certainly be affected by the problems in sampling a mixture of powders. Increasing the sampling size or homogenizing the sample by liquefaction or some other process may lower the error limits in these analyses. One should also be cautious of isotopic fractionation that could occur from the extraction processes used to remove lipid fractions from these samples. Adsorbed solvent from such extractions could also lead to errors in the $^{13}C-^{12}C$ determinations.

With this in mind, these data allow for the estimation of the accuracy of measurements using $^{13}C-^{12}C$ techniques on inhomogeneous mixtures. The use of this technique to measure relative amounts of soy protein in meat products appears to be possible with errors of $\sim$10% expected. The $^{13}C-^{12}C$ ratios for individual pork and beef samples would be expected to show some variance depending on the proportion of soybean protein, corn, and grasses in their diet, and therefore it will be necessary for a substantial data base to accumulate in the literature before routine use can be made of $^{13}C-^{12}C$ ratio variations. It is evident from the foregoing discussion that this method of differentiating between soybean protein and beef or pork protein would not be useful when the livestock are fed primarily on C_3 feeds.

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Kinetics of the Production of Biologically Active Maillard Browned Products in Apricot and Glucose-L-Tryptophan

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Some properties of those Maillard browning products responsible for these adverse effects were studied. The kinetics of the formation of these brown products was also studied using a natural food system (apricot) and a model system (glucose-tryptophan). The water-soluble products responsible for the deterioration of the normal nutritional state were formed in the early stages of browning. Butanol-soluble products attributable to adverse physiological effects were formed in a later stage. Kinetically, a significant proportion of the parent compounds were degraded, and a maximum yield of Amadori compounds was attained even before an appreciable amount of brown color developed. The rate of formation of browning products showed a linear relationship with reaction time and temperature until the parent compounds were no longer available. After depletion of parent compounds, polymerizations between the remaining products and a partial degradation of Amadori compounds occurred. The products became less soluble in polar solvents as further polymerization proceeded.

The nonenzymatic Maillard browning reaction is known to cause the deterioration of the quality of food products during processing and storage. Along with the loss of nutritional value of foods, some studies have shown the possible antinutritive, adverse physiological, and toxic effects of Maillard browned food products after heat treatment or storage (Lang et al., 1959; Adrian, 1973, 1974; Sgarbieri et al., 1973; Lee et al., 1974, 1977a,b; Tanaka et al., 1977). However, there is no report as to specifically which constituents of Maillard browned products might

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be responsible for these adverse physiological effects. Recently, it was found that the water-soluble fraction of the ethanol extract of browned apricots was responsible for the adverse physiological effect shown in the rats fed a browned apricot diet (Lee et al., 1974, 1977a). Furthermore, using a glucose and tryptophan mixture as a model Maillard browning system (Lee et al., 1977b), the fraction containing fructose–L-tryptophan (an Amadori compound) showed a competitive inhibitory effect on disaccharidase activity, as well as on the absorption of L-tryptophan, whereas the fraction free from fructose–L-tryptophan exhibited a noncompetitive inhibitory effect on disaccharidase activity. The objective of the present investigation was to study chemical properties of Maillard browning products responsible for the adverse physiological effects and the kinetics of the product formation using a natural food system (apricot) and a model system (glucose–tryptophan).

MATERIALS AND METHODS

Preparation and Fractionation of Browning Products. Browning products were prepared by dissolving equimolar amounts of D-glucose and L-tryptophan in methanol to a final concentration of 30 mM. The mixture was refluxed until the free glucose level in the reaction mixture no longer decreased. The concentration of glucose was determined enzymatically with Glucostat reagent (Worthington Biochemical Corp., Freehold, NJ). The refluxed mixture was concentrated by rotor-evaporation at 40 °C and applied to a cellulose column (Whatman CM_{11}, fibrous powder (12 × 600 mm) which had been treated previously with water-saturated 1-butanol. The concentrate was fractionated using water saturated 1-butanol as an eluant. Each fraction was analyzed by thin-layer chromatography using silica gel plates with a solvent system consisting of 1-butanol, acetic acid, and water (4:1:1 by volume). The chromatographic spots were detected under UV light. Fructose–L-tryptophan was prepared according to the method of Heyns and Noack (1964). Fructose–L-tryptophan was isolated from the fructose–L-tryptophan-rich fraction by crystallization at refrigeration temperature overnight and purified by repeated recrystallization with 1-butanol and, finally, once with methanol. The purity of every preparation was ensured by chromatographic examination. On the other hand, fluorescent compounds were isolated from fructose–L-tryptophan-free fraction by dry column chromatography (Loev and Goodman, 1967). Isolation was carried out by developing the fructose–L-tryptophan-free fraction with a solvent system consisting of 1-butanol, acetic acid, and water (4:1:1 by volume). When the eluant reached the bottom of the column, the development was complete. The fluorescent fraction detected by UV light was leached out by suspending it in methanol. The concentrates prepared from different fractions were reconstituted with water and then freeze-dried.

From the freeze-dried browned product prepared from the concentrate of the refluxed mixture, water-soluble and ethanol-soluble fractions were separated by extracting initially with water and subsequently with ethanol in which most of the water-insoluble fraction was dissolved. The resulting fractions were further fractionated by extracting them with a mixture of water and 1-butanol (1:1 by volume). Fractions obtained were evaluated on the solubility characteristics of fructose–L-tryptophan, pigments, and fluorescent compounds.

Kinetics of the Maillard Browning of Glucose–Tryptophan. *Effect of Reaction Time.* The reaction mixture was prepared by dissolving equimolar quantities of D-glucose and L-tryptophan in methanol to a final concentration of 30 mM. The reaction was kept at reflux temperature. Over a 60-h period, samples were taken at different intervals for a colorimetric estimation of color intensity, Amadori rearrangement product (fructose–L-tryptophan), total carbonyl compounds, and fluorescent compounds. The concentrations of D-glucose and L-tryptophan were also measured to determine the extent to which the degradation had occurred on a kinetic basis. Each sample was diluted ten times with methanol, and its color intensity was measured spectrophotometrically at 360 nm. Fructose–L-tryptophan and L-tryptophan were measured by a combination of thin-layer chromatography and colorimetric assay (Speis, 1967). The total amount of carbonyl compounds formed during browning was determined as 2,4-dinitrophenylhydrazone derivatives following the method of Berry and McKerrigan (1958). The fluorescent compounds, which appeared as bright blue spots under UV light, were separated and extracted with distilled water and assayed for fluorescence with a fluorescence spectrophotometer (Perkin-Elmer, MDF-2A) at a predetermined wavelength which gave maximum emission. The rate of the formation of each browning product and the degradation of reactants were calculated on the basis of initial velocity.

Effect of Reaction Temperature. The homogeneous reaction mixture was prepared in the same manner as before. Seven milliliters of reaction solutions was transferred into six ampules (Kimax, 10-mL capacity), respectively, and flame sealed. Each sealed ampule was incubated for 24 h in the oven at various temperatures. The formation of browning products and the degradation of parent reactants were measured as described in the previous section. The activation energy was calculated using the Arrhenius equation within temperature range where a first-order reaction occurred.

Kinetics of Apricot Browning during Storage. *Rate of Browning.* Finely ground apricot powder was divided into six portions and rehydrated separately in a growth chamber (Sherer-Gillett, Marshall, MI) to bring the moisture content to approximately 12, 15, 18, 20, 23, and 26%, respectively. Each portion was then transferred into an airtight glass jar and stored at 50 °C for 2 months, ensuring the same moisture level throughout the course of incubation. The differences in the degree of browning among apricots having different moisture contents were determined by measuring color intensity. The colored substances were extracted stirring with a vortex mixer after incubating a mixture of sample and 20% ethanol (1:10, w/v) in a water bath at 70 °C for 5 min. The homogeneous extract was then centrifuged to obtain a clear supernatant and the color intensity was measured at 420 nm.

Characterization of Chemical Changes during Browning. A sample was taken periodically from the lot and kept in the freezer until it was ready for the analyses of total carbonyl compounds, free amino acids, and glucose. The carbonyl compounds were extracted with ethanol which had been prepared free from carbonyl compounds according to the procedure established by Schwartz and Parks (1961). Extraction of the free amino acids followed the method of Hulme (1956). Apricot was extracted three times with 75% ethanol, followed by removal of all colored material and other aromatic substances by passing extracts

Table I. Characteristic Differences in Solubility of Some Browning Products Formed from the Reaction Mixture of D-Glucose and L-Tryptophan[a]

fraction[b]	fructose-L-tryptophan	tryptophan	fluorescent substances no. 2	fluorescent substances no. 3	pigments
W	++	+	+++	+	
W-W	+++	++	+	Tr	+
W-B	+	++++	+++++	++	+++
E	+	+++	+++++	++	
E-W	++	Tr	+	Tr	+
E-B	+	+	+++++++	++	+++++++++

[a] Solubilities are expressed in arbitrary unit based on the size of spot and the intensity of color. [b] W, water-soluble fraction; W-W, water-soluble fraction of water-butanol extract; W-B, butanol-soluble fraction of water-butanol extract; E, ethanol-soluble fraction; E-W, water-soluble fraction of water-butanol extract; E-B, butanol-soluble fraction of water-butanol extract.

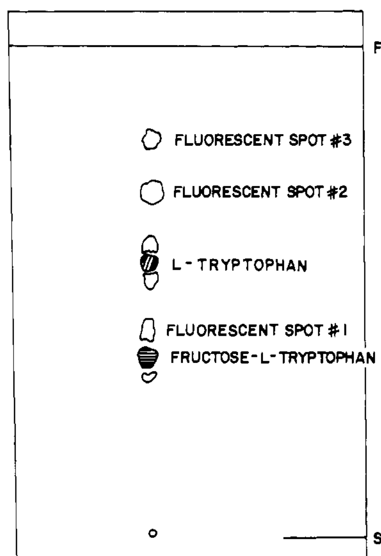

Figure 1. Thin-layer chromatogram of browning products formed from glucose-tryptophan system with solvent butanol-acetic acid-water (4:1:1) on silica gel plate. S, start; F, solvent front.

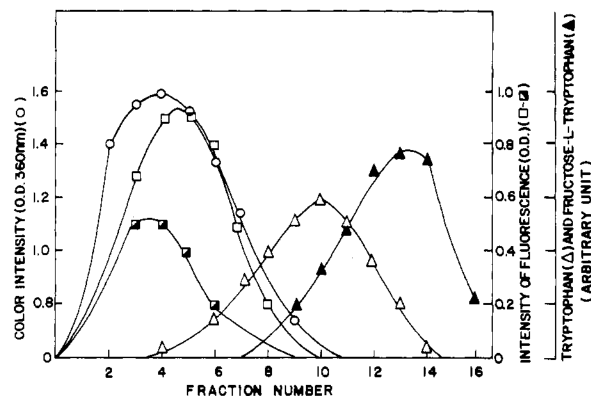

Figure 2. Chromatographic behavior of some browning products formed from glucose-tryptophan system on cellulose column (solvent system: butanol saturated with water). (O) Browning color (360 nm), (□) fluorescent spot no. 2 (excitation, 305 nm; emission, 455 nm); (△) L-tryptophan; (▲) fructose-L-tryptophan; (■) spot no. 3 (Excitation, 380 nm; emission, 450 nm).

through a phosphate-free charcoal column which had been pretreated with 5% acetic acid and then washed free of the acid. Analysis of amino acids was done by using a Technicon Auto-Analyzer (Technicon Instrument Co., Tarrytown, NY). Glucose was extracted with water in the same manner as the extraction of colored substances except using a 10-min incubation period and measured enzymatically with Glucostat reagent (Worthington Biochemical Corp., Freehold, NJ).

RESULTS AND DISCUSSION

A thin-layer chromatogram of the browning products is shown in Figure 1. R_f values of fluorescent spot no. 1, 2, 3, and fructose-L-tryptophan were 0.406, 0.656, 0.756, and 0.343, respectively. Thin-layer chromatographical analysis further showed that tryptophan or glucose alone did not produce these types of fluorescent substances after heat treatment and that there was no contamination of fluorescent substances in the purified fructose-L-tryptophan.

The chromatographic behavior of some browning products on a cellulose column is shown in Figure 2. Most fluorescent substances, as well as browning pigments, emerged early from the column, followed by tryptophan and fructose-L-tryptophan, successively. The elution pattern on the cellulose column thus appears similar to that on silica gel plate except in the cellulose column for the emergence of pigments and fluorescent substances at about the same elution time. A large quantity of fluorescent substances, most of which appeared to be constituted with spot no. 2 fluorescent compound, was separated successfully on a dry column, indicating that fluorescent substances are different from the browning pigments. The products separated in this manner were hardly soluble in water and have a light-brown color due to a slight contamination with brown pigments. Based on the review by Hodge (1953), fluorescent compounds, particularly no. 1 and 2, are probably unsaturated polymers which are developed through the condensation of the intermediate browning products.

The solubilities of various browning products are compared in Table I. Results show that most of the fluorescent compounds can be fractionated in the butanol phase, while fructose-L-tryptophan can be fractionated mostly in the water phase. Browning pigments are shown to be highly soluble in butanol and slightly soluble in water. Therefore, fluorescent substances and browning pigments have similar solubility characteristics.

Kinetics of the formation of browning products and of the degradation of parent compounds are given in Figure 3. The rate of disappearance of glucose is much faster (dv/dt = 10%/h) than that of tryptophan (dv/dt = 2.5%/h). The rate of formation of fructose-L-tryptophan on tryptophan basis was 2.9%/h (dv/dt) with a linear relationship up to 20 h and diminished gradually becoming negative after 30 h of reaction time. The formation of brown color proceeded rapidly at a rate of 0.04 OD/h (dv/dt) up to 45 h after the initial 5-h lag period (dv/dt = 0.02 OD/h). From this point, tryptophan and glucose were no longer available for the formation of color compounds, fructose-L-tryptophan, carbonyl compounds, and fluorescent compounds. The formation of total carbonyl compounds showed a hyperbolic curve which leveled off

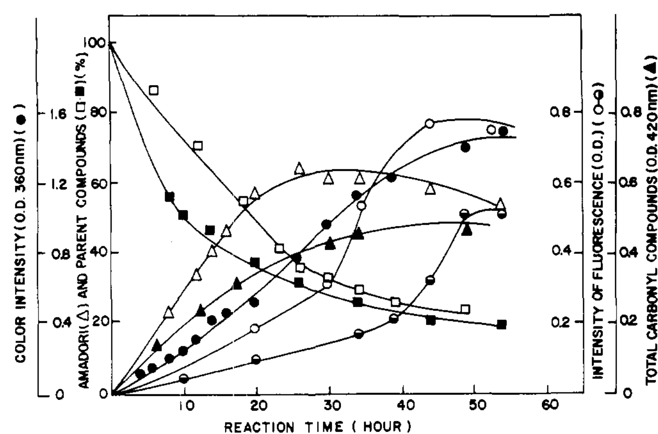

Figure 3. Effect of reaction time on the formation of browning products and degradation of parent compounds in glucose–tryptophan system. (□) L-Tryptophan, (■) D-glucose; (△) fructose–L-tryptophan, (▲) total carbonyl compounds; (○) fluorescent spot no. 2 (excitation, 305 nm; emission, 455 nm), (⊖) fluorescent spot no. 3 (excitation, 380 nm; emission, 450 nm), (●) color intensity. The yield (%) of fructose–L-tryptophan (Amadori compound) was calculated as follows: yield = concn of F–L-T × [(M_r of Trp)/(M_r of F–L-T(366))]/[concn of Trp (incorp.)].

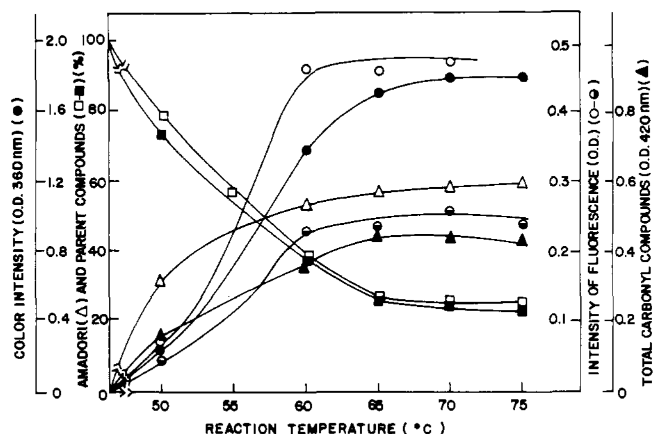

Figure 4. Effect of temperature on the rate of formation of browning products and degradation of parent compounds in glucose–tryptophan system. (□) L-Tryptophan, (■) D-glucose, (△) fructose–L-tryptophan, (▲) carbonyl compounds, (○) fluorescent spot no. 2 (excitation, 305 nm; emission, 455 nm), (⊖) no. 1 (excitation, 330 nm; emission 450 nm), (●) color intensity (360 nm). The yield (%) of fructose–L-tryptophan (Amadori compound) was calculated as follows: yield = concn of F–L-T × [(M_r of Trp)/(M_r of F–L-T)]/[concn of Trp(incorp.)].

after 50 h. The initial rate was found to be 0.022 OD/h (dv/dt). The formation of fluorescent compounds required a relatively long period of initial lag phase showing a sigmoid curve. Similar patterns were observed during the formation of two different fluorescent compounds (spot no. 3 and 2) having dv/dt = 0.0045 and 0.0098 OD/h, respectively, in the lag phase and dv/dt = 0.044 and 0.057 OD/h, respectively, in the logarithmic phase. More rapid uptake of glucose compared to tryptophan indicates that the forward reaction may require more glucose than at a stoichiometrical level. This may explain the higher yield of Amadori compounds from a higher proportion of glucose to amino acid (Lea and Hannan, 1950). A negative rate of the formation of fructose–L-tryptophan observed after 30 h may be attributed to its degradation and partly to the exhaustion of available parent compounds.

The formation of color seemed to depend upon the presence of available glucose and tryptophan. The maintenance of a positive rate of color formation even after a depletion of parent reactants might be due to the degradation product of fructose–L-tryptophan. However, the contribution of fructose–L-tryptophan to the formation of color and carbonyl compounds appears to be minor compared to those of glucose and tryptophan. The leveling off in the rate of formation of carbonyl compounds may indicate that at least some portion of carbonyl compounds may be participating in the condensation process leading to the formation of melanoidin. In uncontrolled systems, the rate of formation and degradation will be varied with different variables for different reaction conditions. These may include the nature and concentration of reactants, reaction temperature, reaction medium, pH, etc. The dependence of the formation of fluorescent substances on the quantity of available parent compounds is not as obvious as in the formation of the other browning products. During the latter stages of the reaction, fluorescence formation and browning proceeded at the same rate in agreement with Overby and Frost (1952).

The effect of reaction temperature on the formation of browning products and the degradation of reactants in the course of browning is shown in Figure 4. These results indicate that the reaction temperature and the reaction time affects Maillard browning in a similar way.

Formation of all browning products reached the maximum level at temperatures ranging between 60 and 70 °C. The rate of the formation of carbonyl compounds gradually decreased with increasing reaction temperature beyond 70 °C. This supports the observation made by Enders (1942) that the level of carbonyl compounds formed in the initial stages were reduced as condensation proceeded for the formation of browning pigments.

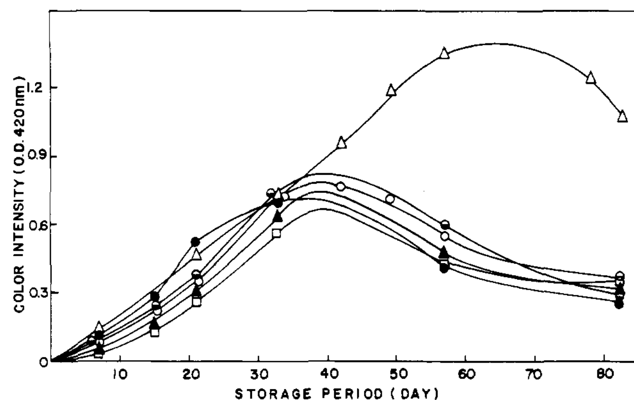

Figure 5. Effect of moisture contents on color formation during storage of apricot. (△) 12% moisture, (○) 15% moisture, (●) 18% moisture, (⊖) 20% moisture, (▲) 23% moisture, (□) 26% moisture.

The activation energies for the formation of fructose–L-tryptophan and for the degradation of glucose and tryptophan were found to be 19.0, 11.7, and 15.8 kcal mol^{-1}, respectively. These results were well reflected in the faster rate of the degradation of glucose than tryptophan and in a much slower rate of the formation of Amadori products than the degradation of parent compounds.

Effect of moisture content on color development as a function of time during browning of apricot is shown in Figure 5. Formation of soluble browning pigments no longer continued after 40 days in apricots having 15% or higher moisture content, while in apricots having 12% moisture it continued until 60 days. The browning pigments became increasingly difficult to solubilize in the solvent system (20% ethanol) used, as polymerization of colored substances was presumed to continue, leading to the formation of higher level polymers. The polymers of higher level are shown to be less soluble and less active than polymers of lower level when formed from the same

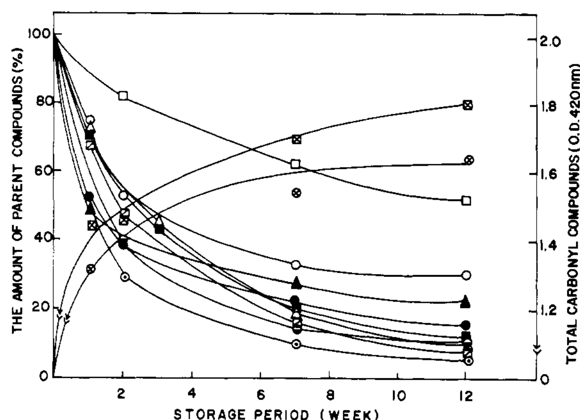

Figure 6. Degradation of amino acids and glucose and formation of carbonyl compounds in the apricot during browning. (○) Glucose (12% moisture), (□) glucose (26% moisture), (▲) glutamic acid, (●) glycine, (■) serine + threonine, (△) asparagine, (⊙) alanine, (⊡) aspartic acid, (⊖) ammonia, (⊠) carbonyl compounds (26% moisture), (⊕) carbonyl compounds (12%).

origin. This polymerization process seemed to be favored in the presence of water since pigments became less soluble as moisture content approached 15% or higher.

Effect of storage on other chemical changes in apricots during browning is presented in Figure 6. Chemical analyses revealed that rapid decreases both in amino acids and in glucose occurred during the first 2 weeks. Glucose was depleted at a slower rate than amino acids and this may be attributed to (1) concentration of glucose in apricot in excess of amounts required to react with amino acids and (2) possible competition for amino acids between glucose and relatively higher concentrations of other sugars present in apricots (Ehearst and Mason, 1967). All amino acids were degraded in a similar way, and some differences in the degradation occurred, depending upon the nature and concentration of amino acids. For instance, asparagine, which accounted for more than half of the total free amino acids, showed the slowest rate of degradation. The acidic amino acids, aspartic acid, threonine, and serine, which occurred in second highest amounts, were degraded at a slower rate than other remaining amino acids. This result is supported by the work of Beacham and Dull (1951) who showed that the rate of browning is proportional to the basicity of the amino compounds. It seems probable that the higher the concentration and the lower the basic strength, the slower the rate of degradation.

The rate of formation of carbonyl compounds appeared to be hyperbolic. A rapid production occurred during the first 2–3 weeks and corresponded well to the rate of degradation of the parent compounds. The differences in accumulation of carbonyl compounds between apricots containing 26 and 12% moisture raises a possibility that the carbonyl compounds formed at the higher moisture are not available to form pigments.

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