Studies on Limit-Peptide Pigments from Glucose–Casein Browning Systems Using Radioactive Glucose

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Protein-bound brown pigments were isolated from glucose–casein reaction systems by the method of Clark and Tannenbaum (1970). Investigations with 2-^{14}C-, 6-^{14}C-, U-^{14}C-, and 6-T-labeled glucose showed high correlation between 6-T loss from incorporated sugar residues and the amount of brown color. A high molecular weight pigment was isolated in which the glucose-derived moiety lost 26% of tritium bound to C-6.

Numerous investigators have used tracer techniques to study the browning reaction. For example, the origin of CO_2 has been shown to be mostly derived from decarboxylation of amino acids (Chichester *et al.*, 1952). The fate of the carbon skeleton of glucose has also been investigated (Stadtman *et al.*, 1952; Wolfrom *et al.*, 1953), and radioactive nondialyzable pigments have been isolated from a glucose–glycine browning system.

In a previous paper (Clark and Tannenbaum, 1970) we presented a general method for isolation and purification of brown limit-peptide pigments from protein–aldose browning systems. This paper presents data on incorporation of glucose labeled with both tritium and ^{14}C into these pigments and thereby extends our present knowledge of pigment structure.

EXPERIMENTAL SECTION

Materials. The casein and glucose were the same as in the previous investigation (Clark and Tannenbaum, 1970).

D-Glucose-*2-^{14}C*, D-glucose-*U-^{14}C*, D-glucose-*6-t* (Nuclear Chicago, Des Plaines, Ill.) and D-glucose-*6-^{14}C* (New England Nuclear, Boston, Mass.) were supplied by the manufacturer as 99% pure.

Preparation of Systems. The casein–glucose model systems were prepared and stored as described previously (Clark and Tannenbaum, 1970) except for the addition of isotopically labeled sugars before freeze-drying. Carbon-labeled glucose and glucose-*6-t* were added to each model system studied to give specific activities of 9.0 or 4.5 μCi T/mmol of glucose and 1.8 or 0.9 μCi ^{14}C/mmol of glucose for the 36 and 55° samples, respectively. The systems were designated CG-37d36-2C6-T, CG-37d36-6C6-T, CT-7d55-UC6-T, and C6-37d55-UC6-T. CG refers to casein–glucose, 7d or 37d to the storage time in days, and 36 or 55 to the temperature of storage at 75% relative humidity. The last term in the code refers to the combination of isotopic labels used, such as 2-^{14}C glucose and 6-*t* glucose for (-2C6-T).

Pigment Purification. Pigments were isolated and purified as previously described (Clark and Tannenbaum, 1970). The procedures include isoelectric washing of the protein, hydrolysis with proteolytic enzymes, and purification by gel permeation chromatography on a 5 × 92 cm column of Bio Gel P-4 (Bio-Rad Laboratories, Richmond, Calif.) in 0.05 *M* NH_4OAc buffer, pH 7.0. A portion of each fraction from the column was reserved for liquid scintillation counting, using a dioxane solvent system containing added water (Rapkin, 1967). The specific activity of each isotope and their ratios (C/T) was determined by appropriate internal standards.

Measurement of Tritium Released from Glucose as HTO. After storage, each 1-g sample was broken into small fragments with a spatula. Two milliliters of distilled water was added, and the sample was resealed, equilibrated for 1 hr at room temperature, frozen, and lyophilized. The distillate from each sample was collected in a separate trap with the lyophilization apparatus of Moss (1964). After lyophilization, the sample was broken into finer pieces, rehydrated with 2 ml of water, and lyophilized again. Radioactivity was measured in portions of the distillate by scintillation counting, and the lyophilization was repeated when tritium activity of the second distillate was greater than 10% of the first.

Measurement of Color Formation. After lyophilization, the color content of the sample was measured. The nonprotein-bound materials were separated from the protein by washing them from the sample at the isoelectric point of casein (pH 4.6), followed by enzymatically solubilizing the protein with pronase (Clark and Tannenbaum, 1970). Color content is equal to the absorbance at 420 nm times the sample volume; the total color included that from the washings as well as the hydrolyzate.

RESULTS AND DISCUSSION

Color formation is presented in Figure 1 as a function of time. After an induction period, the increase is linear. The rate drops off after about 20 days at 55°, suggesting that some precursor becomes limiting at that point and the reaction is complete in about 100 days at 55°. At 36°, the rate of color formation remains approximately constant, increasing linearly with time (total brown color = 283 ml). Whether this rate would eventually reach the same plateau (500 ml expected at approximately 400 days storage) was not determined.

The release of tritium, as HTO, from glucose-*6-t* is represented in Figure 2. The reactions involved in the tritium release are limited to about 12% of the total glucose-*6-t*.

Since brown color formation and HTO are similarly dependent on time, these data are plotted against each other (Figure 3). We performed a regression analysis (Kreyszig, 1967) to determine the best-fitting straight line.

$$\text{total brown color} = 45.8\,(\text{T released}) - 48.6 \qquad (1)$$

The correlation coefficient is 0.991, indicating a relationship between brown color formation and HTO formation ($p < 0.001$; Davies, 1961).

The ^{14}C and T specific activities of the eluates from the gel filtration column are presented in Figures 4 and 5. The ratios of these specific activities, normalized to that of the starting material, are plotted in Figure 6. The elution curve for the most severely stored sample (CG-37d55-UC6-T) is very different from the others, apparently due to tritium loss from the C-6. Assuming that all of the C/T values for the lightly colored, less-severely stored samples

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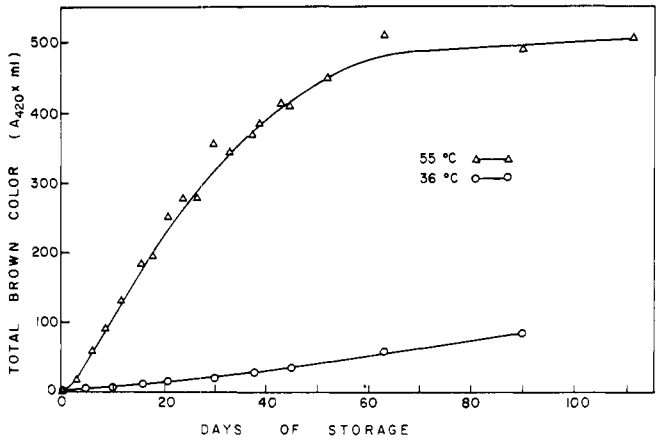

Figure 1. Formation of brown color in model system. Casein-glucose, 2:1. 75% relative humidity.

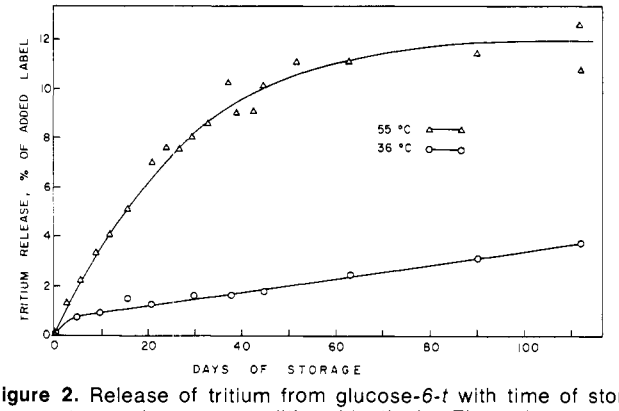

Figure 2. Release of tritium from glucose-6-*t* with time of storage; system and storage conditions identical to Figure 1.

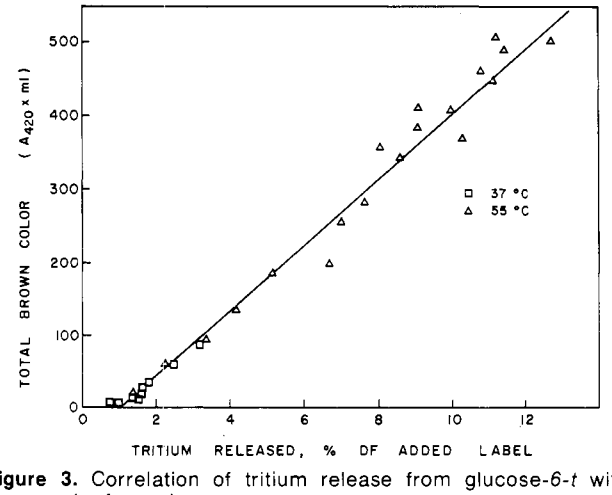

Figure 3. Correlation of tritium release from glucose-6-*t* with brown color formation.

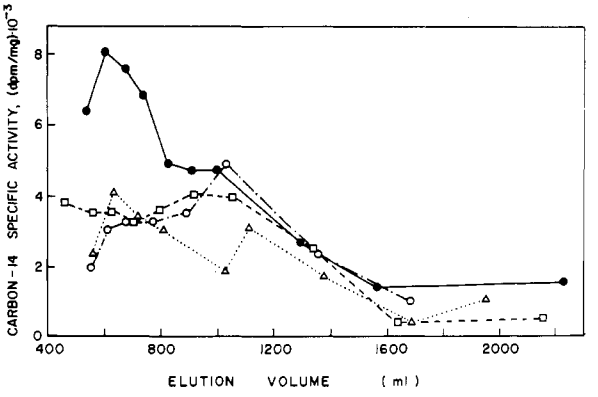

Figure 4. Elution profiles on BioGel P-4 (5 × 92 cm, NH_4OAc pH 7.0) of radioactivity from labeled glucose with casein. ^{14}C specific activity. Numbering code explained in text. CG-37d36-2C6T, □ - - - - □; CG-37d36-6C6T, ○ - - - - ○; CG-7d55-UC6-T, ▽ - - - ▽; CG-37d55-UC6-T, ● - - - - ●.

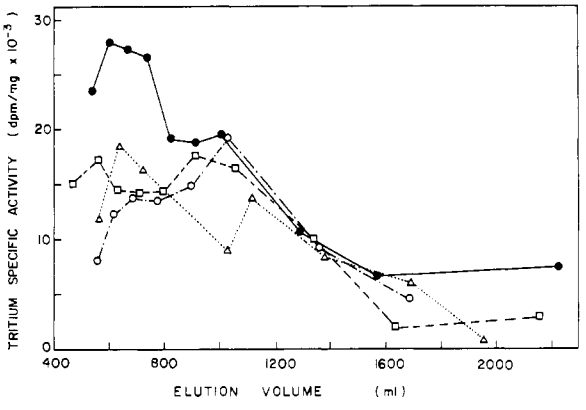

Figure 5. Elution profiles of radioactivity from labeled glucose reacted with casein. Tritium specific activity (column conditions and legend identical to Figure 4).

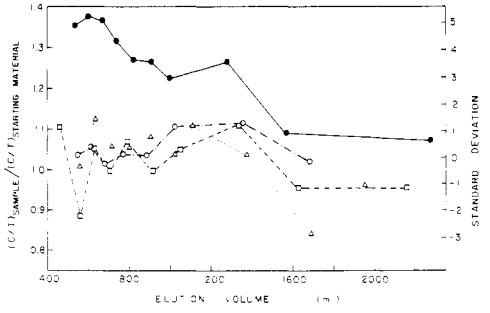

Figure 6. Ratios of specific activities of radioactive labels from starting material and reacted system in relation to elution profile (column conditions and legend identical to Figure 4).

are the same, the mean and standard deviation of these data can be calculated (Davies, 1961). Most of the data for the dark, higher molecular weight pigments from CG-37d55-UC6-T lie outside three standard deviations of this mean (Figure 6), which indicates a very significant difference between the samples. This difference is confirmed by the analysis of counting statistics (Herberg, 1964).

The extent of the tritium loss is calculated from Figure 6, where the C/T ratio for the highest molecular weight fractions is 1.36 times that of the starting material.

$$(\text{C/T sample})/(\text{C/T starting sugar}) = 1.36 \qquad (2)$$

If we assume that the specific activity of the glucose-derived carbon in the pigment is the same as in the starting sugar, then

$$\text{T sample/T starting sugar} = 0.74 \qquad (3)$$

which indicates that 26% of the tritium has been lost from pigment, compared to the carbon content.

This loss represents a nearly threefold concentration of glucose molecules which have lost the 6-T in the pigment compared to only 9.3% 6-T lost from all the glucose present. Therefore, either the glucose that has lost 6-T is selectively incorporated into the pigment or glucose residues which are incorporated into the pigment selectively lose 6-T.

The total loss of tritium from any fraction, as compared to the ^{14}C content, is calculated from eq 4.

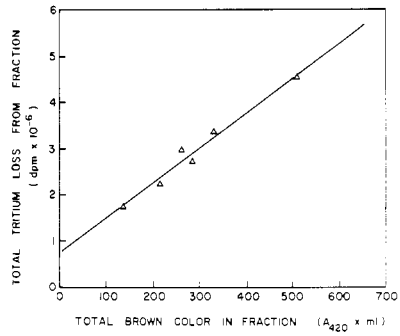

Figure 7. Correlation of release of tritium from glucose-6-*t* and brown color in high molecular weight (> 2000 Daltons) purified pigments (sample CG-37d55-UC6-T, chromatography as in Figure 4).

$$\text{total T loss} = [\text{fractional T loss}]\,[\text{sample weight (mg)}] \times {}^{14}\text{C incorporation (dpm/mg)}/(\text{C/T}) \text{ starting material} \quad (4)$$

where the fractional tritium loss is (from eq 3)

$$1 - (\text{T sample/T starting sugar}) = \text{fractional T loss} \quad (5)$$

A plot of the total tritium loss *vs.* the total color (A_{420} × volume) for each fraction (up to 1100 ml elution volume) is presented in Figure 7. In these fractions, where the tritium loss is large enough to be accurately measured, the tritium loss and the extent of color formation are highly correlated, which indicates that the purified pigments are truly representative of the overall system (Figure 3).

In considering the tritium loss from glucose-6-*t*, one must also consider whether H *or* T or H *and* T are lost from the reaction involving the substituents on the C-6 and whether a kinetic isotope effect might be important.

Since the immediate precursor for the tritium loss is unknown, this discussion is based on typical dehydration and enolization reactions which occur on glucose (Hodge, 1967; Figure 8). In example A, an enediol (I) dehydrates to II. Equilibration (enolization) between II and III in the presence of water would make all of the tritium on C-6 labile. Thus, at equilibrium, any loss in T or H would result in loss of T. In example B, a degradation product, perhaps from hydrolytic ring opening of a 5-hydroxymethylfurfural residue (IV, linked or not linked to the peptide), would give V. V could enolize (V → VI → VII → VIII), releasing only the T *or* H. In the presence of water, any enolization involving C-6 would result, at equilibrium, in loss of T. VIII might react with amines or condense with other sugar residues, which might or might not result in loss of T. Degradation reactions involving substituents on C-6 are thus likely to remove the tritium label. Therefore, the percentage of glucose molecules degraded at C-6 can be assumed to be the same as the percentage which have released tritium.

Tritium, with a molecular weight three times that of the common natural isomer, might react more slowly than H. Goldstein (1966) observed a large kinetic isotope effect when the rate-limiting step in a reaction sequence involved the breaking of a covalent bond of the heavy atom. Isbell *et al.* (1971) found an isotope effect for enolization of sugars in alkaline solutions. The ratio of the rate constants for loss of T and H (k_T/k_H) was 0.13. It must be emphasized that storage time for this browning system was long compared to those for determination of enolization rates. We assume that enolic equilibrium has been reached and there is no isotope effect.

It is important to note that only about one-fourth of the sugar residues in these highest molecular weight CG-37d55 pigments have lost 6-T. This represents, however, a threefold concentration effect compared to the 9.3% tritium released from the whole sample (Figure 2). It is unlikely that a pigment isolated from a sample stored for a longer time (~100 days) would have a much larger 6-T loss, since browning and 6-T loss are more than 80% complete at 37 days (Figures 1 and 2).

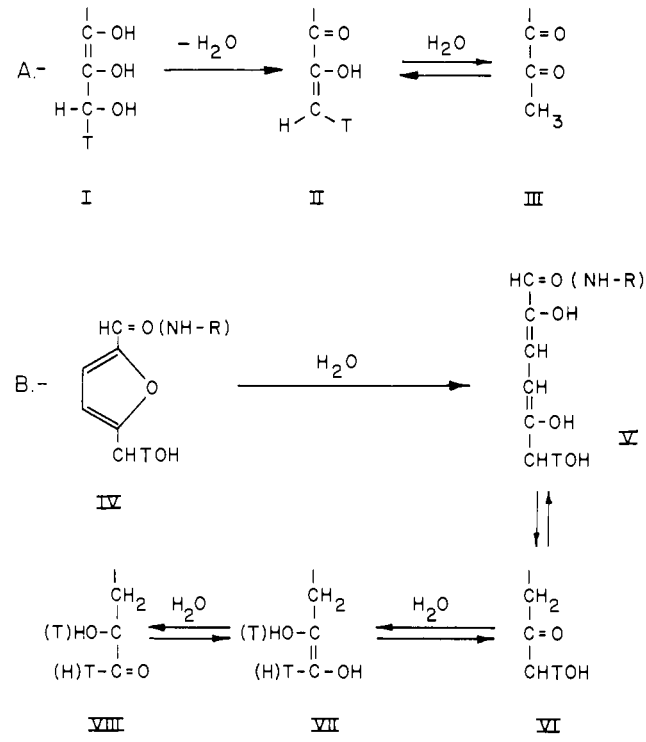

Figure 8. Possible reactions for loss of tritium from glucose-6-*t* residues of glucose–casein reaction.

If the loss of tritium from glucose is either the last slow step in the reaction sequence leading to pigment or if it occurs just after the last slow step, this could explain the high correlation shown in Figure 3. Alternatively, this loss of tritium could be unimportant; the reactions which form color could be competing for the same precursor(s). The tritium loss observed before color formation (Figure 3) could be related to these competing reactions or to an isotope-exchange reaction.

The high correlation of tritium loss and pigment molecular weight shown in Figure 7 indicates that tritium loss from C-6 is either essential for color formation and/or the cross-linkages of the pigment, or that it is a side reaction occurring shortly after the binding and crosslinking reaction(s). It is also possible, but less likely, that unbound sugars which have lost 6-T might be so unstable that they interact at the site of pigment formation. Taken together, the correlations in Figures 3 and 7 are not conclusive, but strongly implicate terminal dehydration of the sugar molecule as a key crosslinking and chromophore-generating reaction.

ACKNOWLEDGMENT

A. V. C. was supported by a Food Science Training Grant No. UI-01-033. The authors would like to thank John Hodge for helpful comments on the manuscript.

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Received for review May 15, 1972. Accepted September 27, 1972.

Isolation and Identification of Some Sulfur Chemicals Present in Two Model Systems Approximating Cooked Meat

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The following two model meat systems were heated: hydrolyzed vegetable protein (HVP)–L-cysteine·HCl–D-xylose–water and L-cysteine·HCl–D-xylose–water. The flavor concentrates were isolated by atmospheric steam distillation, followed by continuous solvent extraction of the distillate. Isolation and identification were accomplished by gas chromatography and coupled gc–mass spectrometry. Identifications were based on I_E values and mass spectra. A total of 24 sulfur compounds were identified in the HVP–L-cysteine·HCl–D-xylose system and 15 were identified in the L-cysteine·HCl–D-xylose system, of which 10 were not present in the former model system.

One of the most challenging problems now confronting the flavor chemist is the successful identification and duplication of cooked meat flavor. The importance of this flavor type for the fabricated foods of today and of the future is clear to all those involved in developing protein foods for affluent as well as developing countries. During the past decade, numerous patents have been granted which encompass the processing of naturally occurring ingredients to produce a meat-like flavor. For instance, U. S. patent no. 3394015 (Giacino, 1968) concerns the processing of thiamine in the presence of cysteine and other amino acids to produce meat flavors. Also, U. S. patent no. 2934437 (Morton *et al.*, 1960) is concerned with the processing of cysteine and other amino acids in the presence of pentose and hexose monosaccharides for the production of meat flavors.

Many workers have speculated as to the importance of sulfur chemicals in meat flavor, but few such speculations have been reported. Brennan and Bernhard (1964) identified hydrogen sulfide and methyl, ethyl, propyl, and butyl mercaptans in the headspace of canned cooked beef. Dimethyl disulfide and dimethyl sulfone were identified by Liebich *et al.* (1972) in boiled beef. In addition, Chang *et al.* (1968) identified 3,5-dimethyl-1,2,4-trithiolan in boiled beef.

It is our opinion that one of the major difficulties in the flavor analysis of cooked beef is that an important part of the meat flavor is due to trace constituents whose identification is made difficult by the large quantities of common lipid-derived aldehydes and ketones formed during the cooking process and present in the flavor isolate. For this reason, we decided to investigate initially a model system in order to give us some insight as to the types of sulfur chemicals present in a nonlipid system.

EXPERIMENTAL SECTION

Two model reaction systems were used throughout this work. In the first system, 3708 g of a carbohydrate-free commercial hydrolyzed vegetable protein, 105.6 g of L-cysteine·HCl (Diamalt A.G.), and 60.0 g of D-xylose (Fisher Scientific) were dissolved in 8076 g of water, refluxed for 4 hr, and then allowed to stand overnight at room temperature. The heated mixture possessed a strong roast odor reminiscent of cooked meat, coffee, and other roasted products. The heated product was atmospherically steam distilled in a 22-l. flask with a Kjeldahl bulb and an ice water cooled condenser. The distillate, 34 l., was collected at a rate of 1 l. per hr in a 5° trap. The distillate was salt saturated and then continuously extracted for 7 hr with 700 ml of distilled diethyl ether in a liquid-liquid extractor. Acidic material was removed by extracting the ether extract with two 0.2-vol of 5% sodium carbonate. The ether extract was dried over anhydrous sodium sulfate and concentrated to approximately 10 ml by careful distillation in a Kuderna-Danish concentrator (Kontes Glass Co., Vineland, N. J.) equipped with a 300-mm × 13 mm i.d. Vigreux reflux column. The concentrate was divided into 17 fractions by small-scale preparative gas chromatography on a 20 ft × ¼ in. o.d. stainless steel column packed with 20% Carbowax 20M on 60–80 mesh AW-DMCS Chromosorb W. The instrument used was an F&M Model 700 gas chromatograph equipped with a thermal conductivity detector. The oven temperature was programmed from 70 to 225° at 2° per min after a 5-min post-injection hold. The temperature of the injector and detector was 230°, and the helium carrier gas flow rate was 80 ml per min. Repetitive 50-μl injections were made and the effluent was collected in 150 mm × 2 mm o.d. glass tubes cooled with crushed Dry Ice.

The second model reaction system consisted of 36.3 g of D-xylose and 181.5 g of L-cysteine·HCl dissolved in 363 g of water. The solution was heated to 121° in a Parr bomb equipped with an agitator and internal cooling coil and held at this temperature for 4 hr. After cooling, the contents were allowed to stand overnight at 4°. The reaction product was extracted with two 300-ml vol of methylene chloride (Matheson, Coleman and Bell, ACS Reagent Grade), and the extract was dried and concentrated in a Kuderna-Danish concentrator as previously described. The concentrate was divided into ten fractions by small-scale preparative gas chromatography on a 10 ft × ¼ in.

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