constant of the corresponding acid have been determined,³⁰ the corresponding data for the meta derivatives have not been reported. It is clear that more study of biphenyl derivatives with functional meta substituents is needed in order to establish whether or not the behavior of *m*-styryl indicates an important second-order dissymmetry of the σ -bond to the substituent. Experiments designed to provide the requisite information are in progress.

We will now return to the para substituents. It is clear that moving the substituent from the meta to the para position has an opposite effect upon the σ -constant in the two cases under consideration. Although the difference in, for example, the acidity constants is small in the case of the styrylbenzoic acids the fact that sign changes can only be accounted for by assuming that there is a small, direct interaction between a carboxyl function and the unsaturated substituent which is destroyed or inhibited in the reaction process. Structure VII illustrates the concept.



(30) E. Tommila, L. Brehmer and H. Elo, Ann. Acad. Sci. Fennicae, Ser. A59, No. 9, 3 (1942). That such an interaction should not be of importance in the acetylenic acid is implicit in our previous discussion. However, the geometry of the molecule will not permit the view that the effects responsible for the influence of *m*-phenylethynyl are alone sufficient to account for the large positive value of the para sigma constant. It is quite possible that the acetylenic function serves as an electron sink and actually exhibits a high conjugative aptitude toward donor systems. Structures such as VIII may account for the preferential stabilization of anions such as the substituted benzoate ion and the transition state for ester saponification.



An alternative to this explanation may be that the change in effective electronegativity of the carbon atom to which the ethynyl group is attached results in the development of a positive charge on the para position.³¹

(31) This situation would be similar to that in pyridine in which a slight assymmetry is induced in the π electron density because of the high electron affinity of the hetero atom.³² It is our opinion that such non-classical inductive effects are not sufficient to account for observed effects.

(32) J. Ploguin, Compt. rend., 226, 245, 339 (1948).

Ames, Iowa

[CONTRIBUTION FROM THE DEPARTMENT OF AGRICULTURAL CHEMISTRY, UNIVERSITY OF NEBRASKA COLLEGE OF AGRICULTURE, AND THE DEPARTMENT OF BIOLOGICAL CHEMISTRY, UNIVERSITY OF ILLINOIS COLLEGE OF MEDICINE]

Studies on Inulin. The Preparation and Properties of Inulobiose¹

By John H. Pazur and Alan L. Gordon²

Received February 23, 1953

Inulobiose, a reducing disaccharide with the probable structure 1-(β -D-fructofuranosyl)-D-fructose, has been isolated from a partial acid hydrolysate of inulin by paper chromatographic methods. The specific rotations of the new compound and of its octaacetyl derivative are -32.5° (c 2.6, water) and -6.5° (c 1.5, chloroform), respectively. The rate constant for the hydrolysis of inulobiose in 0.01 N hydrochloric acid at 70° is 0.042 min.⁻¹ and for the hydrolysis of sucrose under the same conditions it is 0.018 min.⁻¹.

The oligosaccharides produced enzymatically from sucrose⁸ have been shown, by paper chromatographic procedures, to contain sucrosyl and inulobiosyl moieties. In order to establish the structure of these oligosaccharides with finality, it is necessary to have pure inulobiose available as a reference compound. Inulobiose can be prepared from inulin by controlled acid hydrolysis or from fructose and raffinose by enzymatic synthesis.⁸ It is a reducing disaccharide consisting of two fructose residues united through a β -2,1'-D-fructosidic bond. The new disaccharide should prove to be an ideal substrate for the detection of β -D-fructofuranosidase activity⁴ in enzyme preparations.

(1) Published with the approval of the Director as paper No. 602, Journal Series, Nebraska Agricultural Experiment Station.

(2) Research Fellow, University of Illinois, College of Medicine, Chicago, Illinois. The funds for this fellowship were made available through a grant to the University of Illinois College of Medicine from the National Fund for Medical Education.

(3) J. H. Pazur, J. Biol. Chem., 199, 217 (1952).

(4) C. Neuberg and I. Mandl, in J. B. Sumner and K. Myrbäck, "The Enzymes," Vol. 1, Part 1, Academic Press, Inc., New York, N. Y., 1950, p. 527.

The methods for the preparation of inulobiose from inulin as well as the physical and chemical properties of the new compound are described in this paper. Preliminary experiments on the rate of hydrolysis of inulin in 0.01 N hydrochloric acid at 70° showed that the concentration of inulobiose attained a maximum in 30 minutes. The products in the neutralized hydrolysate consisted of fructose, inulobiose, inulotriose and the other homologous members of the inulin series⁵ (Fig. 1). The first members of this series were resolved by descending paper chromatography. The inulobiose was extracted from the chromatograms with water, concentrated in vacuo, and precipitated with acetone. Details of the preparative procedures are presented in the experimental section.

Properties of Inulobiose.—From a hydrolysate of 10 g. of inulin, 0.42 g. of pure inulobiose was obtained. The disaccharide dissolves readily in

(5) The small quantity of D-glucose present in inulin probably results in the formation of oligosaccharides of glucose and fructose residues. However, the concentration of these oligosaccharides is insignificant in comparison to the fructosyl series. water and tastes sweeter than sucrose. The specific rotation of inulobiose is $[\alpha]D - 32.5^{\circ}$ (c 2.6, water). The octaacetyl derivative of inulobiose was crystallized from ethyl alcohol and was also levorotatory; $[\alpha]_D - 6.5^\circ$ (c 1.5, chloroform). The purity of the inulobiose preparation and the

nature of the products of acid hydrolysis were ascertained by paper chromatography. A tracing of the paper chromatogram is reproduced in Fig. 1. Since one characteristic spot was obtained on the chromatogram, the inulobiose is obviously free from contamination by the other homologs of the inulin series. In addition, Fig. 1 shows that fructose and unhydrolyzed inulobiose were the reducing components in a partial acid hydrolysate of inulobiose and that fructose was the only reducing compound produced on complete hydrolysis. Quantitative determination of fructose in the hydrolysate agreed with the calculated yield for a disaccharide of two fructose units. The molecular weight of our product as calculated from its reducing power is 336 and is in good agreement with a molecular weight of 342 for a disaccharide of two hexose units.

The configuration of the fructose units of inulin is known to be fructofuranose and the bond between the monosaccharide units to be β -2,1'-Dfructosidic.⁶ The fructose units in inulobiose, the disaccharide from inulin, may be assumed to be connected through a similar β -2,1'-linkage. On the basis of the foregoing argument and the experimental findings (fructose content, molecular weight, acetylation value, etc.), the tentative structural configuration assigned to inulobiose is $1-(\beta$ -Dfructofuranosyl)-D-fructose.

Previous work⁷ on the rate of hydrolysis of sucrose and inulin indicated that the two compounds were hydrolyzed at approximately the same rate. More recently, experiments on the identification of the hydrolytic products from 1-inulobiosyl-D-glucose³ have shown that the inulobiosyl moiety was hydrolyzed more rapidly than the sucrosyl moiety. The rate constants for the hydrolysis of sucrose and of inulobiose have now been determined at 70° and in 0.01 N hydrochloric acid. The values, 0.018 min.⁻¹ for sucrose and 0.042 min. $^{-1}$ for inulobiose, illustrate the difference in the susceptibility of the two linkages to hydrolysis by acids.

Experimental

Rate of Inulin Hydrolysis.--- A solution of 1 g. of inulin⁸ in 5 ml. of water was added to 5 ml. of 0.02 N hydrochloric acid. The resulting solution was placed in a constant temperature water-bath at 70°. After intervals of 5, 10, 15, 30, 60 and 120 minutes, samples of 1 ml. were removed and neutralized with solid sodium carbonate. 0.02-ml. aliquots of these samples were tested for reducing com-pounds by paper chromatographic methods.⁹ Examination of the sprayed chromatogram showed that with hydrolysis periods up to 30 minutes the concentration of the low molecular weight compounds increased and that with hydroly-



Fig. 1.-A double ascent paper chromatogram of inulin oligosaccharides, of inulobiose and its hydrolytic product and of reference compounds.

sis periods greater than 30 minutes the concentration of fructose continued to increase, whereas that of the oligo-saccharides markedly decreased. Consequently the hy-drolytic conditions of 0.01 N hydrochloric acid at 70° for 30minutes were selected for preparing an acid hydrolysate of inulin.

Preparation of Inulobiose.---A solution of 10 g. of inulin in 100 ml. of 0.01 N hydrochloric acid was placed in a constant temperature bath of 70°. After hydrolysis for 30 minutes it was neutralized with solid sodium carbonate. 2-ml. aliquots of the neutralized hydrolysate were intro-24mi. and dots of the neutralized hydrolysate were into-duced in a continuous streak near one edge of a rectangle of Whatman No. 2 filter paper (57×46 cm.). The paper sheets were placed in a chromatocab¹⁰ arranged for descend-ing paper chromatography. The solvent system employed many fitteral corriding water (64.42 by volume). After was n-butanol-pyridine-water (6:4:3 by volume). After 72 hours the chromatograms were removed from the container and dried at room temperature. Two strips (2 cm. wide) from the chromatograms were sprayed for reducing sugars⁹ and used as markers for sectioning the remainder of the chromatograms.

The sections of the chromatograms containing inulobiose were combined and extracted with water. The water ex-tract was concentrated by distillation under vacuum and the inulobiose precipitated with acetone. The compound was collected on a filter and dried to constant weight in a vacuum oven at 60°. The yield of inulobiose was 0.42 g. Its specific rotation is $[\alpha]_D - 32.5^\circ$ (c 2.6, water).

Molecular Weight Determination.-The reducing power of 2.65 mg. of inulobiose was determined with the alkaline copper reagent 60 as outlined by Shaffer and Somogyi.11 The value for inulobiose expressed in milliequivalents of reducing agent per gram of inulobiose was 15.6. Since glucose,¹³ fructose and maltose¹² yield values of 32.3, 31.8 and 16.4, respectively, the theoretical reducing value expected for inulobiose was 15.9 (one-half of the value for fructose). As already indicated, the molecular weight of inulobiose calculated on this basis was found to be 336; actual molecular weight 342.

Preparation of Octaacetyl Inulobiose.--- A sample of 0.1 g. of pure inulobiose was stirred with 3 ml. of cold pyridine and 2 ml. of cold acetic anhydride. Acetylation was allowed to proceed in the refrigerator for 6 days. At the end of this

⁽⁶⁾ E. G. V. Percival, "Structural Carbohydrate Chemistry,"

Prentice-Hall, Inc., New York, N. Y., 1950, p. 119. (7) H. Hibbert and E. G. V. Percival, THIS JOURNAL, 52, 3995 (1930).

⁽⁸⁾ Reagent Grade, Nutritional Biochemicals Corp., Cleveland, Ohio.

⁽⁹⁾ D. French, D. W. Knapp and J. H. Pazur, THIS JOURNAL, 72, 5150 (1950).

⁽¹⁰⁾ Research Equipment Corporation, Oakland, California

⁽¹¹⁾ P. A. Shaffer and M. Somogyi, J. Biol. Chem., 100, 695 (1933). (12) M. A. Swanson and C. F. Cori, ibid., 172, 797 (1948), have reported that the reducing power of maltose was one half of the value for glucose when measured by this method.

time, the mixture was poured into 200 ml. of cold water which decomposed the excess acetic anhydride. The inulobiose acetate was extracted from this solution with diethyl ether. The residue obtained on evaporation of the ether was dissolved in 5 ml. of hot ethyl alcohol, decolorized with carbon, and allowed to crystallize in a refrigerator. The crystalline acetate was collected on a filter and dried to constant weight; yield 0.08 g., $[a]D - 6.5^{\circ}$ (c 1.5, chloroform). The acetyl groups in the derivative were determined quantitatively by saponification methods¹³; acetyl content, 51.6% (found), and 50.9% (calculated).

Acid Hydrolysis of Inulobiose.—A solution of 1.5 ml. of inulobiose (2.65%) and 1.5 ml. of 0.02 N hydrochloric acid was heated in a constant temperature water-bath of 70°. Aliquots of 0.2 ml. were removed at 0, 15, 30, 60 and 180 minutes and neutralized with sodium carbonate. The compounds present in the hydrolysate were resolved on paper chromatograms. The chromatogram for the 30 and 180 minute hydrolysate is reproduced in Fig. 1.

The remainder of the hydrolysate was cooled and used for qualitative and quantitative identification of the hydrolytic product of inulobiose. As shown in Fig. 1, one monosaccharide was produced on acid hydrolysis of inulobiose. This monosaccharide reacted positively with Benedict and Seliwanoff solutions, moved on paper with an $R_{\rm J}$ value identical with that of fructose, and formed an osazone deriva-

(13) A. Kunz and C. S. Hudson, THIS JOURNAL, 48, 1978 (1926).

tive at the same rate as fructose. Microscopic examination of the osazone crystals showed that the two osazones were identical. The quantitative determination¹⁴ of fructose in appropriately diluted aliquots of the hydrolysate yielded values of 0.114 and 0.118 mg. per ml.; theoretical value from the weight of inulobiose, 0.112 mg. per ml.

Rate Constants for Hydrolysis of Sucrose and Inulobiose. Two ml. of 0.02 N hydrochloric acid was added to 2 ml. of 0.0388 M solution of sucrose or inulobiose. The test tubes containing the solutions were stoppered tightly and heated in a water-bath at 70° . 0.2-ml. samples were removed, in duplicate, at intervals of 0, 5, 10, 15, 20, 30 and 60 minutes and introduced into large test-tubes containing 4.8 ml. of water and 5 ml. of alkaline copper reagent 60. After all the samples had been collected, the reducing values of the aliquots were determined in the usual manner.¹¹ From the increase in reducing values the amounts of sucrose and inulobiose hydrolyzed at the various times were determined. These values and the equation for first-order kinetics were used to calculate the hydrolysis rate constants for the two compounds. At 70° and in 0.01 N hydrochloric acid, k for the hydrolysis of sucrose is 0.018 min. $^{-1}$ and k for the hydrolysis of inulobiose is 0.042 min.⁻¹.

(14) J. H. Roe, J. Biol. Chem., 107, 15 (1934).

LINCOLN, NEBRASKA CHICAGO, ILLINOIS

[BIOCHEMISTRY DEPARTMENT, MEDICAL COLLEGE OF ALABAMA AND SCHOOL OF DENTISTRY, UNIVERSITY OF ALABAMA]

Reactions of Carbohydrates with Nitrogenous Substances.¹ II. Factors Affecting the Darkening of N-D-Glucosylaniline^{2,3}

BY LAWRENCE ROSEN, KENNETH C. JOHNSON AND WARD PIGMAN

Received January 5, 1953

The major factors involved in the darkening of methanolic solutions of N-D-glucosylaniline are the amounts of water and acid present. The rate in the early stages of darkening increases with an increase in the normality of the hydrogen chloride present at any fixed concentration of water. An important relationship at this stage is the molar ratio of hydrogen chloride to N-D-glucosylaniline. When this ratio is less than unity, there is an initial inhibition period in the development of color; when the ratio becomes greater than unity, the inhibition disappears. The darkening of N-D-glucosylaniline as a function of acid concentration at longer time periods shows an optimum at approximately a molar ratio of unity. The effect of water is to inhibit the color development at any given concentration of hydrogen chloride studied. Atmospheric oxygen, copper ion catalysis, and the darkening of the possible hydrolysis products (glucose or aniline) do not contribute significantly to the darkening of N-D-glucosylaniline.

Solid mixtures and solutions of amino acids and sugars or their derivatives spontaneously form dark odorous products on storage. This type of reaction (non-enzymic browning) may be responsible for some of the dark colors, odors and changes in protein solubility that take place during the drying and subsequent storage of foods.⁴ Maillard⁵ was the first to investigate systematically the browning reaction by means of model systems. Such model systems represent a fundamental approach to the complex browning problem because they offer a much more readily controlled

(1) For Paper I of this series, see THIS JOURNAL, 73, 1976 (1951).

(2) This paper is taken in part from the thesis of Lawrence Rosen submitted to the University of Alabama in partial fulfillment for the degree of Master of Science, June, 1952.

(3) Presented in part before the Division of Sugar Chemistry of the American Chemical Society at the 122nd National Meeting, September 16, 1952, in Atlantic City.

16, 1952, in Atlantic City.
(4) For reviews see: J. P. Danehy and W. W. Pigman, "Advances in Food Research," Vol. III, Academic Press Inc., New York, N. Y., 1951, pp. 241-290; E. R. Stadtman, *ibid.*, Vol. I, 1948, pp. 325-372.

(5) (a) L. C. Maillard, Compt. rend., 154, 66 (1912); (b) Compt. rend. soc. biol., 72, 599 (1912). set of reactions. The use of such model systems has become increasingly apparent. $^{6-9}$

The compound N-D-glucosylaniline (N-phenyl-D-glucosylamine) undergoes darkening in acid solution.^{7,10} Cameron^{10b} believed that N-D-glucosylaniline in acetic acid solution underwent hydrolysis to *aldehydo*-glucose and aniline. This open chain form of glucose was thought to be especially reactive and to undergo changes to the darkened material. Cameron^{10b} isolated 2,5-dianilidoquinone from the dark material. However, this aniline oxidation product formed but a small part of the total dark material.

(6) M. L. Wolfrom and associates, (a) THIS JOURNAL, **68**, 2022 (1946); (b) **69**, 2411 (1947); (c) **70**, 514 (1948); (d) **71**, 3518 (1949).

(7) H. H. Beacham and M. F. Dull, Food Research, 16, 439 (1951).
(8) B. Singh, G. R. Dean and S. M. Cantor, THIS JOURNAL, 70, 517 (1948).

(9) C. H. Lea and associates, Biochem. J., 47, 626 (1950); Biochim. Biophys. Acta, 3, 313 (1949); 4, 518, 532 (1950); 5, 433 (1950); 7, 366 (1951); 9, 56 (1952).

(10) (a) C. N. Cameron, THIS JOURNAL, 48, 2233 (1926); (b) *ibid.*, 2737 (1926).