[CONTRIBUTION FROM THE BIOLOGY DIVISION, OAK RIDGE NATIONAL LABORATORY]

Further Studies on the Separation of the Borate Complexes of Sugars and Related Compounds by Ion-exchange Chromatography[1]

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RECEIVED OCTOBER 30, 1952

Further studies have been made on the separation of sugars and related compounds by the ion-exchange chromatography of their borate complexes on strong-base anion exchangers. Sugars known to occur in complex mixtures, such as those obtained from plants, have been studied with regard to their elution characteristics. The following compounds have been included in this study: raffinose, rhamnose, stachyose, sorbose, gentiobiose, melibiose, melezitose, turanose, sedoheptulose, sedoheptulosan, dulcitol, sorbitol and mannitol. A procedure for the removal of borate from the isolated sugar–borate complexes is presented as part of the process for recovery of the pure sugar.

Introduction

A technique for the separation of sugars by the ion-exchange chromatography of their borate complexes has recently been reported by Khym and Zill.[2] The extension of this method to other sugars and sugar alcohols which are known to occur in complex mixtures, such as those obtained from plant sources, represents a part of the present report. It is also our purpose to further demonstrate the apparent relationships which exist among the structural configurations of the polyhydroxy compounds, the borate complexes of the compounds, and the affinities of the complexes for the exchanger. A final object of this study was the development of a procedure for the removal of borate from the isolated sugar–borate complexes as a step in the recovery of the pure sugars.

Experimental

The apparatus used is identical with that previously described.[2] All the sugars with the exception of sedoheptulose were assayed by the anthrone method.[3,4] Sedoheptulose and sedoheptulosan were assayed by the orcinol method.[5] The sugar alcohols were determined by the West and Rapoport modification[6] of the chromotropic acid test. In order to determine the elution characteristics of the individual compounds, separate column runs were carried out. In addition, paper chromatography[7] was used for the identification of sugars isolated by the exchangers. To obtain satisfactory papergrams, removal of cations from the sample is essential, as pointed out and described in the previous paper.[2]

The preparation of ion exchangers (Dowex-1, a quaternary ammonium derivative of a polystyrene resin, was obtained in 200–400 mesh beads from the Dow Chemical Co., Midland, Michigan) and experimental procedure have been described.[2]

The equilibrium mixture of sedoheptulose and sedoheptulosan was obtained by the treatment of sedoheptulosan with hydrochloric acid in a manner identical with that of LaForge and Hudson.[8] The chloride ion was removed from the final mixture by treatment with an anion exchanger (Dowex-3).

Removal of Borate from the Isolated Sugar–Borate Complexes.—The individual fractions of a single sugar were pooled and the cations removed by batchwise treatment with Dowex-50 (hydrogen form), yielding a boric acid solution of the sugar. This operation should be carried out as soon as possible after elution of the individual sugars from the column in order to prevent any decomposition which may result from the alkaline conditions. After removal of the Dowex-50 by filtration the water solution was reduced almost to dryness by distilling *in vacuo* (water-pump) at a temperature of *ca.* 30°. Repeated distillations (*in vacuo*) with 250-ml. portions of absolute methyl alcohol then removed the borate as volatile methyl borate. After the final distillation, water was added and any insoluble residue was removed by filtration through a sintered glass filter. The sugar could then be recovered from the water solution by the ordinary methods of sugar crystallization. In order to determine the completeness of removal of borate, a series of representative samples were prepared and carried through the procedure. The sugar solutions obtained were analyzed spectrographically for boron.

Results

Separation of the Hydrolytic Products of Melezitose.—The separation of glucose and fructose, which results from the complete acid hydrolysis of melezitose, can be accomplished in a simple manner.[2] If melezitose is only partially hydrolyzed (by dilute acid) the resulting mixture of glucose, turanose and residual melezitose can be resolved under the conditions given in Fig. 1.

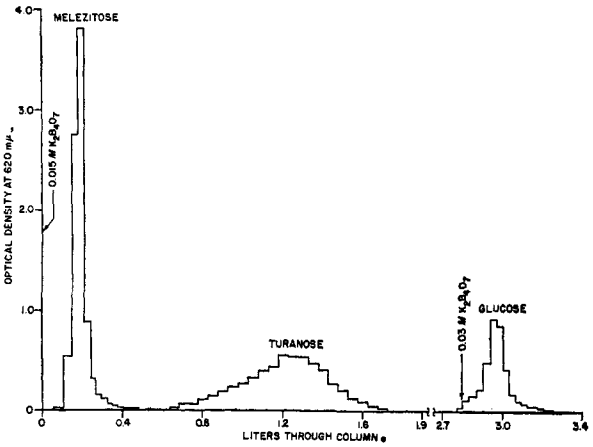

Fig. 1.—Separation of sugars resulting from partial hydrolysis of melezitose: exchanger, 0.85 sq. cm. × 11 cm. strong-base anion resin, *ca.* 300 mesh, borate form; eluting agent, potassium tetraborate as shown at 1 ml./min.; test material, 10 mg. of melezitose, 10 mg. of turanose, 10 mg. of glucose in 10 ml. of 0.01 *M* potassium tetraborate.

Any fructose produced by the hydrolytic cleavage of turanose will be eluted in the same position as turanose under the conditions given in Fig. 1. Separation of these two sugars may be achieved by recycling the mixture with the use of a different elution system as presented in Fig. 2. Turanose is found to be eluted quite easily at a low *p*H with boric acid while fructose exhibits a stronger affinity

(1) Work performed under USAEC Contract No. W-7405-eng-26.

(2) J. X. Khym and L. P. Zill, THIS JOURNAL, **73**, 2399 (1951); **74**, 2090 (1952).

(3) R. Dreywood, *Ind. Eng. Chem., Anal. Ed.*, **18**, 499 (1946).

(4) D. L. Morris, *Science*, **107**, 254 (1948).

(5) A. H. Brown, *Arch. Biochem.*, **11**, 269 (1946).

(6) C. D. West and S. Rapoport, *Proc. Soc. Exptl. Biol. Med.*, **70**, 141 (1949).

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

(8) F. B. LaForge and C. S. Hudson, *J. Biol. Chem.*, **30**, 61 (1917).

for the exchanger than does the turanose. The separation is thereby effected.

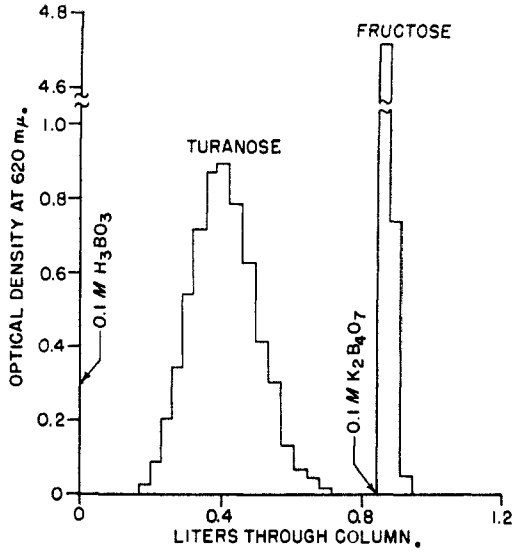

Fig. 2.—Separation of turanose and fructose: exchanger, 0.85 sq. cm. × 11 cm., strong-base anion resin, *ca.* 300 mesh, borate forms; eluting agents, boric acid and potassium tetraborate as shown at 1 ml./min.; test material, 5 mg. of turanose plus 5 mg. of fructose in 10 ml. of 0.01 *M* potassium tetraborate,

Sedoheptulose and Sedoheptulosan.—The behavior of sedoheptulose on an anion exchanger in the borate form and its separation from sedoheptulosan is given in Fig. 3. On the assumption that the free sugar and the sedoheptulosan (when present in equimolar amounts) both produce the same color intensity with the orcinol reagent, the calculated percentage composition of the mixture

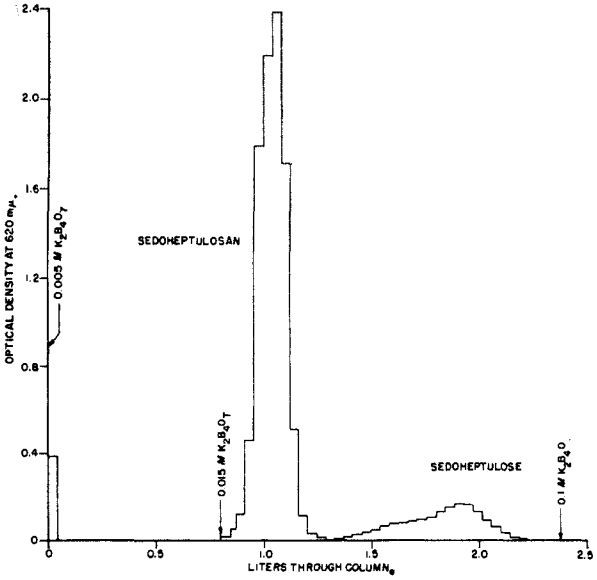

Fig. 3.—Separation of sedoheptulose and sedoheptulosan: exchanger, 0.85 sq. cm. × 11 cm., strong-base anion resin, *ca.* 300 mesh, borate forms; eluting agent, potassium tetraborate as shown at 1 ml./min.; test material, *ca.* 20 mg. of an acid prepared equilibrium mixture of sedoheptulose and sedoheptulosan in 5 ml. of 0.01 *M* potassium tetraborate.

was *ca.* 20% sedoheptulose and 80% sedoheptulosan. This ratio is in good agreement with that obtained by LaForge and Hudson[8] by the use of reduction methods. The presence of a small amount of a third component, as indicated by an initial breakthrough, has not as yet been investigated.

Miscellaneous Sugars.—The volume-to-peak values obtained for several sugars of different structural configuration are given in Table I.

TABLE I

ELUTION ORDER OF VARIOUS SUGARS

Column size: 0.85 sq. cm. × 11 cm. strong-base anion resin; eluting agent, 0.015 *M* $K_2B_4O_7$ with the exception of raffinose which was 0.005 *M* $K_2B_4O_7$.

Sugar	Volume-to-peak (ml.) value
Raffinose	320
Rhamnose	170
Stachyose	200
Sorbose	1580
Gentiobiose	5000
Melibiose	7000

Separation of Mannitol, Dulcitol and Sorbitol.—The separation of the hexahydric alcohols, mannitol, dulcitol and sorbitol is presented in Fig. 4. In order to have conditions comparable to those used during the elution of sugars and polyhydroxy compounds encountered in plants,[9] 800 ml. of 0.005 *M* potassium tetraborate was passed through the column before elution with the more concentrated borate solution was started. Under these conditions the volume-to-peak values are 2260, 2990 and 4000 ml., respectively, for the sorbitol, dulcitol and mannitol.

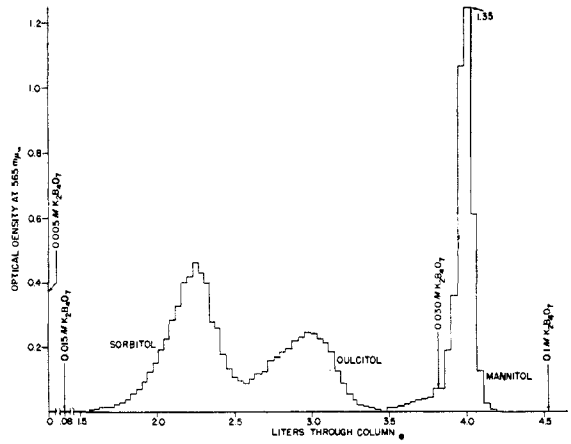

Fig. 4.—Separation of dulcitol, mannitol and sorbitol: exchanger, 0.85 sq. cm. × 11 cm., strong base anion resin, *ca.* 300 mesh, borate form; eluting agent, potassium tetraborate as shown at 1 ml./min.; test material, 5 mg. each of sorbitol, dulcitol and mannitol in 10 ml. of 0.005 *M* potassium tetraborate.

Removal of Borate.—The data showing the completeness of borate removal from several representative sugars by the described procedure are shown in Table II. Recoveries of the individual sugars, after removal of the borate, ranged from 75 to 90%.

(9) G. R. Noggle and L. P. Zill, *Arch. Biochem. Biophys.*, in press.

TABLE II

COMPLETENESS OF BORATE REMOVAL BY DISTILLATION WITH METHYL ALCOHOL

	Sample[a]	No. of distillations with 250 ml. CH_3OH	Boron, μ/ml.
1	50 mg. glucose in 500 ml. distilled water	3	1.3
2	500 ml. 0.03 *M* $K_2B_4O_7$	3	2.0
3	no. 2 + 50 mg. glucose	1	830
4	no. 2 + 50 mg. glucose	2	3.2
5	no. 2 + 50 mg. glucose	3	4.2
6	no. 2 + 10 mg. glucose	3	2.7
7	no. 2 + 50 mg. ribose	3	4.6
8	no. 2 + 50 mg. sucrose	3	3.1
9	no. 2 + 50 mg. raffinose	3	6.3

[a] Sample reduced to dryness under vacuum (20 mm.) at temperature of *ca.* 30° followed by distillation with methyl alcohol, also under vacuum. After the final distillation, the residue was taken up in water, filtered, and made up to 25 ml. for the boron determination in each case.

Discussion

It was suggested in a previous paper[2] that definite equilibria are involved as the sugar–borate complexes move down the column, not only between the borate complex and the exchanger but also with the sugar–borate complex and the various forms of the free sugars. The factors involved in these equilibria, such as mutarotation and pyranose–furanose interconversion, are therefore implicated in the affinity of the various sugar–borate complexes for the exchanger, and thereby also in the particular order of elution of the complexes. The separations which have been presented in the present paper may be interpreted on a similar basis.

Polyhydroxy compounds, such as sucrose, which possess no adjacent *cis*-hydroxyl groups have been shown to form borate complexes which exhibit but slight affinity for an anion exchanger.[2] This behavior is further shown by the rapid elution of melezitose which also possesses no *cis*-hydroxyl groups. Raffinose, which has a pair of *cis*-hydroxyls on the 3,4-positions of the pyranoid galactose moiety, was shown to be eluted with only slightly more difficulty than sucrose. The failure of *cis*-hydroxyls in a compound of such a structural configuration to form a borate complex with a high affinity for the exchanger has also been shown by the study of β-methyl-D-arabinopyranoside[10] whose elution characteristics are almost identical with those of raffinose. Further evidence concerning the formation of borate complexes by the 3,4-hydroxyls in a galactopyranoside has been reported by Macpherson and Percival.[11] They found that α-methylgalactopyranoside had little or no effect on the conductivity of a boric acid solution. This evidence for only slight formation of an ionized complex by such a configuration is then in direct agreement with the weak affinity of such complexes for an ion column. The recent establishment of the structure of sedoheptulosan as 2,7-anhydro-β-D-altroheptulopyranose[12] reveals a pyranose ring containing a pair of *cis*-hydroxyls (on carbons 4 and 5). Presumably, the borate complex of this compound should be similar to that of lactose[2] and indeed the moderately weak affinity of the borate complex of sedoheptulosan for an anion exchanger is evidence for such complex formation. On the basis of the present work and that previously reported,[2,10] it can be stated that compounds containing *cis*-hydroxyl groups in a pyranose ring form complexes which exhibit only weak affinity for a strong-base anion exchanger.

The importance of pyranose–furanose interconversion as a factor in the elution order of the borate complexes is further indicated in the behavior of gentiobiose. This sugar forms a complex which exhibits a very strong affinity for the exchanger. An even more extreme affinity than this is demonstrated in the case of melibiose. Presumably, the affinity in both cases can be attributed to the presence of a 1,6-linkage which permits the formation of the furanose isomer, a structural configuration which has been shown to be most favorable for the formation of the sugar–borate complex.[13] This transformation is not possible in the other disaccharides studied[2] and is reflected in their easy elution from a strong-base resin. The greater volume-to-peak value for melibiose over that of gentiobiose may be tentatively attributed to the presence of an additional pair of *cis*-hydroxyls on the galactose portion of the melibiose. Rhamnose represents a further example indicating the influence of the ring structure upon the elution order. One would expect considerable transition to the furanose form in the presence of borate. However, this compound increases the conductivity of boric acid solution only slightly,[11] and is found to be easily eluted from the exchanger. As an explanation for the slight conductivity increment, Böeseken[13] suggests that only small quantities of the furanose form of rhamnose are present and the transition of the pyranoses is hardly disturbed.

Sorbose, which produces a large increase in the conductivity of boric acid,[11] is found to have a correspondingly high affinity for the exchanger. The large conductivity increment (and high volume-to-peak value), is, like that of fructose,[13] evidence that with the ketoses the accumulation of hydroxyl groups in the vicinity of the reducing carbon atom is the main factor in the formation of the borate complexes. Although conductivity data are lacking for the behavior of the borate complexes of sedoheptulose their behavior on an anion exchanger provides further evidence for the above explanation.

Gentianose, which was not studied, would presumably form a borate complex of the same type as that of sucrose, since it contains no *cis*-hydroxyl grouping. On this assumption, the preparation of gentiobiose from gentianose by partial hydrolysis could be accomplished since all four possible products (residual gentianose, gentiobiose, glucose and fructose) are probably separable by the method

(10) M. A. Chambers, L. P. Zill and G. R. Noggle, *J. Am. Pharm. Assoc.*, in press.

(11) H. T. Macpherson and E. G. V. Percival, *J. Chem. Soc.*, 1920 (1938).

(12) J. W. Pratt, N. K. Richtmyer and C. S. Hudson, THIS JOURNAL, **74**, 2200 (1952).

(13) J. Böeseken in "Advances in Carbohydrate Chemistry," W. W. Pigman and M. L. Wolfrom, Academic Press, Inc., New York, N. Y., 1949, vol. 4, p. 189.

presented. The preparation of turanose from melezitose provides a further example of this type of separation. Preparative isolations of other sugars may be worked out in a similar manner.

The strongly positive effect on the conductivity of boric acid solutions produced by straight chain polyhydroxy compounds such as dulcitol, sorbitol and mannitol[11] is reflected in the comparatively high affinity of their borate complexes for a strong-base resin in the borate form. Their elution order may be directly correlated with the increases in conductivity determined by Macpherson and Percival,[11] but are inversely proportional to the conductivities by Böeseken.[13] The behavior of mannitol is consistent with the postulate that this compound acts as a di-diol and four hydroxyls instead of two are combined with borate to give a bivalent ion.[14] As a quantitative method for the separation and analysis of the hexitols occurring in mixtures such as those from plants, the ion-exchange chromatography of their borate complexes holds considerable promise.

Acknowledgment.—The authors express their appreciation to Dr. G. R. Noggle for the preparation of the equilibrium mixture of sedoheptulose and sedoheptulosan and for helpful discussions and criticism during the course of this work, to Dr. N. K. Richtmyer for the sample of sedoheptulosan, to Dr. M. L. Wolfrom for the sample of stachyose, and to Mr. C. Feldman for carrying out the spectrographic analysis.

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OAK RIDGE, TENNESSEE

[CONTRIBUTION FROM THE FOOD TECHNOLOGY LABORATORY OF THE UNIVERSITY OF MASSACHUSETTS[1]]

Malic Acid–Fructose Reaction

BY GIDEON E. LIVINGSTON

RECEIVED JANUARY 2, 1952

Malic acid and fructose react in an aqueous solution at moderate temperatures in a Maillard-type reaction to form dark-colored end-products. Four soluble colored fractions were separated from the malic acid–fructose mixture by chromatographic means. The absorption spectra of these fractions were determined. General absorption curves in the visible range and absorption maxima at about 280 mμ were found in most cases. The reactivity of related compounds and possible intermediates was determined qualitatively. The effect of atmospheres of oxygen and nitrogen on the discoloration of malic acid–fructose solutions was also investigated.

The conventional Maillard reaction,[2] which involves the interaction of amino acids and reducing sugars in solution, results in the formation of colored end-products. The reaction has been studied extensively in recent years in connection with the non-enzymatic browning of foods in storage or upon heat treatment. The unlikelihood that this particular reaction accounts to any significant extent for the storage darkening of low protein fruit and vegetable products has led to the postulation of other modes of browning including the "ascorbic acid theory" and the "active aldehyde theory" described by Stadtman.[3] From a study of the storage darkening of apricot concentrates, Haas and Stadtman[4] concluded that the over-all browning was the result of at least four distinctly different types of reactions: reactions between (1) nitrogenous constituents and sugars, (2) nitrogenous constituents and organic acids, (3) sugars and organic acids, and (4) organic acids only.

The reaction between nitrogen-free carboxylic acids and reducing sugars in pure solutions was studied by Lewis, Esselen and Fellers.[5] These authors found that a Maillard-type reaction, accompanied by browning and carbon dioxide production, resulted from the interaction of glucose with acetic, oxalic, fumaric, citric, tartaric or lactic acids at *p*H 7.2 when incubated at 100°. They concluded that this type of reaction might be partly responsible for the non-enzymatic browning of many food-stuffs. In the course of an investigation of the browning of apple sauce, the reactions of several natural constituents of apples were studied in model systems containing the pure compounds. It was observed that fructose reacts far more rapidly than glucose with malic acid, the predominant carboxylic acid present, to produce browning. It was found that a solution of malic acid and fructose in 1 *M* concentrations in distilled water, held at approximately 60°, darkened visibly within two or three days with insoluble dark-colored reaction products ultimately being formed. The reaction proceeded more rapidly in higher concentrations or at higher temperatures. At 100°, distinct discoloration developed within a few hours.

The results of some preliminary observations on the reaction are reported in this paper.

Experimental

The relative darkening of glucose, fructose and sucrose, in solution by themselves or in the presence of malic acid, was determined by heating the 1 *M* solutions in a stoppered 100-ml. round-bottom flask partially immersed in an electrically heated water-bath. The temperature was maintained between 60 and 70°. Color changes were determined by visual comparison of the heated solutions with unheated controls. A Model H2 Beckman line meter was used to measure *p*H. The samples were examined for fluorescence by exposure to ultraviolet light in a darkened room. The reagents were *l*-malic acid, C.P. (Pfanstiehl), *d*(−)-levulose, C.P. (Pfanstiehl), dextrose, reagent (Mallinckrodt) and sucrose, reagent (Merck).

A separation of some of the colored reaction products of

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(2) L. C. Maillard, *Compt. rend.*, **154**, 66 (1912).

(3) E. R. Stadtman, *Advances in Food Research*, I, 325 (1948).

(4) V. A. Haas and E. R. Stadtman, *Ind. Eng. Chem.*, **41**, 983 (1949).

(5) V. M. Lewis, W. B. Esselen, Jr., and C. R. Fellers, *ibid.*, **41**, 2591 (1949).