ever, the relatively high *p*H of the washing medium of Doty and Zubay as well as the presence of Versene make it seem unlikely that either DNA-ase I or II act to destroy the gel formed by slow mixing of the sediment with water in the Waring Blendor, during the process of stirring this gel with the motor stirrer in the ice-box. Nevertheless degelation does occur and this process is accompanied by a drop in *p*H of a few tenths of a *p*H unit, indicating a possible hydrolysis of some sort with the liberation of acid groups. It seems quite possible that such hydrolysis could be proteolytic in nature. Trypsin and chymotrypsin are known to destroy the gelability of liver cell nuclei isolated at *p*H 4.0 in dilute citric acid.[8]

Although we have not made extensive physiochemical studies of our material isolated by the method of Doty and Zubay, an examination of a sample by ultracentrifugation[11] indicated that it was similar in properties to the material isolated by them. Moreover the method of isolation is so simple that it would seem difficult to avoid obtaining their product.

In conclusion it seems obvious that the nucleoprotein of Doty and Zubay is a complex material consisting of several components, the main bulk of which appears to consist of DNA and histone. Since the latter two substances comprise macromolecules with opposite charges which would be expected to sediment together, and since the ultracentrifuge ordinarily does not separate DNA into different components, it does not seem surprising that the nucleoprotein material of Doty and Zubay should behave more or less as a single component in the ultracentrifuge, even in the absence of gel formation. It therefore seems surprising that these authors merely on the basis of particle size determination and N/P ratios can believe it likely that they have isolated a structural unit of the chromosome. Previous work on the composition and gelability of cell nuclei and chromosomes has not been mentioned by them, especially the role of the residual protein and the role of enzymes in destroying desoxyribonucleoprotein gels. The phenomenon of gel formation by the nucleoprotein has been lightly dismissed without justification on an experimental or theoretical basis. Neither has the possible role of the Waring Blendor in mechanically disrupting deoxyribonucleoprotein fibers been considered. Therefore the suggestion of Doty and Zubay that a fundamental unit of chromosomal structure has been isolated appears to be without sufficient experimental basis, and the concept that the formation of elastic recoilable gels by isolated nuclei and chromosomes is caused by a firm union of the DNA with the residual protein still appears valid to us.

As a final and very recent piece of evidence which we believe supports this concept indirectly but rather strongly, we refer to the work of Butler, *et al.*,[12] who found that the persistence of a small amount of strongly bound protein in isolated DNA can cause an abnormally high viscosity which can be reduced through the use of chymotrypsin.

(11) We wish to thank Dr. M. Schoenburg for subjecting a sample of the material in question to ultracentrifugation.

(12) J. A. V. Butler, D. M. Phillips and K. V. Shooter, *Arch. Biochem. Biophys.*, **71**, 423 (1957).

ROCHESTER, N. Y.

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY OF THE OHIO STATE UNIVERSITY]

Structures of Isomaltose and Gentiobiose

BY M. L. WOLFROM, A. THOMPSON[1] AND A. M. BROWNSTEIN[1]

RECEIVED NOVEMBER 27, 1957

An unequivocal proof of the structures of isomaltose and gentiobiose, based upon the identification of the hydrolytic fragments of their fully methylated aldonic acids (and methyl glycoside for isomaltose) is described. A method for isolating isomaltose and gentiobiose on a preparative basis from an acid reversion mixture of D-glucose is detailed.

The structure of isomaltose has been known for some time, with a considerable degree of certainty, to be 6-*O*-α-D-glucopyranosyl-D-glucose as shown by the periodate oxidation of the methyl glycoside,[2] isomaltitol,[3] and the free sugar,[4] as well as through the "acetobrominolysis" method of Jeanes, Wilham and Hilbert.[4] The proof of structure of gentiobiose rests upon its definitive synthesis by Helferich and co-workers[5]; the proof by methylation techniques[6] is incomplete.

We wish to report herein the preparation of methyl hepta-*O*-methyl-β-isomaltoside and its hydrolysis by acid to produce 2,3,4,6-tetra-*O*-methyl-α-D-glucopyranose and 2,3,4-tri-*O*-methyl-D-glucose (identified as its aniline derivative). This proof is incomplete in that it assumes the presence of the pyranose ring in the reducing moiety. We wish therefore to describe rigid structural proofs for isomaltose and gentiobiose by the classical methylation procedure which Haworth, Loach and Long[7] applied to the definition of the structure of melibiose, 6-*O*-α-D-galactopyranosyl-D-glucose.

(1) Research Associate (A. T.) and Research Fellow (A. M. B.) of the Corn Industries Research Foundation. Preliminary work was carried out on this problem in this Laboratory by Mr. M. Inatome.

(2) M. L. Wolfrom, L. W. Georges and I. L. Miller, THIS JOURNAL, **71**, 125 (1949).

(3) M. L. Wolfrom, A. Thompson, A. N. O'Neill and T. T. Galkowski, *ibid.*, **74**, 1062 (1952).

(4) Allene Jeanes, C. A. Wilham, R. W. Jones, H. M. Tsuchiya and C. E. Rist, *ibid.*, **75**, 5911 (1953); Allene Jeanes, C. A. Wilham and G. E. Hilbert, *ibid.*, **75**, 3667 (1953).

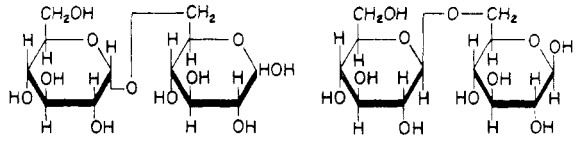
(5) B. Helferich, K. Bäuerlein and F. Wiegand, *Ann.*, **447**, 27 (1926); B. Helferich and W. Klein, *ibid.*, **450**, 219 (1926).

(6) W. N. Haworth and B. Wylam, *J. Chem. Soc.*, **123**, 3120 (1923).

(7) W. N. Haworth, J. V. Loach and C. W. Long, *ibid.*, 3146 (1927).

In our application of this procedure the disaccharide was oxidized to the aldonic acid and this was fully methylated and then hydrolyzed by acid. The resulting 2,3,4,6-tetra-*O*-methyl-D-glucose was identified and the 2,3,4,5-tetra-*O*-methyl-D-gluconic acid fragment was oxidized with nitric acid and isolated after esterification as the known crystalline dimethyl 2,3,4,5-tetra-*O*-methyl-D-glucarate.[8]

The isolation of these fragments from the hydrolyzate of octa-*O*-methylgentiobionic and octa-*O*-methylisomaltonic acids demonstrates unequivocally that the D-glucose units in gentiobiose and isomaltose are joined by (1→6)-linkages with their stereo nature being indicated[2] by the high optical rotation of isomaltose (α-D) and the low rotation of gentiobiose (β-D).



Isomaltose (amorphous) β-Gentiobiose

Hydrol, the residue from the commercial production of D-glucose, is largely a reversion product of D-glucose and has been used as a source of both isomaltose[3] and gentiobiose.[9] These sugars have also been isolated from an acid reversion mixture of D-glucose.[10] The latter material has the advantage that it is free from extraneous impurities which interfere with the isolation. We wish to describe herein a preparative procedure for the isolation of isomaltose and gentiobiose, as their acetates, from the reversion mixture. Gentiobiose can be obtained in a somewhat lower yield from the synthetic mixture by using the acetylation procedure described by Berlin.[9]

Experimental

Methyl Hepta-*O*-methyl-β-isomaltoside.—Using the general methylation procedure of Haworth, Hirst and Webb,[11] 2.5 g. of methyl hepta-*O*-acetyl-β-isomaltoside[2] in 10 ml. of acetone was placed in a three-necked conical flask fitted with a mechanical stirrer. Maintaining an alkaline reaction, dimethyl sulfate (20 ml.) and 30% sodium hydroxide (30 ml.) were added simultaneously and dropwise to the solution over a period of 1.5 hr., with violent agitation, and at a bath temperature of 55°. The temperature of the bath was then raised to 100° for 30 min. After cooling, the solution was neutralized with 2 *N* sulfuric acid and extracted with five 40-ml. portions of chloroform. The combined chloroform extracts were washed with sodium bicarbonate solution and water, dried with anhydrous sodium sulfate and evaporated to a sirup under reduced pressure. This material was then dissolved in 20 ml. of liquid ammonia and further methylated by the general procedure of Muskat.[12] A solution of 200 mg. of sodium in 60 ml. of liquid ammonia was added dropwise until the blue color of the solution persisted for 40 min. Methyl iodide (15 ml.) was added in portions and the solvent allowed to evaporate overnight. An additional 30 ml. of methyl iodide was added and the mixture was refluxed for 3 hr. The reaction mixture was extracted with several portions of dry ether. The combined ether extract was evaporated to a sirup, redissolved in ether, filtered with decolorizing carbon and again evaporated to a sirup; yield 800 mg. A portion of this sirup was dried under reduced pressure over phosphorus pentoxide; $n^{20}D$ 1.4623, $[\alpha]^{30}D$ +80.7° (*c* 3.8, chloroform).

Anal. Calcd. for $C_{12}H_{14}O_3(OCH_3)_8$: C, 52.84; H, 8.43; OCH_3, 54.18. Found: C, 52.77; H, 8.53; OCH_3, 52.3.

Hydrolysis of Methyl Hepta-*O*-methyl-β-isomaltoside.—Methyl hepta-*O*-methyl-β-isomaltoside (560 mg.) was refluxed in 60 ml. of 0.5% hydrochloric acid for 21 hr. The acid was removed by passing the solution through a 25 (diam.) × 100 mm. column of Duolite A-4.[13] The combined solution and washings were then evaporated under reduced pressure to a sirup. Residual water was removed by repeated distillation with methanol; yield 550 mg. This sirup was dissolved in 20 ml. of benzene and placed on a 45 (diam.) × 220 mm. column of Silene EF[14]–Celite[15] and developed with 500 ml. of benzene–*t*-butyl alcohol (100:1 by vol.). After extrusion of the column, two zones appeared at 90 to 130 mm. and 25 to 70 mm., respectively, from the column top on streaking with indicator (0.1 g. of potassium permanganate, 1.0 g. of sodium hydroxide, 10 ml. of water).

The acetone eluate of the lower zone was evaporated to a sirup under reduced pressure, dissolved in ether and brought to crystallization by the addition of low-boiling petroleum ether; yield 45 mg., m.p. 96–100° cor. The product was recrystallized in the same manner; m.p. 98–100° cor., undepressed on admixture with an authentic specimen of 2,3,4,-6-tetra-*O*-methyl-α-D-glucose of m.p. 101–102° cor. The X-ray powder diffraction data were identical with those of a known specimen and were[16]: 11.55vs(3), 9.29vs(2), 7.49m, 5.86w, 4.46s, 4.32vs(1), 4.20vw, 4.02m, 3.89w, 3.66w, 3.39m, 3.29vw, 3.20w, 3.01w.

The carbohydrate material was eluted from the upper zone with acetone and the eluate was evaporated under reduced pressure to a sirup. This sirup was heated in a boiling water-bath for 5 min. with 20 mg. of sodium acetate, 0.3 ml. of acetic acid, 0.2 ml. of aniline and 1.0 ml. of water. It was then evaporated under reduced pressure to dryness and extracted with ether. The ether extract was filtered with carbon and evaporated to a sirup which was crystallized from ether–petroleum ether; yield 39 mg., m.p. 144–146° cor., undepressed on admixture with an authentic specimen of *N*-phenyl-2,3,4-tri-*O*-methyl-β-D-glucopyranosylamine. The X-ray powder diffraction data[16] were identical with those of a known specimen and were: 14.32vs(1), 12.59vw, 11.38vs(1), 8.86m, 7.45w, 6.95s, 6.25w, 5.59vw, 5.05vw, 4.60m, 4.29vs(3), 4.11w, 3.90m, 3.79vw, 3.60m, 3.58vw, 3.43w, 3.35vw, 3.26w, 3.09vw, 3.01w.

The known sample of *N*-phenyl-2,3,4-tri-*O*-methyl-β-D-glucopyranosylamine was prepared, as described above, from known (sirupy) 2,3,4-tri-*O*-methyl-D-glucopyranose, obtained in turn by the hydrolysis of tri-*O*-methyl-1,6-anhydro-β-D-glucopyranose.[17] The known aniline derivative (250 mg.) was purified by chromatography on a Magnesol[18]–Celite[15] (5:1 by wt.) column (45, diam., × 220 mm.), developing with 2000 ml. of benzene–*t*-butyl alcohol (200:1 by vol.). After extrusion, a zone appeared 80 to 130 mm. from the column top upon streaking with permanganate indicator. This material was eluted with acetone and recrystallized from ether–petroleum ether; m.p. 144–146° cor., in agreement with the published value (145–146°),[19] $[\alpha]^{21}D$ −77° (*c* 1.6, chloroform).

Calcium Gentiobionate.—Gentiobiose (33 g.), obtained by deacetylation[20] of β-gentiobiose octaacetate without isolating the sugar in crystalline form, was dissolved in 1500

(8) P. Karrer and J. Peyer, *Helv. Chim. Acta*, **5**, 577 (1922).

(9) H. Berlin, THIS JOURNAL, **48**, 2627 (1926).

(10) A. Thompson, Kimiko Anno, M. L. Wolfrom and M. Inatome, *ibid.*, **76**, 1309 (1954).

(11) W. N. Haworth, E. L. Hirst and J. I. Webb, *J. Chem. Soc.*, 2681 (1928).

(12) I. E. Muskat, THIS JOURNAL, **56**, 693, 2449 (1934).

(13) A product of the Chemical Process Co., Redwood City, Calif.

(14) A product of the Columbia Chemical Co., Barberton, Ohio.

(15) A siliceous filter-aid produced by the Johns-Manville Co., New York, N. Y.

(16) Interplanar spacing, Å., CuK_α radiation. Relative intensity, estimated visually: vs, very strong; s, strong; m, medium; w, weak; vw, very weak. Parenthetical numerals indicate the order of the three most intense lines; 1, most intense.

(17) J. C. Irvine and J. W. H. Oldham, *J. Chem. Soc.*, **119**, 1744 (1921).

(18) A hydrated magnesium acid silicate produced by the Westvaco Chemical Division of the Food Machinery and Chemical Corp., South Charleston, W. Va.

(19) S. Peat, Elsa Schlüchterer and M. Stacey, *J. Chem. Soc.*, 581 (1939).

(20) A. Thompson and M. L. Wolfrom, THIS JOURNAL, **75**, 3605 (1953).

ml. of water containing 57 g. of barium benzoate[21] and cooled to 0°. Bromine (6 ml.) was added and the solution was allowed to come to room temperature and kept in the dark, with occasional vigorous shaking, for 40 hr. The excess bromine was removed by bubbling a rapid stream of air through the mixture. The solution was filtered and nearly, but not quite, all of the barium was precipitated by the addition of sulfuric acid. The solution was filtered and the filtrate was stirred with an excess of basic lead carbonate until *p*H 6–7 had been reached. The solution was again filtered and the excess metallic ions were removed by stirring with Amberlite IR-120.[22] The remaining bromide ions were removed by stirring with silver carbonate and filtering. The solution was again stirred with Amberlite IR-120 to remove any remaining metallic ions, extracted with chloroform, boiled with 10 g. of calcium carbonate and evaporated to about 200 ml. It was then filtered with carbon and evaporated to about 60 ml. The solution was poured slowly with stirring into 2000 ml. of hot methanol. The precipitated calcium salt was filtered and dried at 60°. It did not reduce Benedict solution; yield 33 g.

Methyl Octa-*O*-methylgentiobionate.—The calcium gentiobionate (33 g.) was methylated according to the Haworth[23] method by stirring at 70–80° for 4 hr. during the dropwise addition of 180 ml. of dimethyl sulfate and 144 g. of sodium hydroxide dissolved in 335 ml. of water, being careful to maintain the *p*H above 7. The solution was then boiled for 30 min., cooled to room temperature and acidified to congo red with sulfuric acid. The solution was filtered and extracted several times with chloroform. The aqueous phase was made just alkaline and evaporated to a low volume, the precipitated salts being removed occasionally by filtration. The residual solution from this treatment was remethylated as above with 130 ml. of dimethyl sulfate and 96 g. of sodium hydroxide in 225 ml. of water. The combined chloroform extracts were washed once with water, dried with anhydrous sodium sulfate and evaporated to a sirup; yield 23 g. This sirup was then further methylated with the Purdie[24] reagents by stirring at room temperature for 30 min. with 50 ml. of methyl iodide and 15 g. of silver oxide. The mixture was then filtered, the precipitate washed with ether and the combined filtrate and washings were evaporated to a sirup. This was methylated twice more as above (but at 35° and for 24 hr.). The sirupy methyl octa-*O*-methylgentiobionate was distilled; yield 14.2 g., b.p. 175–180° (0.02 mm.), $[\alpha]^{24}D$ +3° (*c* 4.2, chloroform), $n^{25}D$ 1.4589. The infrared absorption spectra indicated essentially no free hydroxyl group.

Anal. Calcd. for $C_{12}H_{18}O_3(OCH_3)_9$: OCH_3, 57.64. Found: OCH_3, 54.62.

Fragmentation of Methyl Octa-*O*-methylgentiobionate.—A solution of 10 g. of methyl octa-*O*-methylgentiobionate in 1000 ml. of 0.25 *N* hydrochloric acid was refluxed for 22 hr. The course of the reaction was followed polarimetrically and changed from $\alpha^{20}D$ −0.20 → +1.05°, 2-dm. tube. The solution was neutralized with sodium hydroxide and evaporated under reduced pressure to dryness. The residue was extracted several times with chloroform. The chloroform solution was dried with anhydrous sodium sulfate and evaporated to a sirup which crystallized; yield 4.8 g. (96%), m.p. 90–92°, 95–97° after recrystallization from ether–petroleum ether. The X-ray powder diffraction data were identical with those cited above for 2,3,4,6-tetra-*O*-methyl-α-D-glucose.

The residue from the chloroform extraction was made strongly acid, mixed with hot methanol and filtered. The precipitate was washed several times with chloroform and these washings were kept separate. The methanol filtrate was evaporated under reduced pressure to a sirupy mass. This mixture was triturated with chloroform several times. The chloroform washings were combined, dried with anhydrous sodium sulfate and evaporated to a sirup; yield 4.5 g. (85%). This material was dissolved in 40 ml. of nitric acid (sp. gr. 1.2), refluxed for 5 hr. and evaporated to a sirup under reduced pressure. The traces of nitric acid were removed by repeated addition of water and evaporation under reduced pressure. The sirup was finally dried by repeated addition of methanol and evaporation under reduced pressure. The dry residue was dissolved in 100 ml. of dry methanol containing 3 g. of hydrogen chloride and refluxed for 18 hr. The hydrogen chloride was removed by stirring with Duolite A-4.[13] The resin was filtered and the solution was evaporated to a sirup. The sirup was then distilled; yield 2.9 g. (52%), b.p. 125° (0.1 mm.). This material crystallized from ether; m.p. 73–75°, $[\alpha]^{15}D$ +11° (*c* 6.3, water), $[\alpha]^{28}D$ +19.5° (*c* 4.3, chloroform); X-ray powder diffraction data[16]: 10.13vw, 8.28w, 6.75vs(1), 6.27m, 5.98s(2), 5.30m(3), 4.58w, 4.37s(2), 3.97vs(1), 3.75vw, 3.63vw, 3.45w, 3.36w, 3.29vw, 3.12w, 2.98vw, 2.86vw, 2.74vw, 2.65w, 2.46vw, 2.42w, 2.34vw, 2.29vw. These data are in agreement with those found for an authentic sample of dimethyl 2,3,4,5-tetra-*O*-methyl-D-glucarate prepared by the method of Karrer and Peyer.[8]

Calcium Isomaltonate.—Amorphous isomaltose (30 g.), made by the deacetylation[2] of crystalline β-isomaltose octaacetate, was oxidized by the procedure described above for the preparation of calcium gentiobionate; yield 27.5 g.

Methyl Octa-*O*-methylisomaltonate.—The calcium isomaltonate (27.5 g.) was methylated by the Haworth[23] and Purdie[24] procedures as described for the corresponding gentiobiose derivative; yield of methyl octa-*O*-methylisomaltonate 10 g., b.p. 185–190° (0.05 mm.), $[\alpha]^{18}D$ +114.4° (*c* 4.6, chloroform), n^{20} 1.4562.

Anal. Calcd. for $C_{12}H_{13}O_3(OCH_3)_9$: OCH_3, 57.64. Found: OCH_3, 55.63.

Hydrolysis of Methyl Octa-*O*-methylisomaltonate.—Methyl octa-*O*-methylisomaltonate (9.0 g.) was hydrolyzed in 900 ml. of 0.25 *N* hydrochloric acid as described above for the corresponding gentiobiose derivative; rotation change $\alpha^{20}D$ +1.70 → +1.00°, 2-dm. tube; yield of 2,3,4,6-tetra-*O*-methyl-D-glucose 3.7 g. (84%), m.p. 96–98°, X-ray powder diffraction data identical with those of the known substance (see above). The acid fraction was oxidized as above with nitric acid and esterified with dry methanol and hydrogen chloride; yield 3.2 g. (66%), m.p. 72–74°, $[\alpha]^{25}D$ +19° (*c* 2.5, chloroform), $[\alpha]^{28}D$ +10° (*c* 4, water). The X-ray powder diffraction pattern (see above) was identical with that of known dimethyl 2,3,4,5-tetra-*O*-methyl-D-glucarate.

Preparation of Acid Reversion Mixture from D-Glucose.—A solution of 1600 g. of D-glucose in 1600 ml. of water containing 20 ml. of hydrochloric acid (sp. gr. 1.18) was refluxed for 12 hr. After cooling, the acid was removed by passage through a column of Duolite A-4[13] and diluted to 15 liters. Ammonium dihydrogen phosphate and potassium monohydrogen phosphate (10 g. of each) were added, the solution was inoculated with 30 g. of bakers' yeast, and allowed to ferment for one week. The solution was then filtered with the aid of Celite,[15] and evaporated to about one-third volume to remove ethanol.

β-Isomaltose Octaacetate.—The above solution was placed on a carbon[25] (Nuchar C, unground)[26] column (1050 × 180 mm.) and washed with 30 liters of water. The water effluent, which generally contains only D-glucose, was discarded. The developing solution was changed to 3% ethanol, the effluent being discarded until it gave a positive Benedict test. The next 60 liters of effluent was collected and evaporated under reduced pressure to a sirup, the last of the water being removed by repeated additions of methanol and evaporation under reduced pressure; yield 112 g. This sirup was then acetylated with 50 g. of sodium acetate and 800 ml. of acetic anhydride by heating carefully to the boiling point in a 3-liter flask. After the reaction had subsided, the solution was heated again to the boiling point and allowed to cool. The excess acetic anhydride was hydrolyzed by pouring into 5 liters of ice and water and stirring for several hours. The sirupy material was then extracted with chloroform, the chloroform solution was washed successively with water, saturated sodium bicarbonate solution and water, dried with anhydrous sodium sulfate and evaporated to a sirup under reduced pressure. The last trace of chloroform was removed by repeated addition of ethanol and evaporation under reduced pressure. The sirup was dissolved in ethanol. The first crop of crystalline material was β-gentiobiose octaacetate; yield 7 g. The mother liquor was

(21) C. S. Hudson and H. S. Isbell, THIS JOURNAL, **51**, 2225 (1929).
(22) A product of the Rohm and Haas Co., Philadelphia, Pa.
(23) W. N. Haworth, *J. Chem. Soc.*, **107**, 8 (1915).
(24) T. Purdie and J. C. Irvine, *ibid.*, **83**, 1021 (1903).
(25) R. L. Whistler and D. F. Durso, THIS JOURNAL, **72**, 677 (1950).
(26) A product of the West Virginia Pulp and Paper Co., New York, N. Y.

nucleated with β-isomaltose octaacetate and stirred with a magnetic stirrer for 24 hr. The crystalline material was filtered and was twice recrystallized from ethanol. Further crops of β-isomaltose octaacetate were obtained from the mother liquors and were recrystallized from ethanol; combined yield 64 g., m.p. 142–144°, $[\alpha]^{25}D$ +95° (*c* 4.6, chloroform).

β-Gentiobiose Octaacetate.—After the zone containing largely isomaltose had been removed from the carbon column with 3% ethanol, as indicated by a weak Benedict test, the developing solution was changed to 10% ethanol. When the Benedict test again became strongly positive the effluent was collected (20 liters) and evaporated to a sirup under reduced pressure. The sirup was dried by repeated addition of methanol and evaporation under reduced pressure; yield 120 g. This material was acetylated by the procedure described above; total yield 96 g. Pure material was obtained on recrystallization from ethanol; m.p. 191.5–192.5°, $[\alpha]^{27}D$ −4.6° (*c* 3.3, chloroform).

If it is desired to isolate only the gentiobiose from the reversion mixture, the dried unfermentable portion above may be acetylated directly and isolated, in a somewhat lower yield, as the β-octaacetate, as described by Berlin[9] for hydrol; yield 48 g. from 1600 g. of D-glucose, m.p. 190–192°, $[\alpha]^{25}D$ −5° (*c* 3.5, chloroform).

COLUMBUS 10, OHIO

[CONTRIBUTION FROM THE NUTRITION AND PHYSIOLOGY SECTION, RESEARCH DIVISION, AMERICAN CYANAMID COMPANY]

The Synthesis of Some 2-Amino-4-hydroxy-6-polyhydroxyalkyl-pteridines Which Are Active in Supporting the Growth of the Protozoön *Crithidia fasciculata*

BY E. L. PATTERSON, R. MILSTREY AND E. L. R. STOKSTAD

RECEIVED AUGUST 9, 1957

Four 2-amino-4-hydroxy-6-polyhydroxyalkyl-pteridines were prepared. Among these was an apparent correlation between the presence of two adjacent carbinols both having the L-configuration in the alkyl side chain and high potency in supporting the growth of the protozoön, *Crithidia fasciculata*.

A pteridine was isolated from normal human adult urine which was required for growth by the protozoön, *Crithidia fasciculata*. It was identified as 2-amino-4-hydroxy-6-[1,2-dihydroxypropyl-(L-erythro)]-pteridine and named biopterin.[1–3] The same compound identified except for optical configuration was one of the fluorescent substances extracted from *Drosophila melanogaster*.[4] In connection with the work on the proof of structure and synthesis of biopterin several 2-amino-4-hydroxy-6-polyhydroxyalkyl-pteridines have been prepared. This paper describes some of the properties of four of these compounds.

Experimental

The method of synthesis and the subsequent purification procedures outlined in the following example were essentially the same for all the pteridines. The aldoses having the proper configurations which were selected to prepare each of the pteridines are given in Table I along with some of the properties of the products.

The 2-Amino-4-hydroxy-6-[1,2,3-trihydroxypropyl-(L-erythro)]-pteridine (I).—One hundred and fifty g. (1 mole) of L-arabinose, 156 g. (1.7 moles) of anhydrous sodium acetate and 62.5 g. (1 mole) of boric acid were dissolved in 500 ml. of water. One hundred and twenty-six g. (0.89 mole) of 2,5,6-triamino-4-hydroxy pyrimidine (II) in 250 ml. of water prepared as a smooth slurry in a Waring blender was added slowly with good stirring. The *p*H of the pale yellow solution was 5.7. It was heated to 80° with agitation by means of a nitrogen stream, and 117 g. (2 moles) of 85% hydrazine hydrate was added. The *p*H was adjusted from 7.8 to 5.5 with about 100 ml. of acetic acid. The solution was heated to 95° for 1 hour and 15 minutes, and during this time it slowly turned dark brown in color, and a precipitate formed. The reaction mixture was cooled at 0° for a few hours and filtered. The weight of brown colored product was 64 g.

The crude precipitate was suspended in 2.5 l. of hot water which was then saturated with lime. The dark brown insoluble material was filtered off and washed twice with 2.5 l. of hot water and discarded. The filtrate and washings were combined and heated to 80° and stirred while a saturated zinc chloride solution was added slowly until the *p*H dropped to 8.6. The black insoluble material was filtered off and washed twice with 200 ml. of hot water and discarded. The combined filtrate and washings were bright yellow in color. The volume was reduced to 2 l. by distillation at reduced pressure.

A 6 × 16 in. chromatographic column of magnesol[5a] super cel[5b] (2:3 by weight) degassed by stirring in water was poured as a slurry and allowed to pack by gravity. The yellow pteridine solution was put on the column with the aid of 2 p.s.i. air pressure. The column was developed by gradient elution with 20 l. of water and 20 l. of 2 *N* ammonium hydroxide under 3 to 5 p.s.i. air pressure. The eluate was collected in fractions of 800 to 1000 ml., each representing a one-hour interval. The fractions saved were those in which the ratio of the ultraviolet absorption in 0.1 *N* NaOH at 253 mμ to that at 360 mμ fell in the range of 2.5 to 3. The combined selected fractions were reduced to near dryness by distillation at reduced pressure, and after standing overnight in 500 ml. of 3 *N* HCl a magnesol gel that had formed was removed by centrifuging, washed with about 25 ml. of 3 *N* HCl and discarded. The total solids in the combined centrifugate and wash weighed 20 g.

The sample from the magnesol column in 500 ml. of 3 *N* HCl was added to a 3 × 20 in. column of Dowex 50[5c] × 8 200–400 mesh in the hydrogen cycle which had been equilibrated with 3 *N* HCl. The column was developed by gradient elution with 4 l. of 3 *N* HCl and 4 l. of 6 *N* HCl. The flow rate was about 200 ml. per hour, and fractions were collected in two-hour intervals. The desired fractions were selected on the basis of ultraviolet absorption as before. The composite was reduced to dryness by distillation at reduced pressure, and the residue was dissolved in 200 ml. of water. The total solids in the sample weighed 7 g.

Upon the addition of lime to this solution a considerable quantity of material, mostly Dowex 50 resin, precipitated and was filtered off and discarded. The solution was heated to 80°, and saturated zinc chloride was added slowly with good stirring until the *p*H dropped to 8.6. The dark colored insoluble material was removed by filtration and discarded, and the yelllow filtrate was made acid with acetic acid. After standing at 4° overnight the pale yellow precipitate was filtered off and washed three times with acetone and air dried. The product weighed 2.7 g., and by microbiological assay with *C. fasciculata*,[6] it was 35% pure.

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(1) E. L. Patterson, H. P. Broquist, Alberta M. Albrecht, M. H. von Saltza and E. L. R. Stokstad, THIS JOURNAL, **77**, 3165 (1955).

(2) E. L. Patterson, M. H. von Saltza and E. L. R. Stokstad, *ibid.*, **78**, 5871 (1956).

(3) E. L. Patterson, R. Milstrey and E. L. R. Stokstad, *ibid.*, **78**, 5868 (1956).

(4) H. S. Forrest and H. K. Mitchell, *ibid.*, **77**, 4865 (1955).

(5) (a) Magnesol industrial powdered, Westvaco Chemical Division, South Charleston, W. Va.; (b) Johns-Manville Corporation, New York, N. Y.; (c) The Dow Chemical Company, Midland, Michigan.

(6) For a description of the microbiological assay see Harry P. Broquist and Alberta M. Albrecht, *Proc. Soc. Expl. Biol. and Med.*, **89**, 178 (1955).