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.

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[14] H. S. Isbell, J. F. Brewster, N. B. Holt and H. L. Frush, *J. Research Natl. Bur. Standards*, **40**, 129 (1948).

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

Malic Acid–Fructose Reaction

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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 $m\mu$ 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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[1] Contribution No. 826, Massachusetts Agricultural Experiment Station.

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

[3] E. R. Stadtman, *Advances in Food Research*, **1**, 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).

the malic acid–fructose reaction mixture was effected by chromatographic techniques. For this purpose, 500 ml. of a solution 1 *M* with regard to each constituent was heated for 96 hours in a 1000-ml. round-bottom flask topped with a loose glass wool plug. The dark brown sediment formed was recovered by filtration and called fraction A. The filtrate was concentrated under vacuum at a temperature of approximately 45°, until a sirupy residue remained. Enough 95% ethyl alcohol to dissolve the residue was added to permit its transfer to a beaker. The alcohol was then removed by gentle heating in a water-bath. In the course of the alcohol evaporation, a new sediment was deposited, was filtered off and labeled fraction B. When the alcohol was fully removed, a sirupy residue again remained. Acetone was added to the residue and the mixture was vigorously stirred. A portion of the residue dissolved in the acetone, forming a light brown solution. This extract was decanted, and the process repeated with two additional portions of acetone. The acetone solution was chromatographed through a 1.9 by 10 cm. column of a 2:1 mixture by weight of activated alumina (Fisher adsorption grade) and Dicalite (Laboratory Filteraid). The effluent was colorless. Two bands, a brown one near the top, labeled fraction D, and a yellow one near the middle of the column, fraction C, were adsorbed. Fraction C was first eluted with distilled water. Washing the column with 1% H_2SO_4 eluted fraction D.

The acetone-insoluble portion of the residue was dissolved in a small amount of distilled water and passed through an identical chromatographic column. A brown band, fraction F, was retained near the top of the column, while a light yellow solution, fraction E, passed through the column. The column was washed with ethyl ether to remove residual fructose present; fraction E, then eluted with 1% H_2SO_4.

The fractions collected were prepared for spectral transmittance analysis by diluting them with distilled water to approximately the same color intensity and then adjusting them to a *p*H of 7.0 with dilute NaOH or H_2SO_4 as required. Fractions A and B were dissolved in 1 *N* NaOH prior to dilution and neutralization. Only fraction C could not be fully neutralized since a reversible precipitation occurred at *p*H 4.3; the solution was, therefore, used at that *p*H. Light transmittance data were obtained in the range of 220 to 650 mμ with a Beckman DU quartz spectrophotometer.

To determine the effect of atmospheres of nitrogen and oxygen gases upon the discoloration of the malic acid–fructose mixture, a 0.1 *M* solution was prepared. Two 500-ml. erlenmeyer flasks, each containing 300 ml. of solution, were sealed with a one-hole rubber stopper wired tightly to the neck of each flask. A short section of glass tubing leading through the hole in each stopper was connected to a 10-cm. length of Tygon tubing sealed by a pinch clamp. By connecting the tubing to a three-way glass stopcock, it was thus possible to apply a vacuum to the system or to introduce the desired gas. To obtain the oxygen or nitrogen atmospheres used, a vacuum was drawn three successive times and released each time with the proper gas. Each day a sample of the reaction mixture was pipetted from the flask, filtered, when necessary, and its optical density at 450 mμ was obtained with a Coleman Model II Universal Spectrophotometer.

To obtain more information on the mechanism of the malic acid–fructose reaction as well as the extent to which it might apply to related substances, a number of compounds similar in structure to malic acid or fructose, or their known or suspected breakdown products, were held in aqueous solution at 60° for 96 hours and compared against controls. These tests were entirely qualitative with regard to both the concentration of the reactants and the mode of examination, which consisted solely of visual comparison. The reagents used were: *l*-ascorbic acid, U.S.P., Hoffman–La Roche; furfural, C.P., Eimer and Amend; levulinic acid, Grade A, A. E. Staley Mfg. Co.; pyruvic acid, The Matheson Co.; furoic acid, The Quaker Oats Co.; diacetone alcohol (4-hydroxy-4-methyl-2-pentanone) Technical, Eastman; maleic acid, Technical, Eastman; succinic acid, Eastman; dihydroxymaleic acid, Delta Chemical Works; dihydroxytartaric acid, Delta Chemical Works; tartaric acid, C.P., Eimer and Amend; 5-hydroxymethyl-2-furaldehyde, prepared by the method of Middendorp.[6]

Results and Discussion

Upon heating 1 *M* solutions of malic acid, glucose, fructose and sucrose by themselves and as mixtures, it was apparent that only the malic acid–fructose mixture was capable of incurring discoloration within 48 hours at 60–70°. This period of heating did not materially affect the *p*H values of the mixtures, but did result in increased acidity in the case of the pure sugar solutions. Fluorescence after heating was noted only in the case of the malic acid–glucose mixture. This effect has been discussed previously by Pearce,[7] Tarassuk and Simonson[8] and other authors concerned with the Maillard reaction and non-enzymatic browning.

To determine whether the malic acid–fructose reaction was an acid-catalyzed sugar degradation, the *p*H of a 1 *M* solution of fructose was adjusted with hydrochloric acid to the *p*H of the 1 *M* malic acid–fructose solution, *i.e.*, 1.58, and heated as before. No darkening was noted. To determine whether the darkening could occur in the *p*H range of apple sauce, another portion of the malic acid–fructose solution was adjusted with Na_2CO_3 solution to *p*H 3.25, and heated. Darkening occurred. The malic acid–sucrose solution exhibited some darkening, although it appeared to be considerably slower than that of the malic acid–fructose solution. This effect was indeed anticipated since inversion of the sucrose to glucose and fructose was expected in an acid medium.

The separation of colored end products developed in the course of the malic acid–fructose reaction resulted in the isolation of the six crude fractions whose characteristics are summarized in Table I. The absorption maximum in the range of 280–285 mμ, which is evident in all cases, has been associated with the presence of 5-hydroxymethyl-2-furaldehyde (HMF) by various authors.[9–11] The action of heat and acid treatment on fructose solutions have been studied by Wolfrom, *et al.*[12–15] and it is well established that HMF is a product of fructose degradation. It is pertinent, however, that the discoloration of malic acid and fructose takes place under conditions in which no similar discoloration takes place when hydrochloric acid is substituted for the organic acid. It may therefore be supposed that the malic acid reacts with one or more of the fructose degradation products to form the dark-colored compounds responsible for browning.

The rates of darkening of 0.1 *M* malic acid–fructose solutions at 60° under atmospheres of two gases were determined. The sample heated under a nitrogen atmosphere was found to darken

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[6] J. A. Middendorp, *Rec. trav. chim.*, **38**, 1 (1919) (*C. A.*, **13**, 2344 (1919).

[7] J. A. Pearce, *Food Technol.*, **4**, 416 (1950).

[8] N. P. Tarassuk and H. D. Simonson, *ibid.*, **4**, 88 (1950).

[9] M. L. Wolfrom, R. D. Schuetz and L. F. Cavalieri, THIS JOURNAL, **70**, 514 (1948).

[10] B. Singh, G. R. Dean and S. M. Cantor, *ibid.*, **70**, 517 (1948).

[11] V. A. Haas, E. R. Stadtman, F. H. Stadtman and G. Mackinney, *ibid.*, **70**, 3576 (1948).

[12] M. L. Wolfrom and M. G. Blair, *ibid.*, **70**, 2406 (1948).

[13] M. L. Wolfrom, W. W. Binkley, W. L. Shilling and H. W. Hilton, *ibid.*, **73**, 3553 (1951).

[14] M. L. Wolfrom and W. L. Shilling, *ibid.*, **73**, 3557 (1951).

[15] M. L. Wolfrom, H. W. Hilton and W. M. Binkley, *ibid.*, **74**, 2867 (1952).

TABLE I

FRACTIONS ISOLATED FROM THE MALIC ACID–FRUCTOSE REACTION MIXTURE

				Light absorption characteristics		
Fraction	Color	Solubility	Adsorption characteristics	Max. (in mμ)	Min.	Max.
A	Brown-black	Insol. in H_2O, sol. in alkali	......	285	250	...
B	Orange-brown	Insol. in ethanol, sol. in alkali	......	285	250	...
C	Yellow	Sol. in H_2O and acetone, insol. in alkali	Adsorbed on Al_2O_3 from acetone, eluted with H_2O	280	250	...
D	Red-brown	Sol. in H_2O acetone and 1% H_2SO_4	Adsorbed on Al_2O_3 from acetone, eluted with 1% H_2SO_4	285	250	...
E	Yellow	Insol. in acetone, sol. in H_2O	Not adsorbed on Al_2O_3	285	240	355
F	Orange-brown	Insol. in acetone and ethyl ether, sol. in H_2O and 1% H_2SO_4	Adsorbed on Al_2O_3 from H_2O, eluted with 1% H_2SO_4	285	240	...

more rapidly than that heated under oxygen for the first 15 days. Thereafter, while the darkening under oxygen progressed at the same rate, the solution held under nitrogen exhibited a lightening of the color. This appeared to coincide with the formation of the sediment. No specific explanation for this behavior can be advanced at this point, in view of the absence of any proved reaction mechanism. It might be suggested (1) that the one or more intermediate phases of the reaction are essentially non-oxidative and proceed best in the absence of oxygen or (2) that the presence of oxygen favors a number of oxidation reactions which result in the formation of breakdown products non-contributory to the darkening.

The results of the qualitative reaction tests using the compounds cited are summarized in Table II. It was found that fructose, HMF and ascorbic acid reacted equally well with malic acid. Glucose, furfural, furoic acid, pyruvic acid, levulinic and diacetone alcohol were not found to react. On the other hand, maleic acid, hydroxymaleic acid, tartaric acid and hydroxytartaric acid reacted easily with HMF, whereas both maleic and succinic acids did not. It would appear that the five-member furan ring structure with substitution in the 2 and/or 5 position by a $-CH_2OH$ group is required for the compound to react with malic acid. More, however, is involved since glucose is classed among the non-reactive compounds. On the acid side, the presence of hydroxy groups in the 2- or 3-position appears to be required for reaction with HMF.

TABLE II

REACTIVITY OF MIXTURES OF COMPOUNDS IN AQUEOUS SOLUTIONS HELD AT 60° FOR 96 HOURS AS INDICATED BY DARKENING OF THE SOLUTIONS

React with malic acid	Do not react with malic acid	React with hydroxymethylfurfural	Do not react with hydroxymethylfurfural
Fructose	Glucose	Malic acid	Maleic acid
Hydroxymethylfurfural	Furfural	Hydroxymaleic acid	Succinic acid
Ascorbic acid	Furoic acid	Tartaric acid	
	Pyruvic acid	Hydroxytartaric acid	
	Levulinic acid		
	Diacetone alcohol		

Conclusions

It would appear from the preceding data that an interaction of malic acid with fructose or its degradation products is involved in the discoloration of malic acid–fructose solution. HMF, which is formed in the course of the reaction, has been found to darken more rapidly in the presence of malic acid than by itself. It is, therefore, likely that it is one of the important intermediates in the browning of these solutions. By treating various dicarboxylic acids with HMF, it was ascertained that an α-hydroxyl group in the acid is required for the occurrence of the reactions leading to browning.

More extensive studies of this reaction are underway.

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