ml.,[10] and of acetyl-L-hexahydrophenylalaninamide, *i.e.*, $27 \pm 4 \times 10^{-3}$ *M* and 0.6×10^{-3} mole/min./mg. protein-nitrogen/ml., it can be seen that the replacement of a benzyl group by a hexahydrobenzyl group has relatively little effect upon either the K_S or k_3 values, at least at 25° and *p*H 7.9. Since there is reason to interpret the K_S values of these two specific substrates in terms of the corresponding enzyme–substrate dissociation constants, *i.e.*, the k_2/k_1 values,[9,13,29-31] it appears that the π-electrons present in an aromatic side chain are not involved in the combination process. The possible greater affinity of the active site for the corresponding hydroaromatic side chain may be due to the slightly greater effective mass of the latter if, as it seems likely that, van der Waals forces are the principal forces involved in the combination of uncharged specific substrates and competitive inhibitors with the active site of the enzyme.

Experimental[32,33]

N-Acetyl-L-hexahydrophenylalanine.[34]—A solution of 12.6 g. of acetyl-L-phenylalanine in 75 ml. of glacial acetic acid was hydrogenated over platinic oxide at 40 p.s.i. of hydrogen at 25°, the reaction mixture filtered, the filtrate largely freed of solvent, the residue triturated with water, the colorless crystalline solid collected and dried to give 11.8 g. of product, m.p. 182–183°. Recrystallization from water gave a product, m.p. 188–189°, $[\alpha]^{25}D$ −5.5° (*c* 8.2% in ethanol). A portion of the product, m.p. 182–183°, was hydrolyzed with aqueous hydrochloric acid, the hydrolysate neutralized and treated with *p*-toluenesulfonyl chloride and aqueous sodium hydroxide to give *p*-toluenesulfamido-L-hexahydrophenylalanine, m.p. 162–163°, lit.[35] m.p. 160.5°.

Acetyl-L-hexahydrophenylalaninamide.—A solution of 11.8 g. of acetyl-L-hexahydrophenylalanine, m.p. 182–183°, in 80 ml. of absolute ethanol was saturated at 0° with dry hydrogen chloride, the reaction mixture allowed to stand at 25° for 3 days, and then evaporated *in vacuo* to a thick sirup. The sirup was dissolved in 200 ml. of methanol, the solution saturated at 0° with anhydrous ammonia, the reaction mixture allowed to stand at 25° for one week, then evaporated to dryness, the solid residue extracted with chloroform and the residual solid recrystallized twice from water to give 5.6 g. of the desired amide, colorless needles, m.p. 156–157°, $[\alpha]^{25}D$ −16.5 ± 0.7° (*c* 3.8% in ethanol). Recrystallization of this product from ethyl acetate and then from water gave a product of identical m.p.

Anal. Calcd. for $C_{11}H_{20}O_2N_2$ (212.3): C, 62.2; H, 9.5; N, 13.2. Found: C, 62.0; H, 9.6; N, 13.6.

Acetyl-DL-hexahydrophenylalaninamide, m.p. 205–207°, was prepared in an analogous manner from either acetyl-DL-phenylalanine or acetyl-DL-tyrosine except in the latter instance ethanol was used in lieu of glacial acetic acid in the hydrogenation and hydrogenolysis of acetyl-DL-tyrosine.

Enzyme Experiments.—The general technique was identical with that described previously[13] and in every instance a formol titration[13] was used to follow the course of the reaction. All measurements were made at 25° in solutions 0.02 *M* with respect to the amine component of a tris-(hydroxymethyl)-amino methane–hydrochloric acid buffer and the enzyme preparation was Armour lot no. 90402. The enzyme preparation used in experiments no. 53–60 was a sample of the same lot no. which was lost for a period and when recovered was found to possess a diminished activity corresponding to a lesser amount of protein-nitrogen. It will be noted that in all experiments the relative concentrations of E and S were such as to permit the attainment of zone A conditions[11-13] and that there is no question as to the stability of α-chymotrypsin in aqueous solutions at 25° for the periods required in this investigation.[13,36]

(30) H. T. Huang and C. Niemann, THIS JOURNAL, **73**, 3223 (1951).
(31) H. T. Huang, R. V. MacAllister, D. W. Thomas and C. Niemann, *ibid.*, **73**, 3231 (1951).
(32) All melting points are corrected.
(33) Microanalyses by Dr. A. Elek.
(34) D. Shemin and R. M. Herbst, *ibid.*, **61**, 2471 (1939).
(35) P. Karrer and W. Kehl, *Helv. Chim. Acta*, **13**, 50 (1930).
(36) D. S. Hogness and C. Niemann, *ibid.*, **75**, 884 (1953).

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[CONTRIBUTION FROM THE INSTITUTE OF PAPER CHEMISTRY]

The Constitution of Sapote Gum. II. Components of the Methyl Ether Derivative

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Methanolysis of sapote gum methyl ether furnishes a sirupy mixture of glycosidic products which can be separated into two fractions. The components of one of these fractions have been reported previously. The second fraction is composed of compounds containing both the glycosidic methyl group and the uronic acid ester function. Further treatment of this fraction has furnished, in part, the anomeric forms of methyl (methyl 3,4-di-O-methyl-D-glucopyranosid)-uronate. These, characterized by reduction of the methoxycarbonyl group followed by hydrolysis of the glycosidic function to provide 3,4-di-O-methyl-D-glucose, furnish crystalline 3,4-di-O-methyl-D-glucuronic acid upon removal of the ester and the aglycone methyl group by hydrolysis. The remainder of the second fraction has not been resolved completely. Prolonged aqueous hydrolysis thereof furnishes 3-O-methyl-D-xylose as the alkylated sugar moiety. The molar ratio of the components of sapote gum methyl ether has been estimated and some conclusions are drawn as to the molecular architecture of the macromolecule.

Methanolysis of sapote gum methyl ether produces a sirupy mixture of glycosidic products which can be separated into two fractions. The components of one of these fractions[1] have been identified as being derived from 3-O-methyl-D-xylose, 2,3,4-tri-O-methyl-D-xylose and 2,3,4-tri-O-methyl-L-arabinose. The components of the second fraction contain both the glycosidic methyl group and the uronic acid ester function. Upon further treatment of this fraction with methanolic hydrogen chloride and eventual vacuum distillation of the products of the reaction, methyl (methyl 3,4-di-O-methyl-D-glucopyranosid)-uronate in its anomeric forms was obtained as one of the components. The latter was characterized by reduction of the methoxycarbonyl group to the primary alcohol function followed by hydrolysis of the glycosidic methyl group to provide the known crystalline compound, 3,4-di-O-methyl-D-glucose. When both the ester group and the aglycone function are removed from the glycosiduronate by hydrolysis, the product of the reaction, namely, 3,4-di-O-methyl-D-glucuronic acid, may be obtained in crystalline form. It reacts in stoichiometric proportion with one mole of periodic acid establishing the presence

(1) E. V. White, THIS JOURNAL, **75**, 257 (1953).

of a glycol type structure and, therefore, the only possible positions of the O-methyl substituents. Certain of the uronic acid units resident in the sapote gum molecule are, therefore, united at the 2-position by glycosidic linkage to other carbohydrate units. This type of linkage has not been reported previously in the uronic acid units of the polysaccharides, although Lythgoe and Trippett[2] have established its presence in the biouronate component of glycyrrhinic acid.

The aldobiosiduronate components of the second fraction have not been completely resolved and, indeed, may prove to be a mixture of closely related compounds. Prolonged hydrolysis furnishes 3-O-methyl-D-xylose as the alkylated sugar moiety thus demonstrating that either or both the 2- and 4-positions are glycosidically engaged with alkylated glucuronic acid residues. These, in the original polysaccharide, are evidently of two types, one of which bears an O-methyl substituent.[3]

A quantitative evaluation of the components of the methyl ether derivative is a matter of some difficulty because decomposition inevitably accompanies all attempts to effect hydrolytic cleavage of the resistant glycosidic linkage of the glycuronic acid residues. A successful hydrolysis could, of course, be achieved by the usual methods if the uronic acid groupings or the esters thereof were reduced to the alcoholic function prior to the hydrolysis reaction. Unfortunately, the concomitant decrease in solvent solubility of the polysaccharide ether, brought about by a replacement of the solubilizing methoxycarbonyl groups, interferes radically with quantitative reduction of the macromolecule. This difficulty was overcome by a partial methanolysis which effected both a decrease in the molecular weight of the polysaccharide ether and an increase in the solubility of the products through methyl glycosidation and simultaneous esterification. The resulting sirupy mixture of glycosidic products was then reduced using lithium aluminum hydride, and finally hydrolyzed without difficulty to the corresponding reducing sugars. An examination of the sirup by paper chromatography, using the previously isolated components or derivatives thereof as reference standards, revealed four distinctly separate bands whose elution product could be determined reproducibly upon a micro scale.[4] The molar ratio of the components by this method was: 3-O-methyl-D-xylose, 2.80 parts; 3,4-di-O-methyl-D-glycuronic acid determined as 3,4-di-O-methyl-D-glucose, 1.11 parts; 2,3,4-tri-O-methyl-L-arabinose plus a possible unidentified sugar, 2.12 parts; and 2,3,4-tri-O-methyl-D-xylose, 0.97 part.

The possibility for error in so complex an evaluation is not to be underestimated. The final result is ultimately dependent upon the stoichiometric character of three successive reactions, namely, simultaneous esterification and glycosidation, reduction and hydrolysis. Two of these reactions may be accepted as quantitative without serious question but that of the reduction step has yet to be established. Furthermore, the chromatographic band containing 2,3,4-tri-O-methyl-L-arabinose may actually contain a second, as yet unidentified, component of very similar or identical R_f value. For the present, therefore, it seems best to consider the molar ratio of the components as an approximation. On this basis, the end or terminal groups of the sapote system are units of D-xylose and L-arabinose as evidenced by their fully alkylated derivatives in the methanolyzate. Other units of D-xylose serve as branch points in the system with side chains originating at either the 2- or 4-positions thereof as described by the 3-O-methyl-D-xylose component. The remainder of the molecule may be composed of glucuronic acid residues some of which are united to other parts of the system by an unusual 1,2-linkage. These latter units provide the only evidence of linearity thus far encountered in the sapote polysaccharide.

It is not possible, of course, at the present time, to decide upon the manner in which the different components are united with each other to form the molecular architecture of the original polysaccharide. A clue thereto is to be found in the precise structure of the aldobiosiduronate components of the second fraction from which, by hydrolytic cleavage, 3-O-methyl-D-xylose was identified as the alkylated sugar moiety.

Experimental Part

Methylation of Sapote Gum and Partial Methanolysis of the Methyl Ether Derivative.—Sapote gum methyl ether was prepared by the procedure described previously.[1] In this instance, the product was isolated by warming a solution of the dialyzed, deionized ether derivative to 70° and filtering off the resulting precipitate. The latter was dissolved, while moist, in methanol and then dried as a film on a glass surface.

A partial methanolysis of the product was achieved by following the previous procedure.[1] The resulting sirup was divided arbitrarily into part A and part B.

Separation of the Aldobiosiduronate and Glycosiduronate Components.—Part A, 20 g., was dissolved in 200 ml. of 3 *N* barium hydroxide and warmed at 60° for two hours to saponify the ester grouping and form the corresponding barium salts. Excess alkali was neutralized with carbon dioxide gas and the precipitated barium carbonate was removed by filtration. The filtrate was then freed from barium ion on a column of Amberlite resin IRA 120 followed by absorption of the acidic components of the solution on an adequate column of Amberlite resin IR 4B. The eluate was not investigated. The absorbed acids were then eluted from the column with *N* ammonium hydroxide solution, the excess of ammonia being removed from the eluate by distillation under reduced pressure. The solution was finally deionized with Amberlite resin IRA 120 and the eluate concentrated to a sirup. The latter was dried, dissolved in ether, and treated with an ethereal solution of diazomethane in order to esterify the carboxyl residues. After removal of excess reagent and solvent, the resulting sirup was distilled fractionally at 0.2 mm. to furnish fraction I, 0.5 g. (b.p. 85–100°); fraction II, 2.5 g. (b.p. 100–110°); fraction III, 5.3 g. (b.p. 160–185°).

Isolation and Identification of 3,4-Di-O-methyl-D-glucuronic Acid.—Fraction I, 0.5 g., proved to be a part of fraction II. Upon treatment with methanolic ammonia in the usual manner, an amide of methyl 3,4-di-O-methyl-D-glucopyranosiduronic acid was obtained. When recrystallized from ethyl acetate it melted at 192°.[2]

Anal. Calcd. for $C_9H_{17}O_6N$: OMe, 39.6. Found: OMe, 39.4.

Fraction II, 2.0 g., was hydrolyzed for 24 hours on a boiling water-bath with 15 ml. of *N* sulfuric acid in order to remove both the methyl ester group and the aglycone func-

(2) B. Lythgoe and S. Trippett, *J. Chem. Soc.*, 1983 (1950).

(3) E. Anderson and H. D. Ledbetter, *J. Am. Pharm. Assoc.*, **40**, 623 (1951).

(4) E. L. Hirst, L. Hough and J. K. N. Jones, *J. Chem. Soc.*, 928 (1949).

tion. The reaction mixture was then cooled, neutralized with barium carbonate, and filtered. The filtrate was freed from barium ion on a column of Amberlite resin IRA 120 and the eluate concentrated under reduced pressure to a sirup, yield 1.5 g. The latter was extracted with acetone and, upon removal of excess solvent, the solution furnished crystalline 3,4-di-O-methyl-D-glucuronic acid with m.p. 184°, $[\alpha]^{20}D$ 60° (*c* 3.0, equilibrium in water).

Anal. Calcd. for $C_8H_{14}O_7$: OMe, 27.9. Found: OMe, 27.1.

A clue to the structure of the compound was provided by its reaction in stoichiometric proportion with 1.0 mole of periodic acid. Neither the isomeric 2,3-di-O-methyl nor the 2,4-di-O-methyl derivative is capable of reacting with this reagent.

When 1.0 g. of the corresponding glycosiduronate, fraction II, was dissolved in 20 ml. of anhydrous tetrahydrofuran and added dropwise to a well-stirred solution containing 0.75 g. of lithium aluminum hydride in 30 ml. of tetrahydrofuran maintained at 25°, the ester function was smoothly reduced to the primary alcohol group. The excess of reagent was destroyed by careful addition of moist tetrahydrofuran followed by an excess of water in order to decompose the addition product complex and precipitate the hydroxides of lithium and aluminum. These were removed by filtration. The filtrate was then evaporated under reduced pressure to a sirup. This was extracted with acetone and the solution, after concentration, was extracted with ether. After removal of solvent, the residue was distilled at 0.2 mm. The yield, 0.7 g., had b.p. 108–110°. Upon hydrolysis of the aglycone group with *N* sulfuric acid followed by isolation of the product in the usual manner, a sirup was obtained. The yield was 0.6 g. This was extracted with propyl acetate and crystallized from the same solvent to furnish 3,4-di-O-methyl-D-glucose with m.p. 125°[5]; $[\alpha]^{20}D$ 73° (*c* 3.0, equilibrium in water).

One-tenth of a gram of the crystalline compound was treated in the usual manner with 1.1 moles of aniline in absolute ethanol. Upon removal of solvent and crystallization from ether, the aniline derivative of 3,4-di-O-methyl-D-glucose was obtained. It melted at 175°,[6] unchanged upon admixture with an authentic specimen.

Examination of Fraction III.—Fraction III has not been completely characterized. It gives evidence of being a mixture of two aldobiosiduronates and a small amount of entrained fraction II. Repeated treatment with 7% methanolic hydrogen chloride, followed by isolation of the products in the usual manner, eventually furnished a sirup distilling under high vacuum in the boiling range of fractions I and II. The distillate thus obtained was treated as described for part A except that the glycoside-containing effluent from the column of Amberlite resin IR 4B was examined. The solution was evaporated to a sirup under reduced pressure, extracted with acetone, and dried. When redissolved in acetone, 3-O-methyl-D-xylose crystallized in good yield; it melted at 104°,[7] unchanged upon admixture with an authentic specimen.

The acidic products from the hydrolysis of fraction III which were adsorbed on the Amberlite resin column, IR 4B, were not investigated.

Estimation of the Molar Ratio of the Components of Sapote Gum Methyl Ether.—Part B of the sirup, obtained upon partial methanolysis of the ether derivative, was reduced with lithium aluminum hydride following the previously described procedure. The reaction products were isolated as before and then treated under reflux with 3% methanolic hydrogen chloride to complete the methanolysis reaction initiated upon the original ether derivative. The reaction products were isolated in the usual manner, dissolved in sufficient *N* sulfuric acid to provide a 10% solution, and heated under reflux for five hours. The reaction mixture was then cooled, neutralized with barium carbonate, and filtered. The filtrate, evaporated under reduced pressure to a sirup, was separated from residual inorganic salt by extraction with acetone. The components of the latter solution were separated into four distinct bands by paper partition chromatography, using the strip technique, with a mixture of ethyl acetate, acetic acid and water (9:2:2) as the developer. The reference standards were the compounds or derivatives thereof previously isolated and described as the components of the methyl ether derivative. The aqueous eluates of the different bands were analyzed by the alkaline iodine method.[4] The average values from five determinations, calculated on a basis of seven molar parts, were as follows: 3-O-methyl-D-xylose, 2.80 parts; 3,4-di-O-methyl-D-glucuronic acid determined as 3,4-di-O-methyl-D-glucose, 1.11 parts; 2,3,4-tri-O-methyl-L-arabinose plus a possible unidentified compound, 2.12 parts; and 2,3,4-tri-O-methyl-D-xylose, 0.97 part. There was some indication that the band containing the alkylated arabinose fraction also contained a second component of very similar R_f value but this, if actually present, could not be separated by the present chromatographic method. No evidence was obtained to support the concept that the compound in question could be 2,3,4-tri-O-methyl-D-glucose derived from the reduction of the corresponding glucuronic acid derivative although the presence of the latter in the hydrolyzate is suspected.

(5) D. J. Bell and G. D. Greville, *J. Chem. Soc.*, 1902 (1950).

(6) The specimen sample was kindly supplied by G. D. Greville.

(7) P. A. Levene and A. L. Raymond, *J. Biol. Chem.*, **102**, 331 (1933).

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