Studies with Radioactive Tracers. XVII. Model Browning Reactions between Glycine and D-Glucose[1]

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ABSTRACT

Air-dried solutions of glycine and D-glucose on filter paper were baked at 120°C. for selected time intervals. The reactions were followed by using glycine-1-^{14}C, glycine-2-^{14}C, or uniformly labeled D-glucose-^{14}C as one of the reactants. The Amadori rearrangement product, 1-deoxy-1-glycino-D-fructose, was formed in the initial stages of the reaction and was isolated in crystalline form after heating for 20 min. Changes in radioactivity contents with lengths of heating in the volatile, basic water-soluble, nonbasic water-soluble, and water-insoluble fractions were also determined. The results are compared with previous data from breadmaking studies and discussed in relation to Maillard browning reactions.

Radioactive tracer and paper chromatographic studies have shown that, during breadmaking, glycine-1-^{14}C (1) and glycine-2-^{14}C (2) take part in Maillard-type browning reactions which initially involve reactions between amino acids and reducing sugars (3). It is of interest to investigate these reactions in simpler model systems in order to obtain data for comparison with behaviors in the actual baking of bread. Nonenzymatic browning reactions, including model reactions between amino acids and sugars, have been reviewed by Reynolds (4,5). However, much of the previous work has been carried out in solution at moderate temperatures. It is important to study model reactions under conditions similar to those actually encountered in the food systems being investigated. Thus, for example, Richards (6) has studied the reaction between glycine and D-glucose in the "dry" state at 70% r.h. and 37°C. in order to simulate the conditions of storage of dried food. In

[1]Contribution from the Department of Chemistry and Chemical Engineering, University of Saskatchewan, Saskatoon, Saskatchewan, Canada. For Paper XVI, see ref. 2.

the present work, the reaction of glycine and D-glucose is investigated under conditions chosen to approximate those in bread-baking. The radioactive tracer technique is applied by the use of glycine-1-^{14}C, glycine-2-^{14}C, or uniformly labeled D-glucose-^{14}C as one of the reactants.

MATERIALS AND METHODS

Studies with ^{14}C-Labeled Compounds

The glycine-1-^{14}C, glycine-2-^{14}C, and uniformly labeled D-glucose-^{14}C were obtained from commercial sources. More work was done with labeled glycine than with labeled D-glucose. A typical procedure in the investigation of the basic components from the reaction between labeled glycine and D-glucose is given below.

A 1.0-ml. solution containing 50 mg. of D-glucose and 10 mg. of glycine-1-^{14}C or glycine-2-^{14}C (total activity of about 10,000 c.p.m. per experiment) was added onto a 10 × 10-cm. sheet of Whatman No. 3 MM filter paper and then allowed to air-dry at room temperature. The dried paper was heated in an oven at 120°C. for a desired length of time. The temperature of 120°C. was chosen to approximate the interior crumb temperature during baking. If an oven temperature of 220°C. as used in the actual baking of bread was applied directly, browning occurred rapidly giving results similar to those obtained from prolonged heating at 120°C. The resulting baked paper was cut into small pieces, soaked in water, and filtered. The extraction was repeated three times. The combined extract was passed through a column (1.0 cm. diam. × 10 cm.) packed with a strongly acidic ion-exchange resin (Dowex 50, H^+ form). The basic components adsorbed by the resin were eluted either with 25 ml. of 5N NH_4OH (6) or with 100 ml. of 0.2N trichloroacetic acid (TCA) (7). The NH_4OH eluate was air-dried at room temperature and the residue redissolved in 0.2 ml. of water. Aliquots of this solution were then subjected to descending paper chromatography on Whatman No. 1 filter paper, using as solvent a mixture of 1-butanol:acetic acid:water in a ratio of 4:1:1 (v./v.) (2). When the elution was effected by TCA, the TCA was first removed from the eluate by continuous ether extraction (48 hr.) before the solution was air-dried, redissolved in 0.2 ml. of water, and chromatographed as described. The distribution of radioactivity on each paper chromatogram was measured by a paper chromatogram scanner which records the radioactivity vs. the position along the paper strip (1,2).

The radioactivity of the volatile, basic water-soluble, nonbasic water-soluble, and water-insoluble fractions in the model reactions between glycine and D-glucose was measured according to the following procedures.

When glycine-1-^{14}C or glycine-2-^{14}C was used as the active reactant for each experiment, a 0.1-ml. aliquot of solution containing 1.0 mg. of labeled glycine (about 2,000 c.p.m.) and 10 mg. of D-glucose was soaked onto a small disc of filter paper (Whatman No. 3, 2.8 cm. diam.) which was then air-dried and heated at 120°C. for a desired period varying from 5 min. to 48 hr. The radioactivity on the paper was measured before and after heating with a windowless gas flow counter. The percentage of the volatile ^{14}C-components was calculated from the difference in activities before and after heating. Each paper disc was then subjected to extraction by 5-ml. portions of water, the extraction being repeated five or six

times until the residual radioactivity remaining on the disc became constant. The percentage of water-insoluble C^{14}-components formed during the heating was calculated by the ratio of this residual activity to the original activity. The water extracts were combined, passed slowly through Dowex 50 (H^+ form) in a small column (1 cm. diam. X 5 cm.) and washed with 25 ml. of water. The effluent and washing were combined as the nonbasic water-soluble fraction. The basic materials remaining on the column were eluted with 25 ml. of 5N NH_4OH. The nonbasic or basic fraction was evaporated to dryness at room temperature, redissolved in 0.2 ml. of water, and then transferred to a paper disc of the same size as described above. The radioactivity of each of the paper discs was then measured and the percentage of the nonbasic water-soluble, or basic water-soluble, fraction was calculated.

If D-glucose-^{14}C were used directly in similar experiments as described in the preceding paragraph, the high activity of the unreacted sugar could mask the changes arising from the Maillard type of reactions. In order to follow the variations in the radioactivity derived from D-glucose-^{14}C, a purified sugar-glycine interaction product, to be designated as component A (later identified as 1-deoxy-1-glycino-D-fructose), was utilized in the experiments outlined below.

A 1.0-ml. solution containing 50 mg. of D-glucose-^{14}C (about 20,000 c.p.m.) and 10 mg. of glycine was added onto a 10 X 10-cm. sheet of Whatman No. 3 MM filter paper, which was air-dried and heated at 120°C. for 20 min. The basic material, consisting chiefly of component A and isolated by TCA elution from the ion-exchange column, was redissolved in 1.5 ml. of water. This solution was used as the starting material for investigating activity changes in the sugar moiety. For each experiment, a 0.1-ml. aliquot was soaked onto a disc of filter paper, heated for a desired period, and the activities in the four fractions were determined as described earlier in this paper.

Isolation of Component A, 1-Deoxy-1-glycino-D-fructose

Component A, noted as a product formed in the initial stages of reaction between glycine and D-glucose involving a radioactive reactant, was isolated from large-scale experiments with inactive materials.

A solution of 15 g. of glycine and 75 g. of D-glucose in 400 ml. of water was soaked onto 24 pieces of Whatman No. 3 MM filter paper (25 X 28 cm.), which were then air-dried and heated at 120°C. for 20 min. The paper was cut into small pieces and extracted four times with 1-liter portions of methanol. Preliminary experiments have shown that methanol would be a better solvent than water since it could extract component A while minimizing the extraction of the glycine. After the extract was evaporated at room temperature to give a syrupy mass, 400 ml. of water was added to dissolve the material. The solution was passed slowly through a 5 cm. diam. X 36 cm. column packed with Dowex 50, H^+ form. To remove any residual sugar and nonbasic materials, the column was washed thoroughly with 5 liters of distilled water before elution with 4 liters of 0.2N TCA. After the TCA in the eluate was removed by 48 hr. of continuous ether extraction, the aqueous solution was evaporated at room temperature to give about 20 g. of yellowish syrup. This syrup was dissolved in 250 ml. of water and decolorized three times, each time with 10 g. of activated charcoal. The resulting solution was evaporated at

room temperature to about 150 ml., heated to about 60°C., and then an equal volume of hot methanol was added. After cooling in a refrigerator overnight, a yield of 16.5 g. of grayish crystals was obtained. Paper chromatographic examination of this product showed it to be component A (positive color spots with alkaline $AgNO_3$ and with ninhydrin) contaminated with a weak spot (positive color with ninhydrin) corresponding to glycine or an impurity with the same R_f value as glycine. The final purification was carried out by chromatographing all the material on large pieces of paper, with the strips containing only the desired component A cut out and extracted with water. The extract was concentrated and after addition of hot methanol and cooling overnight as described above, 10.8 g. of crystalline material, m.p. 157°C. (decomp), was obtained.

An attempt was made in isolating component A from bread made with glycine added to the baking formula. Each loaf of bread was baked from 100 g. of flour as in the previous work (1,2), except that the radioactive glycine was replaced by 1.0 g. of ordinary glycine. Each loaf was divided into two portions, each portion being extracted in a Waring Blendor with 500 ml. of water for 30 min. followed by centrifuging. The combined aqueous extracts from 35 loaves of bread was passed through a 5 cm. diam. × 50 cm. Dowex 50, H^+ form, column, washed with copious amounts of water, and then eluted with 10 liters of 0.2N TCA. After the TCA was removed by continuous ether extraction and the water evaporated off at room temperature, about 70 g. of syrup was obtained. This syrup was transferred to the top of a cellulose column (6 cm. diam. × 50 cm.) and then eluted with water-saturated 1-butanol for 10 days. About 30 g. of materials with R_f values higher than that of glycine was eluted. Glycine itself moved nearly to the bottom of the column while the materials with R_f values similar to that of component A remained at the upper portion of the column. The top 10 cm. of the cellulose column was then cut out, extracted with water, and evaporated at room temperature to give about 1 g. of syrup. This material was dissolved in 2 ml. of water and streaked across pieces of Whatman No. 3 MM paper, together with samples of component A applied to either side of the streak as reference points. After chromatographing, the strips containing component A from the syrup, but not from the reference material, were cut out, extracted with water, and evaporated at room temperature. Only about 15 mg. of syrupy material was obtained.

RESULTS AND DISCUSSION

The Basic Components from Reactions of Glycine with D-Glucose

The appearance of the curves recording the activity distributions on the paper chromatograms from the reaction of glycine and D-glucose, with one of the reactants labeled with ^{14}C, would, of course, be dependent on the conditions of the experiment, the size of sample chromatographed, and the time allowed (usually 2 to 4 days) for the chromatogram to develop. Figure 1 shows some typical results for the NH_4OH-eluted basic components from the reaction of glycine-2-^{14}C and D-glucose. It can be seen from Fig. 1 that in the initial stages of the reaction, besides unreacted glycine-2-^{14}C (peak G), a distinct product, component A, was formed. On longer heating at 120°C., the amount of unreacted glycine was found to decrease and, eventually, component A also became indistinguishable while appreciable amounts of active material appeared near the spot where the sample

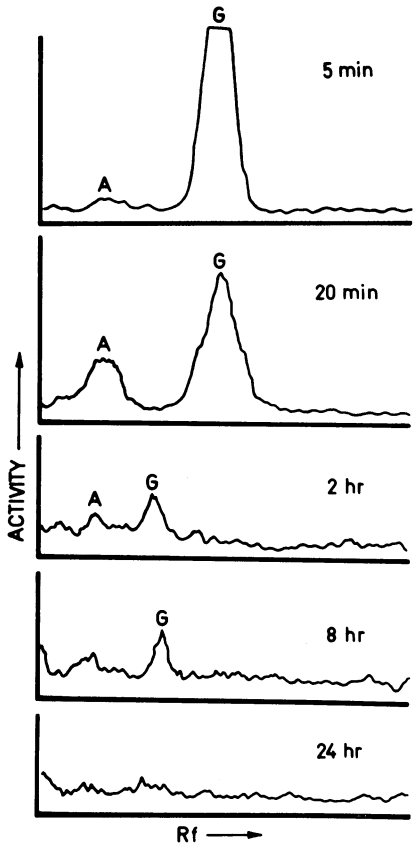


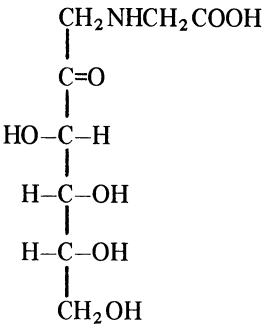
Fig. 1. Activity distributions on paper chromatograms of the basic components derived from heating glycine-2-^{14}C with D-glucose at 120°C. for various lengths of time.

was originally applied. These results suggested that on prolonged heating (8 or 24 hr.), the initially formed component A has changed and, presumably, the much less mobile pigments from the final stages of the browning reaction have been formed.

Further investigations with a reaction time of 20 min. were carried out. With glycine-1-^{14}C, essentially the same activity distribution was observed as in the 20-min. reaction with glycine-2-^{14}C. When the elution was effected with 0.2N TCA rather than with 5N NH_4OH, the glycine peak essentially disappeared, leaving only component A as a distinct product. Thus 0.2N TCA appeared to be a more selective solvent, capable of eluting component A and leaving practically all of the unreacted glycine on the ion-exchange column. When D-glucose-^{14}C was used instead of labeled glycine as the radioactive reactant, after 20 min. of heating at 120°C., a peak showing component A was also obtained. Thus A was apparently some product of interaction between glycine and D-glucose.

From examination of the crystalline product isolated from the large-scale experiment with inactive glycine and D-glucose, component A was identified as 1-deoxy-1-glycino-D-fructose (D-fructoseglycine) (I), the Amadori rearrangement

product (3–8) formed from N-D-glucosylglycine which would be the first product of condensation between glycine and D-glucose.



I

I is shown by the open chain structure, although it may exist in equilibrium with the cyclic pyranose and furanose forms[2].

The identity of component A as I was based on the following observations: m.p. 157°C. (decomp), lit. (9) m.p. 157°C. (decomp); $[\alpha]_D^{25}$ -66°, lit. (7) $[\alpha]_D^{25}$ -65°. The analysis calculated for $C_8H_{15}O_7N$ was C, 40.50; H, 6.37; N, 5.91; that found was C, 40.12; H, 6.34; N, 6.02. On hydrolysis by heating component A with 1N HCl in a sealed tube at 100°C. for 1 hr., the paper chromatogram of the hydrolysate showed the presence of glycine and 5-hydroxymethylfurfural (HMF) as described by Richards (6) for 1-deoxy-1-glycine-D-fructose. Also treatment of component A with phenylhydrazine gave a crystalline product which melted alone and on admixture with D-glucosazone at 203°C., again as reported by Richards (6) for 1-deoxy-1-glycino-D-fructose. Thus under the conditions used in the present model reactions between glycine and D-glucose, the Amadori rearrangement product, I, was the only isolable product formed in the initial stages of the reaction.

From Fig. 1, the $R_{glycine}$ value (R_f relative to that of glycine as 1.00) of peak A (compound I) was estimated as 0.42. It is of interest to note that from the previous breadmaking studies using glycine-2-^{14}C (2), the chromatogram of the basic components of subfraction 2 derived from the crumb extract (subfraction Cb-B-2, Fig. 2B of ref. 2), obtained in the same way as in the present work, showed four active peaks besides that of unchanged glycine-2-^{14}C. The $R_{glycine}$ values for these peaks were 0.13, 0.44, 0.73, and 0.87. Since the $R_{glycine}$ value of one of these peaks was very close to that of I (0.44 compared to 0.42), the possibility existed that I might be present in the crumb of the finished bread. As described earlier in the present paper, the attempted isolation of I from 35 loaves of bread baked with added glycine gave only about 15 mg. of syrupy material. This material,

[2]From consideration of the infrared spectrum, Richards (6) has suggested the enol form as the structure of I. However, in a private communication quoted by Reynolds (4), this suggestion has been withdrawn.

however, did give glycine and HMF on acid hydrolysis as expected for I (6). Thus it may be concluded that compounds similar to the Amadori rearrangement products from reactions of reducing sugars and amino acids could be present in the finished bread; but the actual amounts of such initial products of the Maillard browning reaction that could survive in the bread instead of undergoing further transformations would likely be extremely small.

Changes with Heating Time in the Activities of Various Fractions from Reactions between Glycine and D-Glucose

Variations with lengths of heating time at 120°C. in the radioactive contents of volatile, basic water-soluble, nonbasic water-soluble, and water-insoluble fractions from the reaction between glycine-2-^{14}C or glycine-1-^{14}C with D-glucose, or from heating the basic components (chiefly compound I) derived from the reaction of glycine with D-glucose-^{14}C, are summarized in Tables I, II, and III.

From Table I, it is seen that with short reaction times, most of the activity was found in the basic water-soluble fraction. This is not surprising since unchanged glycine-2-^{14}C and the initial products of reaction such as I would be included in this fraction. With increasing time of heating, the activity in the basic fraction decreased steadily to about 22% after 48 hr. No loss of activity as volatile components was noted in these experiments, indicating negligible losses of the C-2 carbon of glycine under these conditions. The amount of activity in the nonbasic water-soluble fraction increased to about 25% within the first hour and then showed no marked changes on longer heating. On the other hand, activity in the water-insoluble fraction rose steadily, reaching about 41% after 48 hr. of heating. These results are consistent with the occurrence of Maillard-type reactions. As suggested by Hodge (10), the Maillard browning reactions may be discussed in terms of the initial, intermediate, and final stages of reaction. The active materials in the water-insoluble fraction in Table I likely would consist chiefly of polymeric

TABLE I. RADIOACTIVITY IN VARIOUS FRACTIONS FROM HEATING OF GLYCINE-2-C^{14} WITH D-GLUCOSE

Time of Heating hr.	Activity Content, %[a]			
	Volatile	Basic water-soluble	Nonbasic water-soluble	Water-insoluble
1/12	0	80.9	6.0	1.0
1/6	0	74.9	10.7	1.3
1/4	0	67.5	18.2	2.2
1/3	0	62.3	20.7	9.5
1/2	0	56.1	23.2	10.4
3/4	0	51.3	26.0	11.9
1	0	49.8	24.6	12.9
2	0	47.0	22.9	14.8
4	0	42.7	27.0	18.0
8	0	38.4	24.8	26.1
24	0	33.4	26.7	29.3
48	0	21.7	25.5	40.8

[a] In this and subsequent tables, the original activity on the paper disc before heating was taken as 100%. In most cases, the total recovery of activities in the four fractions was less than 100%. This could be due to losses of uneluted material remaining in the ion-exchange column or to losses arising from manipulation of the samples prior to the counting of the basic and nonbasic water-soluble fractions, or both.

TABLE II. RADIOACTIVITY IN VARIOUS FRACTIONS FROM HEATING OF GLYCINE-1-C^{14} WITH D-GLUCOSE

Time of Heating hr.	Activity Content, %			
	Volatile	Basic water-soluble	Nonbasic water-soluble	Water-insoluble
1/12	0	83.0	10.6	1.6
1/6	0.6	73.1	16.0	1.8
1/4	11.5	53.4	18.3	2.5
1/3	19.6	45.6	20.6	2.8
1/2	25.4	37.8	17.2	3.0
3/4	31.5	28.8	16.3	3.2
1	38.5	25.6	19.0	5.2
2	53.1	21.4	19.0	4.4
4	64.3	15.4	13.9	4.9
8	69.0	12.8	12.7	6.8
24	71.5	9.5	11.8	8.6
48	73.2	8.3	10.9	12.1

TABLE III. RADIOACTIVITY IN VARIOUS FRACTIONS FROM HEATING OF THE BASIC COMPONENTS, CHIEFLY 1-DEOXY-1-GLYCINO-D-FRUCTOSE, DERIVED FROM GLYCINE AND D-GLUCOSE-^{14}C

Time of Heating hr.	Activity Content, %			
	Volatile	Basic water-soluble	Nonbasic water-soluble	Water-insoluble
1/12	0	63.4	21.4	0.5
1/6	0.3	56.3	22.4	0.8
1/4	0.5	50.2	18.3	1.3
1/3	0.7	44.8	20.1	1.2
1/2	1.1	38.6	22.4	3.3
3/4	1.7	29.3	20.6	4.0
1	2.0	25.8	31.9	3.8
2	2.3	23.4	24.7	5.9
4	5.8	20.2	33.7	9.0
8	9.8	15.3	32.0	13.7
24	11.6	13.9	29.2	23.0
48	12.7	10.5	31.2	33.4

pigments from the final stages of the browning reactions. The nonbasic water-soluble fraction would include much of the products formed in the intermediate stage reactions, and these, on the one hand, could be derived from the decomposition of the basic components of the initial stage reaction and, on the other hand, could also be converted to insoluble pigments. Thus it is not surprising that a more or less steady concentration was observed for this fraction after heating for about 1 hr.

When the radioactive reactant was glycine-1-^{14}C (Table II), the most pronounced difference from the data obtained with glycine-2-^{14}C (Table I) was the rapid increase of the volatile activity. This was most probably due to the loss of the C-1 carbon as CO_2, presumably via Strecker degradation. The Strecker degradation involving glycine itself would also give rise to volatile formaldehyde. However, no activity was found in the volatile fraction from the studies with glycine-2-^{14}C

(Table I). These findings thus suggested that such degradations would take place after the initial formation of reaction products between glycine and D-glucose and/or that any formaldehyde which might have been produced could immediately condense with other substances to form nonvolatile materials. The first alternative might be the more probable one since initial products such as I have been isolated and since it has been suggested (10) that Strecker degradations occur in the intermediate stages of the browning reactions. From the previous baking studies with glycine-1-^{14}C and glycine-2-^{14}C (1,2), similar conclusions regarding the Strecker degradation have also been reached.

Because of the continual loss of volatile activity in the reaction between glycine-1-^{14}C and D-glucose, the amount of activity in the basic water-soluble fraction was found to decrease more sharply than was noted in the analogous data from glycine-2-^{14}C. Furthermore, the amount of active materials in the nonbasic water-soluble fraction, in contrast to the results obtained with glycine-2-^{14}C, did not reach a plateau, but instead continued to decline slowly on prolonged heating. Since the amount of volatile activity lost after 48 hr. of heating was about 73%, the processes leading to the loss of C-1 from glycine must be of major importance. However, some activities remained in the other three fractions even after 48 hr., indicating the incorporation of the C-1 carbon of glycine into the products of the intermediate and final stages of the Maillard reaction and suggesting that the final products of browning must be derived from a multitude of processes.

The results given in Table III are of interest in that, first of all, they indicated that the basic components derived from the reaction of glycine with D-glucose-^{14}C, such as compound I, could undergo extensive transformations simply by heating. Such transformations led to the production of insoluble materials as well as other degradation products. Apparently the degradation of these basic components could give nonbasic materials very easily, since more than 20% of the activity appeared in the nonbasic water-soluble fraction after a reaction time of only 5 min. This observation is not surprising since the ^{14}C-label was originally introduced as D-glucose-^{14}C, which likely could break down to give radioactive nonbasic materials. Furthermore, the results in Table III also indicated that the decomposition of compounds such as I gave small amounts of volatile materials derived from the sugar moiety, whereas the C-2 carbon of glycine could not participate in the formation of such volatile compounds.

Acknowledgment

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