room temperature and atmospheric pressure in the presence of 0.8 g. of platinum oxide (Adams catalyst). In the course of the reduction the starting material gradually dissolved. The absorption of hydrogen stopped after five hours when 1.1 moles of hydrogen had been consumed. After removing the catalyst by filtration the solution was concentrated at reduced pressure to 25 ml. On cooling, the 1,2,3,4,6-pentaacetyl-inositol crystallized in fine needles that were filtered after standing at 0° overnight; yield 2.73 g., m. p. 174–178°. From the mother liquor, after concentration to 5 ml., an additional 0.64 g. of the product with a slightly lower melting point was obtained (total yield, 84%). After recrystallization from anhydrous ethanol the substance melted at 177–179°.

Anal. Calcd. for $C_{16}H_{22}O_{11}$: C, 49.22; H, 5.69. Found: C, 49.03; H, 5.95.

170 mg. of the substance was acetylated to the hexaacetate by treatment with 1 ml. of a mixture of 95% acetic anhydride and 5% concd. sulfuric acid; yield 180 mg. The hexaacetate, after recrystallization from ethanol, melted at 214–215°; admixture of inositol hexaacetate prepared from meso-inositol produced no depression of the melting point.

1,2,3,4,6-Pentaacetyl-inositol-5-diphenylphosphate (II).—To a solution of 5 g. of 1,2,3,4,6-pentaacetyl-inositol in 15 ml. of anhydrous pyridine, 10.35 g. (3 moles) of diphenylchlorophosphonate[14] was added. The mixture was kept at 80° for twenty hours with careful exclusion of moisture. On cooling in an ice-bath crystals of pyridine hydrochloride separated from the yellow colored solution. After dilution with 15 ml. of pyridine and addition of 0.2 ml. of ice-water to hydrolyze the excess of acid chloride the mixture was added to 1000 ml. of ice-water with vigorous stirring. From the emulsion, after about ten minutes, crystals appeared that were filtered after standing at 0° for two hours, washed with ice-water and cold ethanol, and dried *in vacuo* over sulfuric acid. Recrystallization of the crude product (5.95 g.) from 100 ml. of anhydrous ethanol gave 5.15 g. (64%) of pure 1,2,3,4,6-pentaacetyl-inositol-5-diphenylphosphate which crystallized in fine needles melting at 192–193°.

Anal. Calcd. for $C_{28}H_{31}O_{14}P$: C, 54.02; H, 5.02; P, 4.97. Found: C, 53.96; H, 5.15; P, 5.01.

The substance is easily soluble in acetone, ethyl acetate and chloroform, less soluble in methanol and ethanol, and only sparingly soluble in ether.

1,2,3,4,6-Pentaacetyl-inositol-5-phosphoric Acid (III).—A suspension of 4.5 g. of 1,2,3,4,6-pentaacetyl-inositol-5-diphenylphosphate in 90 ml. of anhydrous ethanol was hydrogenated at room temperature and atmospheric pressure in the presence of 0.2 g. of platinum oxide. The starting material went into solution as the reduction proceeded. The absorption of hydrogen stopped abruptly after ninety minutes when the theoretical quantity (8 moles) had been consumed. The catalyst was removed by filtration and the filtrate was concentrated at reduced pressure to 20 ml. On standing at 0° rapid crystallization occurred (prisms). After gradual addition of 20 ml. of petroleum ether (b. p. 50–60°) and standing at 0° for two hours the crystals were filtered and washed with a mixture of equal parts of ethanol and petroleum ether; yield 2.76 g. (80%). The substance melted at 233–234°; recrystallization did not change this melting point.

(19) All melting points are corrected.

Anal. Calcd. for $C_{16}H_{23}O_{14}P$: C, 40.85; H, 4.93; P, 6.59. Found: C, 40.99; H, 5.01; P, 6.53.

The substance is soluble in water, methanol, ethanol and acetone, insoluble in chloroform and ether. An aqueous solution reacts strongly acid.

Sodium Inositol-5-phosphate.—To 2.5 g. of 1,2,3,4,6-pentaacetyl-inositol-5-phosphoric acid dissolved in 25 ml. of cold anhydrous methanol 13.3 ml. of a 1 *N* solution of sodium methoxide in dry methanol (2.5 moles, 2 moles required for neutralization of the free acid groups) was added. After five minutes an amorphous precipitate of sodium inositol-5-phosphate appeared. The mixture was allowed to stand at 0° overnight. The product was separated by centrifuging and was washed on the centrifuge with dry methanol until the supernatant reacted neutral (four to five washings). After two additional washings with dry ether the substance was dried *in vacuo*. It weighed 1.59 g. (97%).

Anal. Calcd. for $C_6H_{11}O_9PNa_2$: P, 10.19. Found: P, 10.09.

The fine white powder is slightly hygroscopic. A 5% solution in water has a *p*H of 8.0.

Inositol-5-phosphoric Acid (IV).—This substance was prepared by passing a solution of 1 g. of the sodium salt of inositol-5-phosphoric acid in 10 ml. of water through a column containing 15 g. of moist Amberlite-IR-100-AG. The effluent was concentrated at reduced pressure to 3 ml. On addition of ethanol to incipient turbidity spontaneous crystallization occurred. After standing overnight at room temperature the crystals were collected on the filter and washed with ethanol; yield 0.70 g. (82%). For recrystallization the substance was dissolved in 2 ml. of water and 10 ml. of ethanol was added gradually. The colorless hexagonal plates melted with decomposition at 198–200° when rapidly heated and at 195–197° on slow heating.

Anal. Calcd. for $C_6H_{13}O_9P$: C, 27.69; H, 5.04; P, 11.92. Found: C, 27.63; H, 5.06; P, 11.96.

The substance showed no optical activity in concentrations up to 10%. It was very soluble in water, but insoluble in all organic solvents. A 0.2 molar aqueous solution had a *p*H value of 1.4. The salts of inositol-5-phosphoric acid were soluble in water, with the exception of the lead salt, which was formed by addition of lead acetate in excess to an aqueous solution of the free acid or its sodium salt. The barium salt was prepared by neutralizing a solution of the inositol-5-phosphoric acid with barium hydroxide (phenolphthalein) and adding an equal volume of ethanol. The amorphous precipitate was centrifuged, washed with several portions of 50% ethanol and finally with anhydrous ethanol, and dried *in vacuo*. The product contained water of crystallization which was removed only by heating at 120° and 0.1 mm. pressure for three hours.

Anal. Calcd. for $C_6H_{11}O_9PBa$: Ba, 34.7. Found: Ba, 34.6.

Cleavage of the phosphoric acid ester was effected by heating 0.25 g. of inositol-5-phosphoric acid in a sealed tube with 20 ml. of 2 *N* hydrochloric acid at 130° for twenty-four hours. After this period of time the solution was evaporated to dryness and the partly crystalline residue was dissolved in 3 ml. of water. On addition of ethanol to incipient turbidity meso-inositol crystallized spontaneously; yield 158 mg. (91%). The crude product (m. p. 222–226°), on acetylation with 1.2 ml. of a mixture of acetic anhydride (95%) and concd. sulfuric acid (5%), gave 345 mg. (91%) of inositol hexaacetate which, after recrystallization from ethanol, melted at 214–216°. Admixture of hexaacetate prepared from meso-inositol produced no depression of the melting point.

Biological Experiments

The action of *Acetobacter suboxydans* upon inositol-5-phosphoric acid was studied manometrically. Sodium inositol-5-phosphate, in quantities varying from 10 to 100 micromoles and adjusted to *p*H 6 with $^1/_{15}$ molar phosphate buffer, was not attacked by a suspension of resting

bacteria (about 10 mg. dry weight) while in experiments with meso-inositol (20 micromoles) the calculated amount of oxygen was consumed within thirty minutes.

In the bioassay with *Saccharomyces cerevisiae* the method of Jurist and Foy[20] was used. The activity of inositol-5-phosphoric acid was 4 ± 2% of that shown by free meso-inositol. In bioassays with *Neurospora crassa*, carried out according to Beadle,[21] the substance was about 10% as active as inositol. All comparisons were made on the basis of the molecular weight. Hydrolysates of inositol-5-phosphoric acid, prepared by heating the sample with 2 *N* hydrochloric acid at 130° for twenty-four hours and subsequent concentration to dryness and neutralization with dilute sodium hydroxide, showed a 100 ± 3% activity for the inositol calculated to be present in such solutions.

Pentaacetyl-scyllitol Phosphoric Acid.—The synthesis of this substance started from the mixture of inositol- and scyllitol-pentaacetates as obtained by carrying out the hydrogenation of scyllo-meso-inosose pentaacetate in acetic acid instead of methanol. The product crystallized in apparently homogeneous needles melting at 161–163°.

Anal. Calcd. for $C_{16}H_{22}O_{11}$: C, 49.22; H, 5.69. Found: C, 49.23; H, 5.52.

Acetylation of 300 mg. of this substance with 1.5 ml. of a mixture of 95% acetic anhydride and 5% concd. sulfuric acid gave 200 mg. of inositol hexaacetate which, after recrystallization from ethanol, melted at 215–216°, and 50 mg. of scyllitol hexaacetate melting at 299–300° after recrystallization from acetic anhydride. In the bioassay with *Saccharomyces cerevisiae* the mixture of meso-inositol- and scyllitol-pentaacetates, after hydrolysis with 2*N* hydrochloric acid, had only 74% of the activity shown by a hydrolysate of pure 1,2,3,4,6-pentaacetyl-inositol. These results indicate that the mixture contained about 25% scyllitol-pentaacetate. All attempts to separate scyllitol-pentaacetate from the inositol isomer failed.

Phosphorylation with diphenylchlorophosphonate, as described before, gave a mixture of pentaacetyl-inositol-diphenylphosphate and the corresponding scyllitol derivative in 65% yield. After recrystallization from ethanol the product melted at 173–181°.

Anal. Calcd. for $C_{28}H_{31}O_{14}P$: C, 54.02; H, 5.02; P, 4.97. Found: C, 53.81; H, 5.21; P, 4.96.

Attempts to separate the two isomers at this stage by fractional crystallization from ethanol or acetone–water and by fractional extraction with ether were unsuccessful.

3.1 g. of this product was hydrogenated as has been described for the pure pentaacetyl-inositol-diphenylphosphate. When the reduction neared completion fine crystals of pentaacetyl-scyllitol phosphoric acid separated. After warming to dissolve the product the catalyst was removed by filtration and the solution was concentrated at reduced pressure to 20 ml. On standing at room temperature pentaacetyl-scyllitol phosphoric acid separated in extremely fine needles; yield 0.43 g. (18%). From the mother liquor, after further concentration and standing at 0°, 1.60 g. (68%) of 1,2,3,4,6-pentaacetyl-inositol-5-phosphoric acid was obtained which was converted to free inositol-5-phosphoric acid as described before. Pentaacetyl-scyllitol phosphoric acid, after recrystallization from dry methanol, melted at 252–253° with decomposition.

Anal. Calcd. for $C_{16}H_{23}O_{14}P$: C, 40.85; H, 4.93; P, 6.59. Found: C, 41.03; H, 5.14; P, 6.50.

This substance, after removal of the substituents by hydrolysis with hydrochloric acid, was inactive in the bioassay with *Saccharomyces cerevisiae*.

Scyllitol Phosphoric Acid (V).—From 0.80 g. of pentaacetyl-scyllitol phosphoric acid, by catalytic saponification with 2.5 moles of sodium methoxide, 0.51 g. (98%) of sodium scyllitol phosphate was obtained as a white slightly hygroscopic powder.

Anal. Calcd. for $C_6H_{11}O_9PNa_2$: P, 10.19. Found: P, 9.88.

The free scyllitol-phosphoric acid was prepared from 0.4 g. of the sodium salt by removal of the cation with Amberlite-IR-100-AG. From the concentrated aqueous solution, on gradual addition of five volumes of ethanol, the free acid separated in fine crystals; yield 0.28 g. (82%). After recrystallization from water–ethanol the substance melted at 212–214° with decomposition.

Anal. Calcd. for $C_6H_{13}O_9P$: C, 27.69; H, 5.04; P, 11.92. Found: C, 27.61; H, 5.02; P, 11.89.

The substance is insoluble in all organic solvents. An aqueous solution reacts strongly acid (in 1 per cent. solution, *p*H 1.8). The lead salt is the only salt of scyllitol phosphoric acid that is insoluble in water. The barium salt is precipitated from an aqueous solution as a white amorphous powder by addition of 1 volume of ethanol.

Hydrolysis of 50 mg. of scyllitol-phosphoric acid by heating with 10 ml. of 2 *N* hydrochloric acid at 130° for twenty-four hours yielded 33 mg. (95%) of scyllitol, m. p. 345–350°. The crude product, on acetylation, gave 65 mg. (82%) of scyllitol hexaacetate that melted at 298–300°.

Acknowledgment.—The author wishes to express his gratitude to Professor H. O. L. Fischer who has made it possible for him to work on this problem. He is indebted to Dr. H. K. Mitchell, California Institute of Technology, for carrying out the bioassays with *Neurospora crassa*, and to Mr. H. C. Stancer, University of Toronto, for the bioassays with *Saccharomyces cerevisiae*.

Summary

Inositol-5-monophosphoric acid and scyllitol monophosphoric acid have been prepared in good yields by a method involving relatively few steps. Inositol-5-phosphoric acid is resistant to the attack by *Acetobacter suboxydans* and shows only a low activity in the bioassay with *Saccharomyces cerevisiae* and *Neurospora crassa*.

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