Factors Affecting the Maillard Browning Reaction between Sugars and Amino Acids. Studies on the Nonenzymic Browning of Dehydrated Orange Juice

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Color formation in sugar–amino acid mixtures has been used as an index to determine the effect of various factors in contributing to the storage deterioration through the nonenzymic Maillard browning reaction of such food products as dehydrated orange juice. Of the free amino acids present in orange juice, 4-aminobutyric acid was found to be particularly significant in causing the rapid and extensive formation of colored products. It is shown that the initial phase of this reaction, leading to loss of D-glucose and 4-aminobutyric acid, can be readily monitored quantitatively by gas–liquid chromatography of the per(trimethylsilyl)ated reaction mixtures; good correspondence is observed between the results from model systems and those obtained with dehydrated orange juice.

It is well known that natural food flavors in fruits and numerous other foodstuffs are sensitive and readily subject to deterioration. This is a major problem in the storage of fresh foods and of foods preserved by canning, concentrating, and the like. The pH of many food products is slightly acid. There are two general types of complex reactions occurring during the storage life of fresh and preserved foods where sugars and amino acids are present. These are the essentially dehydrating reactions favored by acidity (Wolfrom *et al.*, 1948, 1949, 1952) and the Maillard type of nonenzymic browning reactions (Maillard, 1912, 1916; Reynolds, 1963, 1965) taking place between the amino acids (free or combined as proteins or peptides) present and the reducing sugars. The Maillard reaction is acid–base catalyzed. Both reactions are considered to arise through a 3-deoxyhexosulose type of intermediate (Wolfrom *et al.*, 1952; Anet, 1960, 1965), and both take place through a process of β elimination. The first type of reaction (action of acids), leading to volatile furans and related products, has been especially studied by gas–liquid chromatographic methods (Shaw *et al.*, 1967; Tatum *et al.*, 1967). Work in this laboratory has been concerned mainly with the Maillard reaction in model systems. In a recent report (Wolfrom *et al.*, 1974) it has been shown that the initial steps in the Maillard reaction from D-glucose, involving formation of a D-glucosylamine, and its conversion into a 1-(N-substituted)amino-1-deoxy-D-fructose, can be conveniently monitored quantitatively and the intermediates studied by gas–liquid chromatography (glc) of the trimethylsilylated reaction mixture. A simple preparative route to the subsequent intermediate in the Maillard reaction, the unstable 3-deoxy-D-*erythro*-hexosulose, has also been developed (El Khadem *et al.*, 1971).

With particular reference to the problem of flavor deterioration on storage in dehydrated orange-juice "crystals" produced by the foam-mat process (Bissett *et al.*, 1963; Berry *et al.*, 1967), the present report is concerned with evaluating various factors in the interaction of reducing sugars with amines leading through the Maillard sequence to colored products absorbing maximally at 490 nm, and with examining the applicability of the glc method (Wolfrom *et al.*, 1974) for monitoring the initial stages of the reaction in dehydrated orange juice and model systems for it. The incidence of deteriorative reactions in dehydrated orange juice leading to volatile furan-type products has been noted (Berry and Tatum, 1965; Tatum *et al.*, 1967) and detailed information on the chemical composition of orange juice is given in the monograph edited by Sinclair (1961). Curl (1949) has described model experiments on mixtures simulating the composition of orange juice, containing sucrose, D-glucose, D-fructose, and various amino acids, together with citric and ascorbic acids and potassium citrate, and has recorded loss of ascorbic acid and increase of color density as a function of time of storage at 49°. Related studies have been reported by Joslyn (1957). The present work was designed to examine systematically the influence of individual components and variables in this system as they affect color development, and to establish which sugar–amino acid reactions might merit detailed investigation at their initial stages by the glc method.

EXPERIMENTAL SECTION

Measurement of Color Formation in Model Systems. Mixtures of sugars and amino acids, together with other constituents as indicated, were kept at 65° (unless otherwise stated) under nitrogen for various intervals of time and the optical densities at 490 nm of appropriately diluted aliquots were determined in 1-cm cells with a Beckman DU spectrophotometer, Model 4200. Results are shown in Figures 1–8.

Effect of Inhibitors on Color Formation in a Model System. The model system contained, in addition to D-glucose and the amino acid, the proportions of citric acid, potassium citrate, sodium benzoate, and L-ascorbic acid employed by Curl (1949) in simulating the composition of orange juice as treated for storage. A mixture of D-glucose (360 mg), L-arginine (348 mg), citric acid (36 mg), potassium citrate (25.2 mg), sodium benzoate (3.6 mg), and L-ascorbic acid (1.8 mg) made up to 5 ml with water was heated under nitrogen in a sealed tube for 2 hr at 100°. The solution was then diluted and its optical density at 490 nm was determined. Similar experiments were performed in which various molar proportions (0.01, 0.05, 0.1, 0.5, and 1.0) of the potential inhibitors sodium hydrogen sulfite, hydroxylamine hydrochloride, *p*-tolylsulfonylhydrazine, and semicarbazide hydrochloride were also included. The results are shown in Figure 9.

The results of experiments with a similar model system, without inhibitors, to examine the behavior of D-glucose and D-fructose separately in their interactions with some amino acids are shown in Figure 10.

Determination of Loss of D-Glucose in the Browning Reaction. The trimethylsilylation procedure and quantitative glc analytical method of Wolfrom *et al.* (1974) were used, with addition of D-glucitol as an internal standard before the trimethylsilylation step. Results from experiments with freeze-dried mixtures of D-glucose and 4-aminobutyric acid at 100° are shown in Figure 11, and Figure

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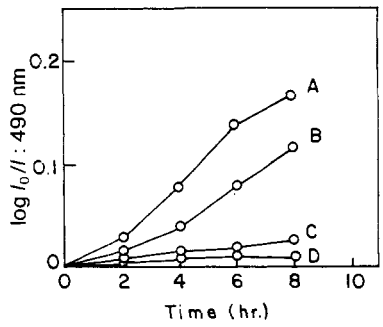

Figure 1. Effect of molar ratio of amino acid to sugar on the browning reaction. D-Glucose–glycine ratio: A, 1:5; B, 1:1; C, 2:1; D, 10:1. Water content 65%, reaction temperature 65°.

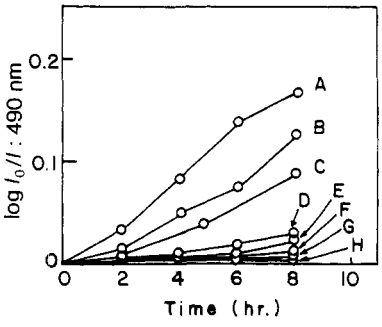

Figure 2. Effect of water content on the browning reaction. D-Glucose–glycine (1:5 molar ratio) at 65°. Water content: A, 65%; B, 70%; C, 72%; D, 76%; E, 80%; F, 83%; G, 85%; H, 95%.

12 shows results of similar experiments in which citric acid, potassium citrate, and L-ascorbic acid were also present.

Loss of D-Glucose from Dehydrated Orange Juice. Orange juice "crystals" were prepared by the foam-mat process on a crater type of dryer by the procedure of Berry *et al.* (1967) from a 58° Brix concentrate of Valencia orange juice. The drying time was 12.5 min at 77°. Samples of this material (40 mg) in sealed tubes were heated for the prescribed period at 100° and then D-glucitol (5 mg) was added as an internal standard, followed by dry pyridine (2 ml) and a mixture of *N,O*-bis(trimethylsilyl)acetamide (2 ml), *N*-(trimethylsilyl)imidazole (2 ml), and chlorotrimethylsilane (0.4 ml), and the mixture was kept for 2 hr at 0° before analysis by glc according to the procedure of Wolfrom *et al.* (1974). The results are shown in Figure 13.

RESULTS AND DISCUSSION

By examining color development (absorbance at 490 nm), in various sugar–amino acid mixtures stored at 65° to simulate conditions of accelerated storage deterioration of such food products as dehydrated orange juice, it was possible to estimate the effect of various factors on the rate and extent of the nonenzymic browning reaction, as indicated by the appearance of colored end products of the browning reaction sequence.

In conjunction with these data for the overall browning reaction, selected studies were made affording quantitative data on the initial phases of the browning reaction, leading to loss of D-glucose from the system, as determined by the glc method of Wolfrom *et al.* (1974). These examples include the D-glucose–4-aminobutyric acid system, a similar mixture also including citric acid, potassium citrate, and ascorbic acid as a model (Curl, 1949) for dried citrus juice, together with an actual example of dehydrated orange juice powder produced by the foam-mat process (Berry, *et al.*, 1967).

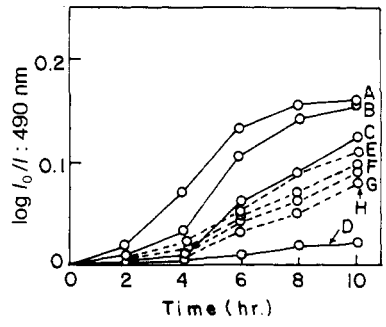

Figure 3. Effect of hydrogen ion concentration on the browning reaction. D-Glucose–glycine (1:1 molar ratio, 19% total w/v) at 65°. Potassium citrate was added in A (0.75 molar ratio to sugar, pH 7.21), B (0.5 molar ratio to sugar, pH 7.04), C (0.1 molar ratio to sugar, pH 6.56), and D (0.01 molar ratio to sugar, pH 6.12), and sodium hydroxide added in E (pH 7.5), F (pH 7.0), G (pH 6.5), and H (pH 6.0).

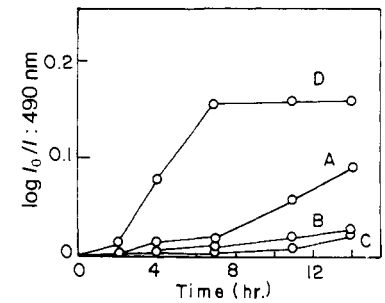

Figure 4. Effect of citric and ascorbic acids on the browning reaction. D-Glucose–glycine (1:1 molar ratio, 22.5% total w/v) at 65°: A, no added acid; B, 1 molar equiv of citric acid/mol of sugar added; C, 1 molar equiv of ascorbic acid/mol of sugar added; and D, 1 molar equiv of potassium citrate/mol of sugar added.

Effect of Various Factors on Color Development in Sugar–Amine Mixtures. *Amino Acid–Sugar Ratio.* Color development in D-glucose–glycine mixtures, containing 65% of water and stored at 65°, showed a marked dependence on the relative proportion of the amino acid, as established in earlier studies (Reynolds, 1963). It can be seen (Figure 1) that the color production is relatively modest at D-glucose–glycine ratios of 10:1 or even 2:1, but that development of color is rapidly accelerated at a ratio of 1:1 or 1:5. The molar ratio of reducing sugars to free amino acids in orange juice is about 5:1.

Water Content. At 65° the 1:5 D-glucose–glycine system (Figure 2) showed progressive decrease of color formation as the water content was increased from 65 to 95%. Color development rose particularly sharply with decrease in water content from 80 to 65%, a significant consideration with regard to the possible incidence of browning reactions in dehydrated foods. Color retardation by increase in the content of water in a 1:5 D-xylose–glycine system has been noted by Wolfrom *et al.* (1953).

Effect of pH. In a 1:1 D-glucose–glycine system containing 19% of solids (Figure 3) the initial pH was adjusted to values between 6 and 7.5 by addition of either sodium hydroxide or of potassium citrate. As anticipated from earlier literature on the browning reaction, the rate of color formation showed a progressive increase with increase of pH in the mixtures where sodium hydroxide was used. Somewhat surprisingly, however, potassium citrate appeared to exert a specific rate-increasing effect considerably in excess of that anticipated from the pH level alone, suggesting that citrate ion (or conceivably, potassium ion) may exert an accelerating effect on the browning reaction. Color formation was low, under the conditions of this experiment, at pH values in the range 3–4 encountered in orange juice.

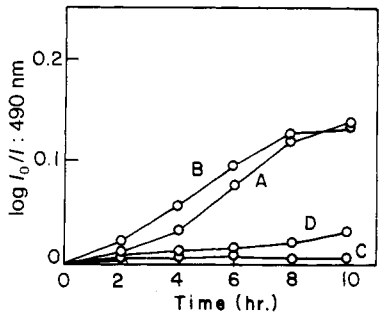

Figure 5. Behavior of various sugars in the browning reaction. Glycine plus sugar (5:1 molar ratio, 30% total w/v) at 65°. Sugars used were: A, D-glucose; B, D-fructose; C, sucrose; D, equimolar D-glucose, D-fructose, and sucrose.

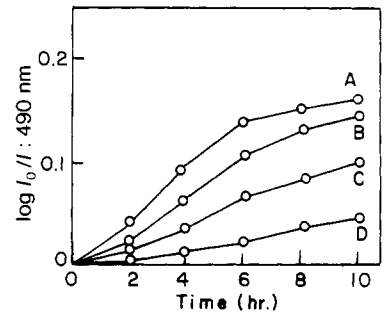

Figure 8. Behavior of D-glucose and L-arginine in the browning reaction (7.2% w/v, 65°). Molar ratio of sugar to amino acid: A, 1:1; B, 2:1; C, 4:1; D, 10:1.

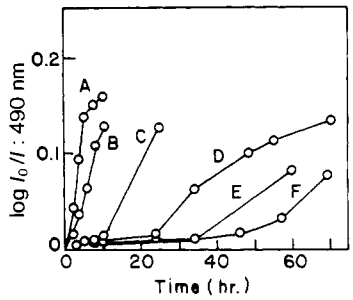

Figure 6. Behavior of different amino acids in the browning reaction. D-Glucose plus amino acid (1:1 molar ratio, 7.2% w/v) at 65°: A, L-arginine; B, 4-aminobutyric acid; C, glycine; D, DL-alanine; E, DL-serine; F, L-proline. DL-Aspartic acid, L-glutamic acid, and L-glutamine showed behavior similar to glycine up to 10 hr.

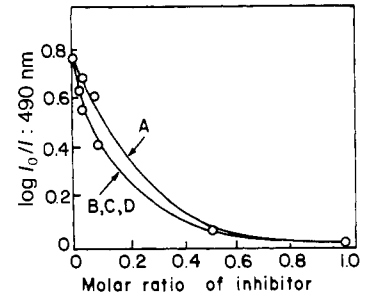

Figure 9. Effect of inhibitors on the browning reaction between D-glucose and L-arginine (1:1) in a simulated orange juice system during 2 hr at 100° (see text): A, sodium hydrogen sulfite; B, hydroxylamine hydrochloride; C, *p*-tolylsulfonylhydrazine; and D, semicarbazide hydrochloride.

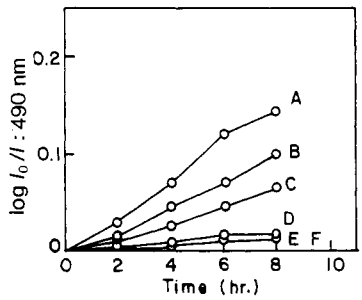

Figure 7. Behavior of D-glucose and 4-aminobutyric acid in the browning reaction (7.2% total w/v, 65°). Molar ratio of sugar to amino acid: A, 1:5; B, 1:2.5; C, 1:1; D, 2:1; E, D-glucose–2-aminobutyric acid (1:5); F, D-glucose–glycine (1:1).

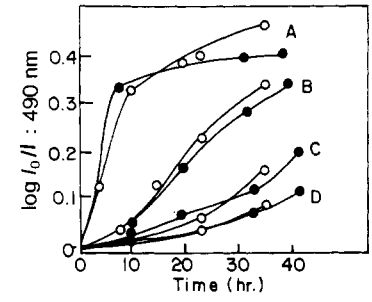

Figure 10. The browning reaction in a simulated orange juice system during 40 hr at 65° (see text). Mixtures (1:1) of D-glucose (open circles) or D-fructose (closed circles) with: A, L-arginine; B, 4-aminobutyric acid; C, glycine; and D, L-lysine.

Effect of Organic Acids. Comparing the color development in the 1:1 D-glucose–glycine system alone with that to which had been added 1 molar equiv of citric acid, ascorbic acid, and potassium citrate, respectively (Figure 4), it can be observed that the browning reaction is markedly inhibited by the two acids, whereas potassium citrate markedly enhances color development. These observed differences may arise merely because of pH changes caused by the added acid or salt; further studies with buffered solutions would be warranted to evaluate the possibility of specific inhibition of the browning reaction by ascorbic acid.

Influence of Different Sugars. The browning reaction in a 1:5 D-glucose–glycine system (30% solids) was compared with similar systems in which D-glucose had been replaced by D-fructose, sucrose, or an equimolar mixture of D-glucose, D-fructose, and sucrose (Figure 5). In the unbuffered system employed, D-fructose showed the anticipated somewhat higher initial rate of browning than D-glucose; this difference might be reversed in the buffered media generally characteristic of foodstuffs.

The browning reaction was quite insignificant in the mixture where sucrose was used; at the pH (6.1) of this experiment, hydrolytic splitting of the glycosidic group to generate D-glucose and D-fructose can be expected to be negligible and sucrose is observed not to enter into the browning reaction, as expected by virtue of its possessing no free reducing group.

Influence of Different Amino Acids. In a 1:1 D-glucose–amino acid mixture (7.2% solids), a comparison was made (Figure 6) between nine different amino acids that are known to occur free in orange juice. Of these nine, L-arginine and 4-aminobutyric acid gave the most intense and rapid color formation, and were quantitatively much more effective than glycine or any of the other amino acids examined. As both 4-aminobutyric acid and L-arginine are relatively abundant in orange juice and in many other fruit juices (Sinclair, 1961), it was considered that these amino acids may play an important role in the deterioration of orange juice on storage, meriting detailed study of the D-glucose–4-aminobutyric acid and D-glucose–L-arginine systems.

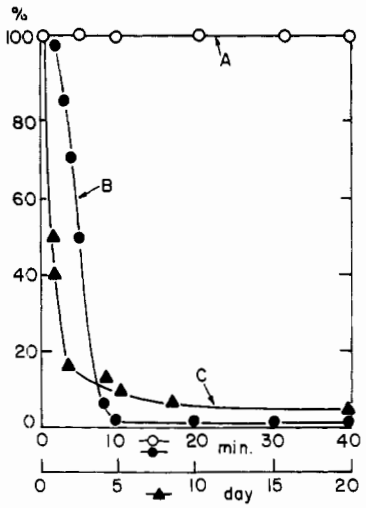

Figure 11. Per cent loss of D-glucose with time in a freeze-dried mixture: A, D-glucose only at 100°; B, 1:1 D-glucose–4-aminobutyric acid at 100°; and C, 1:1 D-glucose–4-aminobutyric acid at 35°.

D-Glucose–4-Aminobutyric Acid System. Figure 7 shows comparisons of the browning reaction of 1:5, 1:2.5, 1:1, and 2:1 mixtures of D-glucose and 4-aminobutyric acid, together with that of 1:1 D-glucose–glycine and 1:5 D-glucose–2-aminobutyric acid, all at a concentration of 7.2% solids. It can be seen that 4-aminobutyric acid is approximately ten times as effective on a molar basis as glycine or 2-aminobutyric acid in generating browning color. This high quantitative significance of 4-aminobutyric acid prompted the detailed study of its reaction with D-glucose that is described in a separate report (Wolfrom *et al.*, 1974).

D-Glucose–Arginine System. For D-glucose–L-arginine mixtures (7.2% solids) in 1:1, 2:1, 4:1, and 10:1 molar ratios, Figure 8 shows progressive decrease of the extent of browning as the content of L-arginine was decreased. It has been reported (Willits *et al.*, 1958) that arginine, which contains a guanidino group at the 5 position of the 2-amino acid, is less effective in generating browning color than lysine (which contains a side-chain amino group). Nevertheless, of the group of amino acids studied here (Figure 6) the effect of arginine in promoting the browning reaction was surpassed only by that of 4-aminobutyric acid.

Browning in the Presence of Inhibitors. Color development in a 1:1 D-glucose–L-arginine mixture also containing citric acid, potassium citrate, sodium benzoate, and L-ascorbic acid (to simulate the composition of orange juice; Curl, 1949) was observed (Figure 9) to be markedly inhibited by sodium hydrogen sulfite and by various hydrazines that presumably interact with the carbonyl group of the sugar and thereby prevent the normal sequence of the browning reaction. An equimolar proportion of any of the four inhibitors examined led to essentially complete suppression of the browning reaction as measured by color development during 2 hr at 100°.

Browning in Model Systems for Orange Juice. A model system (Curl, 1949) as in the previous experiment was used, except that inhibitors were omitted and the four amino acids L-arginine, 4-aminobutyric acid, glycine, and L-lysine were used in a 1:1 molar ratio with either D-glucose or D-fructose, and the mixtures were kept at 65°. The results (Figure 10) showed little difference in behavior between the two sugars, but that L-arginine gave the most rapid and pronounced color development; in this system L-lysine was not particularly effective and in fact caused less color development than did glycine. As anticipated from the higher acidity of this mixture, the overall rate of color development was lower than with comparable mixtures containing no citric acid.

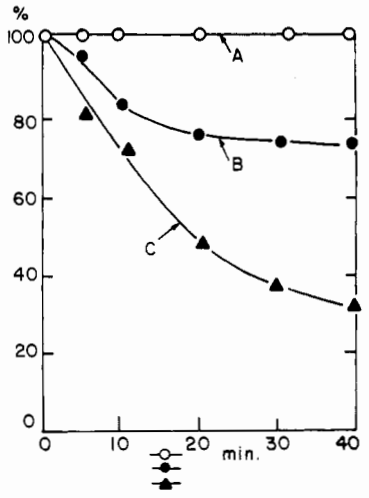

Figure 12. Per cent loss of D-glucose with time in a freeze-dried simulated orange juice system at 100° (see text): A, D-glucose and no amino acid; B, 1:0.105 D-glucose–4-aminobutyric acid; and C, 1:0.308 D-glucose–4-aminobutyric acid.

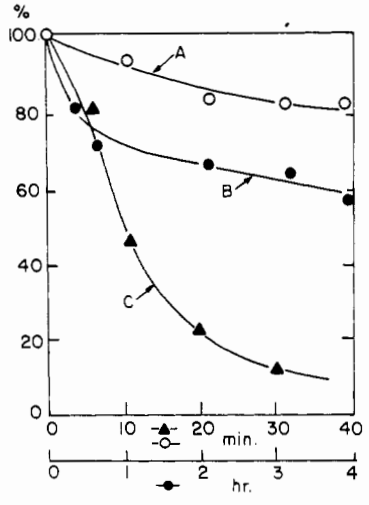

Figure 13. Per cent loss of D-glucose from dried orange juice (foam-mat process) at 100°: A, no pretreatment, 0–40 min; B, no pretreatment, 0–4 hr; and C, after addition of 8.5% (wt) 4-aminobutyric acid (~equimolar with D-glucose present), 0–40 min.

Rate of Loss of Initial Reactants in Model Browning Reaction Systems and in Dehydrated Orange Juice. The glc technique (Wolfrom *et al.*, 1974) was used to monitor quantitatively the loss of D-glucose and of amino acid from the reaction mixtures to provide an index of progress of the initial phases of the browning reaction sequence.

In the simplest system, a freeze-dried 1:1 mixture of D-glucose and 4-aminobutyric acid was kept at 100° and aliquots were assayed at intervals for D-glucose (see Figure 11). The D-glucose had essentially all reacted after the elapse of 10 min. Similar results were observed at a reaction temperature of 35°, except that about 5 days were required for the D-glucose concentration to fall to ~5% of its initial level. When the amino acid was omitted, no loss of D-glucose was observed during several hours at 100°.

In a model system for simulating dehydrated orange juice (*cf.* Curl, 1949), a mixture of D-glucose and 4-aminobutyric acid together with citric acid, potassium citrate, and L-ascorbic acid (together with D-glucitol as an internal standard for glc) was constituted in water and then freeze dried and heated at 100°. The results are shown in Figure 12. At a ratio of 1:0.105 D-glucose–4-aminobutyric

acid (a ratio within the range found in orange juice) the content of amino acid fell to zero after 10 min with a corresponding decrease by ~20% in the content of D-glucose and a considerably slower loss of D-glucose thereafter. When the amino acid was omitted there was no observed loss of D-glucose during 40 min at 100°. Increasing the molar proportion of amino acid to 0.308 caused a more pronounced loss of D-glucose, falling to 40% of its original value after 40 min at 100°.

The validity of this model system, and the analytical procedure used for monitoring the loss of D-glucose, in relation to the thermal deterioration of an actual sample of dehydrated orange juice is illustrated in Figure 13. Dried orange juice powder prepared by the foam-mat process was kept at 100° and assayed by glc for content of D-glucose. The curve for loss of D-glucose showed a decrease by ~20% during the first 40 min, falling by another 20% after a total of 4 hr. The curve is observed to be closely similar to that given in the model system (Figure 12) for D-glucose and 4-aminobutyric acid present in the ratio generally found in orange juice. Greatly accelerated loss of D-glucose was observed in the dehydrated orange juice when ~1 molar equiv of 4-aminobutyric acid (with respect to D-glucose) was added before heating; the content of D-glucose fell to <10% of the original level after 40 min at 100°.

The foregoing results indicate that interactions between D-glucose and amino acids, especially 4-aminobutyric acid and L-arginine, probably play a significant role in reactions leading to deterioration of dehydrated orange juice, and demonstrate that loss of D-glucose takes place under accelerated conditions of storage deterioration. Work with model systems has established that D-glucose interacts with amino acids to give 1-(N-substituted)amino-1-deoxy-D-fructoses by way of a glycosylamine intermediate. It remains to be established, on an isolative basis, the postulated formation and subsequent decomposition of such intermediates during the initial stages of storage deterioration of dehydrated orange juice and other food products where this same degradative sequence is presumed to operate.

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Disc Gel Electrophoresis. A Technique Involving Fluorescent Staining Prior to Separation

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Disc gel electrophoresis of proteins prestained with a fluorescent indicator, Fluram, is described. Less than 1 μg of protein is readily discerned on gels. Speed of analysis is a special advantage over conventional procedures which involve staining and destaining after electrophoresis. Gels do not have to be removed from support tubes and visualization of bands under long-wavelength uv light is possible as soon as electrophoresis is completed. Proteins appear as sharp, fluorescent bands. The procedure is especially applicable at high polyacrylamide concentrations in gels.

Polyacrylamide disc gel electrophoresis is a widely used technique for protein studies. High acrylamide concentrations, sometimes desired for effective separations, result in strong gels which cannot be removed from electrophoresis tubes without fracturing the gel and altering the integrity of separated bands of protein. Moreover, fixation, staining, and destaining procedures for visualization of protein bands can be inordinately lengthy. Described herein is a fluorescent method which overcomes some of these difficulties. Using Fluram (Hoffman La Roche, Inc.), fluorophores of proteins are formed prior to electrophoresis, and after separation individual bands can be visualized without removing the gel from the tube and

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