hours. The oxidation was stopped by addition of 0.4 ml. of oxalic acid solution (10% in glacial acetic). The reaction mixture was filtered and the filtrate was concentrated by vacuum distillation to approximately 0.1 ml. Next 0.1 ml. of 0.1 *N* hydrochloric acid was added and hydrolysis was effected at 100° for 3 hours. Paper chromatograms of the hydrolysate and reference materials were prepared and sprayed with aniline oxalate reagent[25] for locating pentoses and with copper sulfate and molybdic acid reagents[9] for locating the reducing compounds. The apparent R_f values (2 ascents of solvent) of the carbohydrates in the hydrolysate of compound I and reference compounds were: arabinose, 0.74; pentose in hydrolysate, 0.74; glucose, 0.68; galactose, 0.61; other reducing product in the hydrolysate 0.61. From the oxidized and hydrolyzed compounds II and III, reducing products with R_f values of 0.61 and 0.62 were produced; R_f value of galactose under the same conditions was 0.61.

Other samples of the oligosaccharide (0.4 to 0.8 mg.) were oxidized with lead tetraacetate in the Warburg apparatus as outlined by Perlin.[11] Lead tetraacetate consumption was determined by an iodometric procedure and formic acid production was measured manometrically by its conversion to carbon dioxide. Values for the oligosaccharides at 15 to 50 minute reaction periods are recorded in Table II. These values have been corrected for the contribution of the galactosyl moiety to the reaction by determining the lead tetraacetate consumption for methyl α-D-galactoside and *p*-nitrophenyl β-D-galactoside.

LINCOLN, NEBRASKA

(25) R. J. Block, E. L. Durrum and G. Zweig, "Paper Chromatography and Paper Electrophoresis," Academic Press, Inc., New York, N. Y., 1955, p. 133.

[CONTRIBUTION FROM THE CANCER RESEARCH AND CANCER CONTROL UNIT OF THE DEPARTMENT OF SURGERY, AND THE DEPARTMENT OF BIOCHEMISTRY AND NUTRITION, TUFTS UNIVERSITY SCHOOL OF MEDICINE]

The Presence of Maltose, Maltotriose and Maltotetraose in Liver[1]

BY WILLIAM H. FISHMAN AND HSIEN-GIEH SIE

RECEIVED APRIL 1, 1957

In the course of a search for hepatic glucosiduronic acids, material was isolated from fresh rat liver which had the physical and chemical properties of a mixture of oligosaccharides. This mixture was separated into three major components and a number (5–8) of minor constituents by a combination of column and paper chromatographic techniques. The three major components were positively identified as maltose, maltotriose and maltotetraose and the remaining components have been described as members of an ascending homologous series of glucosyl oligosaccharides. The product of acid hydrolysis of the original mixture was isolated and identified as glucose.

Originally, we were conducting a search for physiologically produced glucosiduronic acids in liver as an extension of studies on glucuronylation of steroids[2,3] and on enzyme-catalyzed glucuronyl transfer.[4,5] In this connection, it was found that glucosiduronic acids, *e.g.*, 8-hydroxyquinoline glucosiduronic acid, could be adsorbed from aqueous solution on charcoal and also could be readily eluted from this material by hot alcohol. When this charcoal treatment was applied to zinc hydroxide filtrates[4] of liver homogenate, a creamy-white solid ("X") was obtained whose amount exceeded by far the weight expected from analytical values[6] for glucosiduronic acid.

This was the point of departure of the present investigation which has now culminated in the isolation and identification of the main components of "X" as a homologous series of glucosyl oligosaccharides.

Experimental

Isolation of Liver Oligosaccharides.—The liver of a freshly-killed rat was cooled immediately in ice. An aqueous homogenate (*i.e.*, 1 g. per 10 ml. of ice-cold H_2O) was prepared by means of a chilled glass homogenizer (one-half minute). This mixture was then deproteinized by adding to each 10 ml. of homogenate first 1 ml. of $ZnSO_4$ (10%) and then dropwise 1 ml. of NaOH (0.5 *N*) solution with intermittent agitation. After filtration, the protein-free filtrate was treated with charcoal[7] (1.5 g. per gram of original liver tissue) for at least 30 minutes with intermittent stirring. The charcoal was removed by filtration and was washed repeatedly with water (250–500 ml., total volume). Elution was done with at least 100 ml. of hot ethyl alcohol. Evaporation of the solvent yielded a dry creamy-white solid ("X"). Since "X" was completely soluble in alcohol and it dialyzed readily through a cellophane membrane, it was clearly not glycogen. Its aqueous solutions exhibited reducing power (which increased substantially upon acid hydrolysis) and instability to alkali; optical rotation was $[\alpha]^{24}D$ +134.3° (H_2O).[8] Purified "X" acetate contained (%) C, 49.17; H, 5.59; O, 45.25; acetyl, 46.7; $[\alpha]^{24}D$ +116.5° ($CHCl_3$); mol. wt. (Rast), 779. Tests for nitrogen and phosphorus were negative.

Separation of these oligosaccharides was effected by means of both column and paper chromatography. Thus, 150 mg. of "X" was placed on a 2 × 18 cm. charcoal:Celite column prewashed with the elution mixture and water. First, 250 ml. of H_2O was passed through and discarded. Then, the material was eluted with an ethyl acetate–alcohol–water (3:1:1) mixture and 5-ml. fractions were collected. These were analyzed for hexose.[9] As indicated in Fig. 1, the plot of sugar *versus* tube number indicated three peaks. The contents of the tube corresponding to each peak was examined by descending paper chromatography (Whatman no. 1, solvent system 4 (Table I), ammoniacal silver nitrate[10]). The fastest moving spot (A) was the first to be eluted from the column. "B" and "C" travelled on paper, also in order of their elution from charcoal. Upon prolonged paper chromatography of "X" it was possible to observe 9 spots (Fig. 2) and the $\log_{10}(1/Rf - 1)$ of these spots was plotted as a function of hexose units per molecule (Fig. 3).

(1) These data were presented on December 8, 1956 at the Ninth Annual Meeting of Scientific Advisors, Harvard Club, Boston, and at the Annual Meeting of the American Society for Biological Chemists, April 19, 1957, Chicago, Illinois (W. H. Fishman and H.-G. Sie, *Federation Proc.*).

(2) W. H. Fishman and H.-G. Sie, *J. Biol. Chem.*, **218**, 335 (1956).

(3) H.-G. Sie and W. H. Fishman, *ibid.*, **225**, 453 (1957).

(4) W. H. Fishman and S. Green, THIS JOURNAL, **78**, 880 (1956).

(5) W. H. Fishman and S. Green, *J. Biol. Chem.*, **225**, 435 (1957)

(6) W. H. Fishman and S. Green, *ibid.*, **215**, 527 (1955).

(7) Activated charcoal (Howe and French, Inc., Boston) was previously washed with copious amounts of hot alcohol and water. No oligosaccharides were found in the alcohol eluate of the charcoal before it was employed in this study.

(8) We wish to thank Dr. R. J. Jeanloz of the Massachusetts General Hospital for extending the hospitality of his laboratory to us for measurements of optical rotation. His interest in this work is appreciated.

(9) Z. Dische, *J. Biol. Chem.*, **181**, 379 (1949).

(10) S. M. Partridge, *Biochem. J.*, **42**, 238 (1948).

The three major components (A, B, C) *were isolated and* separated by repeated fractionation on charcoal. Traces of "A" were removed from "B" and "C" by yeast fermentation. In order to obtain "C" free from all traces of "B," the "C"-rich material was chromatographed on paper (solvent 4), and the "C" spot eluted at its expected location. A residue "D" containing only the slower moving components (at least 5 in number) also was prepared.

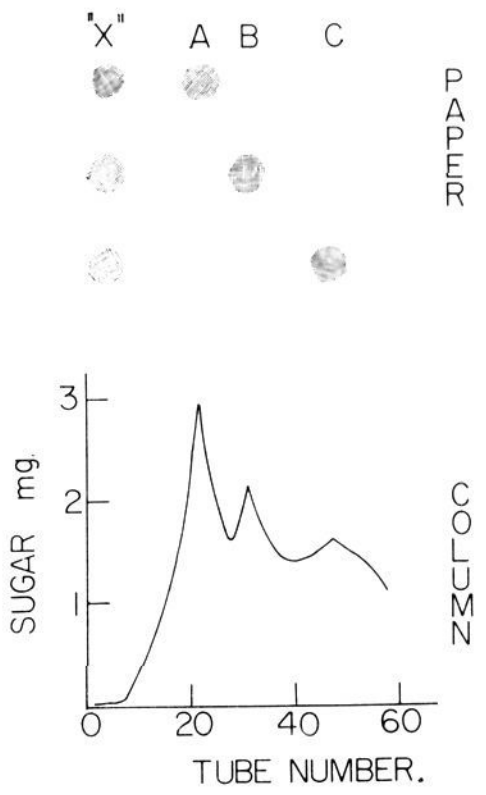

Fig. 1.—Paper and column chromatography of the "X" liver carbohydrate fraction.

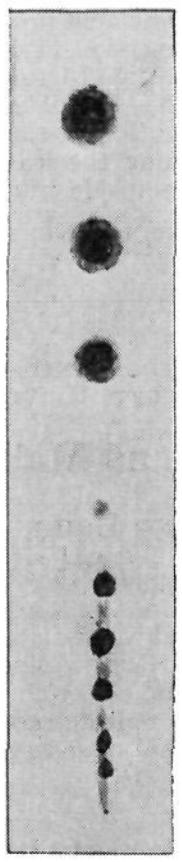

Fig. 2.—Paper chromatogram of "X," run 64 hours, descending chromatography (solvent 4).

TABLE I[a]

"X" was prepared as described. Approximately 30 to 50 γ of this material was spotted on Whatman no. 1 paper along with the reference carbohydrates and glucose. Following descending chromatography for a variable period of time (*vide infra*), the sugars were located on the paper with ammoniacal $AgNO_3$. R_{G1}, defined as the ratio of the distance travelled by the unknown to the distance travelled by glucose, was computed. The composition of the solvent systems and the time period of chromatography in each case was (1) ethyl acetate:acetic acid: H_2O; 3:1:3 (top layer) 7 hours; (2) *n*-butyl alcohol:ethanol:H_2O; 4:1:5 (top layer), 65 hours; (3) *n*-butyl alcohol: pyridine:H_2O; 6:4:3, 19 hours, and (4) ethyl acetate:pyridine:H_2O; 2:1:2 (top layer) 19 hours. The following oligosaccharides were excluded from consideration on the basis of paper chromatography: lactose, gentiobiose, cellobiose, isomaltose, turanose, isomaltotriose, panose, melezitose and raffinose. In the solvent 4 system, but not in the three others, melibiose corresponded in location with the "B" component. However, "X" gave a negative mucic acid test.

R_{G1} VALUES

Solvents	"A"	Maltose	"B"	Maltotriose	"C"	Maltotetraose
1	0.63	0.65	0.42	0.42	0.25	0.25
2	.46	.46	.20	.19	.09	.08
3	.79	.80	.63	.64	.46	.48
4	.81	.81	.63	.63	.45	.44

[a] The reference sugars, maltose, maltotriose and maltotetraose were prepared by partial acid hydrolysis (0.4 *N* H_2SO_4, 1 hr. 90°) of pure corn amylose. Authentic specimens of each sugar were the kind gift of Dr. Dexter French.

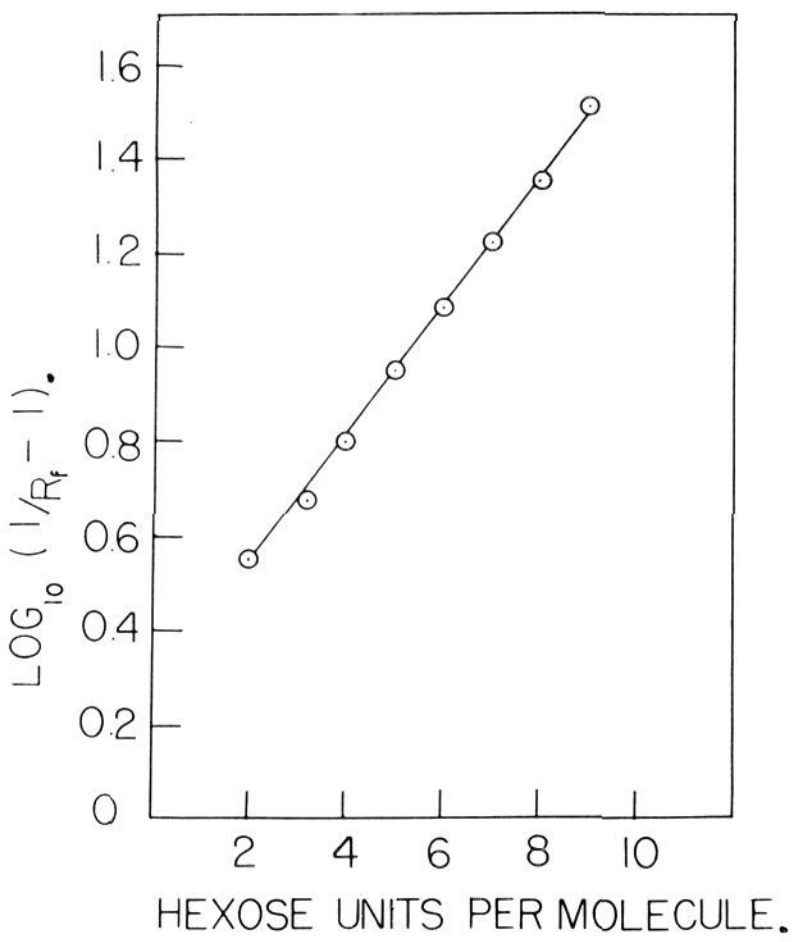

Fig. 3.—A plot of $\log_{10}(1/Rf - 1)$ *vs.* hexose units per molecule.

Identification.—Acid hydrolysis of A, B, C and D produced only D-glucose (osazone m.p. 203–205°, quant. orcinol reaction[11] location on paper[12]). Data obtained by titration with alkaline hypoiodite[13] and the increase in reducing power[14] on acid hydrolysis, indicated that A was a di-, B a tri- and C, a tetrasaccharide, each with one reducing group. The chromatographic data which were obtained by the use of four different solvent systems and of authentic reference glucosyl oligosaccharides are shown in Table I.

Additional data supporting the identification of A as maltose are the following: $[\alpha]^{24}D$ +126.5° (H_2O), maltose, +130.4°, its heptaacetate, calcd. for $C_{26}H_{36}O_{18}$: C, 49.0; H, 5.70; O, 45.30; acetyl, 47.3. Found: C, 49.44; H, 5.73; O, 44.84; acetyl, 48.6. Chromatographic evidence for maltose and maltotriose was also obtained in extracts of rat gastrocnemius muscle.

Effect of Isolation Procedure on Glycogen.—To 300 ml. of a dilute solution of glycogen (1 mg./3 ml.) (General Biochemicals, Inc.) was added dropwise with stirring 10 ml. of 10% $ZnSO_4$ and 10 ml. of 0.5 *N* NaOH solution. After filtration, 15 g. of charcoal was added to the filtrate with occasional stirring. This charcoal was collected on a filter and then washed with 1 liter of distilled H_2O. It was eluted with 250 ml. of hot alcohol and evaporated to dryness. The residue was dissolved in 0.1 ml. of water and chromatographed on paper using the solvent 4 system. No spot for reducing sugar appeared.

A Simple Physical Separation of Glucosyl Oligosaccharides from Liver Homogenate.—All operations were carried out at +4°. Fresh rat livers (37 g.) were homogenized in 200 ml. of ice-cold water for 1 minute in a chilled glass homogenizer. The mixture was centrifuged in a high speed refrigerated centrifuge for 30 minutes and the supernatant dialyzed against 1 liter of distilled water in the cold room (+5°) for 16 hours and against a fresh 1-liter volume for an additional 72 hours. To both dialysates were added 15 g. of charcoal. The charcoal was collected, washed with distilled water and eluted with hot alcohol. The residue after evaporation to dryness was dissolved in the minimum

(11) J. Bruckner, *Biochem. J.*, **60**, 200 (1955).

(12) The mobility on paper (R_f) of the monose product matched that of glucose in 4 different solvent systems. Its location did not correspond with those of D-ribose, L-arabinose, D-lyxose, D-fructose, D-galactose, D-mannose, L-sorbose, L-rhamnose and D-aldoheptose.

(13) P. F. Jorgenson, *Dansk. Tids. Farm.*, **24**, 1 (1950).

(14) G. Neolting and P. Bernfeld, *Helv. Chim. Acta*, **31**, 286 (1947).

amount of water and chromatographed on paper. The first dialysate (16 hours) contained maltose and maltotriose and the second (72 hours), maltotetraose in addition.

Discussion

Liver Glucosyl Oligosaccharides.—The data prove the existence in an aqueous homogenate of fresh liver of maltose, maltotriose, maltotetraose and a number of higher glucosyl homologs. This statement is based on the following observations. The active material ("X") was isolated from liver by a procedure (adsorption on and elution from charcoal) known to be applicable to oligosaccharides. Its properties (solubility in water and in alcohol and its passage through cellophane) excludes high molecular weight polysaccharides such as glycogen. It formed a polyacetate whose elemental analysis, optical rotation and molecular weight suggested a mixture of oligosaccharides. It was unstable in alkali and its reducing power increased greatly following acid hydrolysis. The major components of this mixture were isolated by column and paper chromatography. Minor components were prepared as a mixture (D). It was possible to prepare a pure acetyl derivative of the fastest component and its elemental analysis and rotation corresponded to maltose. Acid hydrolysis of "X" and of its isolated components A, B, C and D yielded only glucose which was identified by valid criteria. The first three members of this series were identified beyond question as maltose, maltotriose and maltotetraose (Table I). When the $\log_{10}(1/Rf - 1)$ of the spots in Fig. 2 was plotted against hexose units per molecule, a straight line resulted (Fig. 3) which indicates a homologous series.[15-17]

The origin of these oligosaccharides, present in liver homogenate, is the subject of current research in this Laboratory. Certain of these experiments to be reported elsewhere indicate that the question of the significance of liver glucosyloligosaccharides deserves more than passing attention.

Acknowledgments.—This investigation was supported by grants-in-aid from the American Cancer Society, Inc., New York (MET-44), and the American Cancer Society, Massachusetts Division (656-Cl.). We are indebted to Drs. Allene Jeanes, S. C. Pan and H. S. Isbell for gifts of rare sugars.

(15) A. J. P. Martin, *Biochem. Soc. Symp.*, **3**, 4 (1949).

(16) E. C. Bate-Smith and R. G. Westall, *Biochim. Biophys. Acta*, **4**, 427 (1950).

(17) D. French and G. M. Wild, THIS JOURNAL, **75**, 2612 (1953).

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[CONTRIBUTION FROM THE RESEARCH DEPARTMENT, CIBA PHARMACEUTICAL PRODUCTS, INC.]

The Alkaloids of *Tabernanthe iboga*. Part III.[1] Isolation Studies

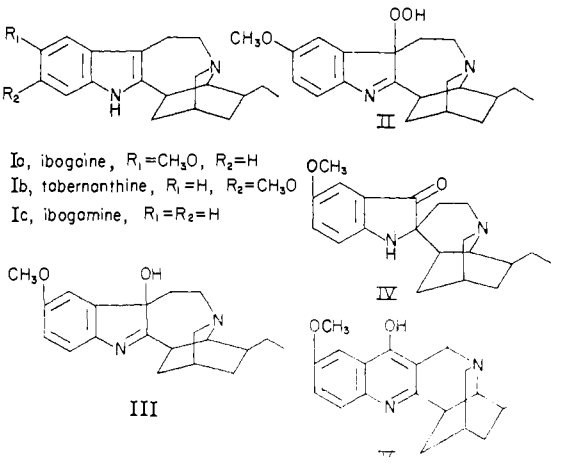
BY D. F. DICKEL, C. L. HOLDEN, R. C. MAXFIELD, L. E. PASZEK AND W. I. TAYLOR

RECEIVED AUGUST 5, 1957

The present investigation of *Tabernanthe iboga* root records the isolation of twelve compounds, which includes not only the four known alkaloids ibogamine, ibogaine, tabernanthine and iboluteine but also iboquine, desmethoxyiboluteine, the hydroxyindolenine derivatives of ibogaine and ibogamine, voacangine, gabonine, kisantine and kimvuline. Of these, the latter three have not been described previously.

The current interest in *Tabernanthe iboga* initiated a more detailed investigation of the alkaloidal content of the root. Emphasis was placed both on the preparation of the major alkaloids in a state of high purity and on the isolation of the greatest possible number of minor alkaloids.

The three indole alkaloids ibogaine (Ia), tabernanthine (Ib) and ibogamine (Ic) known from the earlier investigations of *Tabernanthe iboga* root[2] are presumed to differ only with respect to a methoxyl on ring A.[3] A fourth alkaloid, iboluteine (IV), has been isolated from a plant extract[4] and has been shown to be a 5-methoxypseudoindoxyl related to ibogaine.[5] Catalytic oxidation of ibogaine followed by reduction and alkaline rearrangement led to its formation in good yield. Two intermediates of the reaction sequence, the hydroperoxy (II), and the hydroxyindolenine (III) derivatives of ibogaine were obtained in crystalline form. Desmethoxyiboluteine was prepared by a similar treatment of ibogamine but without isolation of the intermediate compounds. Air oxidation of ibogaine yielded not only iboluteine but also iboquine (V) which was formulated as a 6-methoxyquinolol.[4]



(1) The structural formulas used in this paper are based on evidence presented in Part II, W. I. Taylor, THIS JOURNAL, **79**, 3298 (1957).

(2) T. A. Henry, "The Plant Alkaloids," J. A. Churchill Ltd., London, 1949, p. 768.

(3) P. Lebeau and M. M. Janot, "Traité de Pharmacie chimique," Vol. 4, Masson et Cie, Paris, 1956, pp. 2 982–2988.

(4) R. Goutarel and M. M. Janot, *Ann. pharm. franc.*, 11, 272 (1953).

(5) R. Goutarel, M. M. Janot, F. Mathys and V. Prelog, *Helv. Chim. Acta*, **39**, 742 (1956).