STUDIES WITH RADIOACTIVE TRACERS

X. The Fate of Glycine-1-^{14}C during Breadmaking[1]

Y. H. Liau and C. C. Lee

ABSTRACT

Bread was made with 200 mg. of glycine-1-^{14}C per 100 g. flour as an additional ingredient in the baking formula. Some conversion to active carbon dioxide occurred, but none of the volatile condensates showed appreciable radioactivity. About 20 and 40%, respectively, of the original activity remained in the crust and crumb. Aqueous extracts of the crust and crumb were separated into basic, acidic, and neutral fractions, and the basic fraction, which contained all amino compounds including glycine, showed the highest activity. Paper-chromatographic studies indicated that, besides unchanged glycine-1-^{14}C, at least 10 and 20 well-resolved active components were present in the basic fractions from the extracts of crust and crumb, respectively. Some of these components are hydrolyzable, giving rise to new products or regenerated glycine. These findings demonstrated the occurrence, during breadmaking, of condensation reactions involving glycine. The results are interpreted as strong evidence in support of the Maillard type of browning reaction's taking place during the baking of bread.

The initial stages of the Maillard browning reactions involve condensations between amines and reducing sugars (1,2). It is generally believed that crust browning in bread is of the Maillard type involving amino acids (3–5). Nineteen free amino acids have been identified in the dialyzable fraction from fermented dough (3), and the decrease of free amino acid content in bread crust has been interpreted as supporting the participation of amino acids in browning reactions (4,5). The addition of 0.2 to 0.8 g. of glycine per 100 g. flour in the making of bread has been found to cause a pronounced browning of the crust and a decrease in loaf volume (6). In the present study, the fate of glycine-1-^{14}C (200 mg. per 100 g. flour) during breadmaking was investigated.

Materials and Methods

The flour used, the baking formula, and the methods of fermentation, baking, and collecting of the various fractions were the same as described in the preceding paper (7), with the exception that in the baking formula ordinary sucrose was used in place of sucrose-^{14}C, and 200 mg. of glycine-1-^{14}C[2] containing 0.2 mc. of activity was present as

[1]Manuscript received March 14, 1966. Contribution from the Department of Chemistry and Chemical Engineering, University of Saskatchewan, Saskatoon, Saskatchewan, Canada.

[2]Obtained from New England Nuclear Corp.

an additional ingredient. Radioactivity determinations were carried out by oxidizing appropriate aliquots of each sample to carbon dioxide and counting the gas in a vibrating reed electrometer,[3] giving absolute activities in millimicrocuries (mμc).

The crust and crumb were extracted with water and the aqueous extract separated into basic, acidic, and neutral fractions by treatment with ion-exchange resins as previously described (7). Glycine itself would be included in the basic fraction, since all amino compounds were adsorbed by the first column. These fractions were subjected to ascending paper-chromatographic studies. The solvent systems employed (8) were 90% phenol-water (4:1) and 1-butanol-acetic acid-water (4:1:1) for the basic fraction, chloroform-95% ethanol-90% formic acid (65:33:2) and phenol-water-formic acid (75:25:1) for the acidic fraction, and ethyl acetate-pyridine-water (8:2:1) and benzyl alcohol-1-propanol-water-85% formic acid (72:50:20:20) for the neutral fraction. The activity distribution on each chromatogram was measured by a scanner[4] which records the activities *vs.* the positions along the paper strip.

Results and Discussion

Activity Distributions. The distributions of ^{14}C-activity in the various fractions obtained in the present work are shown in Tables I

TABLE I

ACTIVITY DISTRIBUTIONS IN BREADMAKING USING GLYCINE-1-^{14}C

FRACTION	ACTIVITY		PERCENT	
	Loaf I	Loaf II	Loaf I	Loaf II
	mμc	mμc	%	%
Glycine-1-^{14}C	200,000	200,000	100.0	100.0
Fermentation condensate	trace	trace	...	
Fermentation CO_2	2,926	2,662	1.5	1.3
Oven-vapor condensate	65	46	0.03	0.02
Oven-vapor CO_2	9,000	10,200	4.5	5.1 (20.7)[a]
Crust distillate[b]	trace	17		0.01
Crust residue[c]	46,240	38,780	23.1	19.4 (21.1)[a]
Crumb distillate[b]	trace	trace	...	
Crumb residue[c]	81,870	81,200	40.9	40.6 (30.7)[a]
CO_2[d]	258	205	0.1	0.1
Total recovery			70.1	66.5

[a] Percentage activity in the fraction from loaf III which was baked for 60 min. instead of the usual 25 min.
[b] From vacuum distillation described earlier (7).
[c] Residue after vacuum distillation.
[d] Collected during vacuum distillation of crumb.

and II. It may be worth noting that of all the volatile materials recovered, only carbon dioxide showed significant amounts of activity.

[3] The Dynacon electrometer supplied by Nuclear-Chicago Corp.
[4] The Actigraph II supplied by Nuclear-Chicago Corp.

TABLE II

ACTIVITY DISTRIBUTIONS IN AQUEOUS EXTRACTS OF VACUUM-DISTILLED CRUST AND CRUMB OF LOAF I

	TOTAL	EXTRACT	FRACTION FROM EXTRACT		
			Basic	Acidic	Neutral
		Crust[a]			
Activity (mμc)	46,240	32,756	12,191	550	1,030
Relative distribution (%)	100.0	70.8	26.4	1.2	2.2
Percent based on original glycine-1-^{14}C	23.1	16.4	6.1	0.3	0.5
		Crumb[b]			
Activity (mμc)	81,870	77,230	48,137	356	1,324
Relative distribution (%)	100.0	94.3	58.8	0.4	1.6
Percent based on original glycine-1-^{14}C	40.9	38.6	24.1	0.2	0.7

[a] Only 42% of the activity in the aqueous extract of the crust was recovered after treatment with ion-exchange resins.

[b] Only 64% of the activity in the aqueous extract of the crumb was recovered after treatment with ion-exchange resins.

This is not surprising, as the glycine was labeled in the carboxyl group and any products which might result from reactions of the fragment after decarboxylation would not be radioactive. The total active carbon dioxide in the proofed dough amounted to about 1% based on the original glycine-1-^{14}C. This value was obtained from counting the carbon dioxide liberated when aqueous suspensions of appropriate aliquots of the dough were treated with sulfuric acid. During baking, some 4 to 5% of the total glycine-1-^{14}C activity appeared as oven-vapor carbon dioxide, which suggested further decarboxylation during baking. This was confirmed by the observation that when a loaf was baked for a prolonged period of 1 hr. instead of the usual 25 min., a sharp increase in the activity of the oven-vapor carbon dioxide was observed (Table I).[5]

The total recoveries of activity in loaves I and II were only 70.1 and 66.5%, respectively. Presumably, the loss may be largely untrapped carbon dioxide. As discussed in the preceding paper, such losses may be due to certain manipulations carried out in the open hood, as well as to the possibility of leakage from the oven during baking.

Of the total activity originally added as glycine-1-^{14}C, about 20 and 40%, respectively, were found in the crust and crumb. Of these residual active materials, water extraction removed about 71% from the crust and about 94% from the crumb (Table II). The lesser solubility of the

[5] A major source of the active carbon dioxide could be due to the fact that amino acids may undergo a Strecker degradation with loss of carbon dioxide and the formation of an aldehyde; in the case of glycine-1-^{14}C, active carbon dioxide and inactive formaldehyde would result.

active components in the crust may be regarded as in accord with the presence of some polymeric products from the last stages of the Maillard browning reactions. When the aqueous extracts of the crust and crumb were separated into basic, acidic, and neutral fractions, the basic fractions, which would include any unchanged glycine-1-^{14}C, showed by far the highest amounts of radioactivity (Table II).

Paper-Chromatographic Studies. The basic, acidic, and neutral fractions from the aqueous extracts of the crust and crumb were investigated by paper chromatography. Although the chromatograms of the neutral fractions gave colored spots corresponding to glucose, fructose, and maltose, these spots showed no radioactivity. Actually, when the solvent systems described earlier in this paper were used, essentially all of the activity in each neutral fraction remained unresolved at the original spot.

The chromatograms of the acidic fractions did show a number of active peaks, due presumably to different organic acids; but none of these acids could be identified. Note, however, that more active peaks appeared in the chromatograms of the acidic fraction from the crumb extract than in the chromatograms of the acidic fraction from the crust extract.

For simplicity, the basic fractions derived from the aqueous extracts of the crust and crumb, respectively, will be designated Ct-B and Cb-B. These fractions were first chromatographed using the phenol-water solvent system. Practically every part of the resulting chromatograms was ninhydrin-sensitive, and the radioactivity of each portion of the chromatograms was well above background, as shown in Figs. 1 and 2. These observations suggested the overlapping of many amino-compounds in the chromatograms. For further investigations, each chromatogram was divided into five subfractions (Ct-B-1 to Ct-B-5 and Cb-B-1 to Cb-B-5), as indicated in Figs. 1 and 2. In each case, subfraction 3 proved to be glycine-1-^{14}C and it constituted about 70 and 90%, respectively, of the entire activity in Ct-B and Cb-B. Since 6 and 24% of the original total activity (Table II) were recovered in Ct-B and Cb-B, respectively, subfractions Ct-B-3 and Cb-B-3 indicated that of the 200 mg. of glycine-1-^{14}C initially added to the baking formula, at least one-quarter remained unchanged in the finished bread.

To obtain larger quantities of the various subfractions, samples of Ct-B and Cb-B were streaked on sheets of Whatman No. 3MM paper, the chromatograms were developed by the phenol-water system, and bands of the subfractions were cut out, eluted with water, and concentrated under reduced pressure. These concentrates were examined by ascending chromatography on Whatman No. 1 paper using the

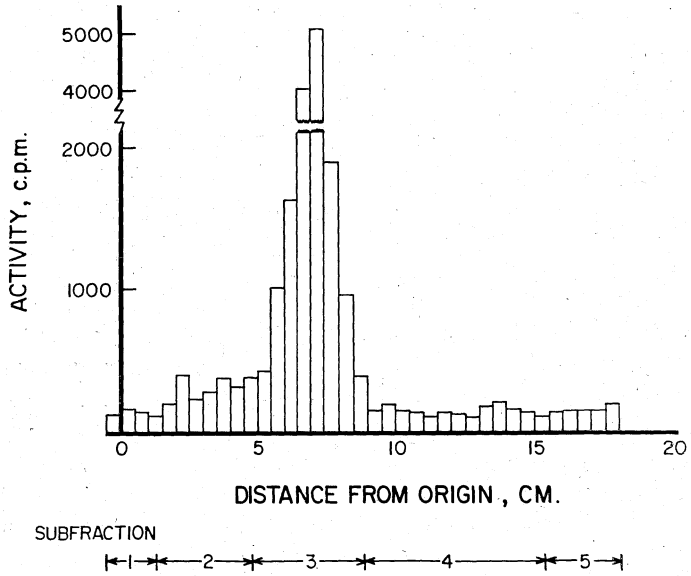

Fig. 1. Histogram showing the distribution of activity on the chromatogram of the basic fraction from the crust extract (Ct-B) developed by the phenol-water solvent system.

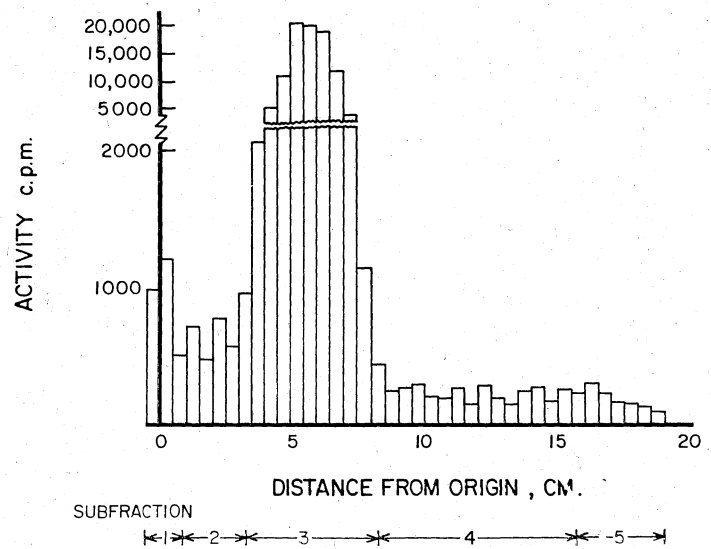

Fig. 2. Histogram showing the distribution of activity on the chromatogram of the basic fraction from the crumb extract (Cb-B) developed by the phenol-water solvent system.

1-butanol-acetic acid-water (BAW) system. In some cases where the R_f values of the active components were low, better separations were achieved by multiple development which involved redeveloping the air-dried chromatograms, several times if necessary, in the same solvent system. For example, Fig. 3-A shows the activity distribution of the subfraction Ct-B-2 on a chromatogram developed four successive times by the BAW system. Other examples are given in Figs. 4-A and 5-A

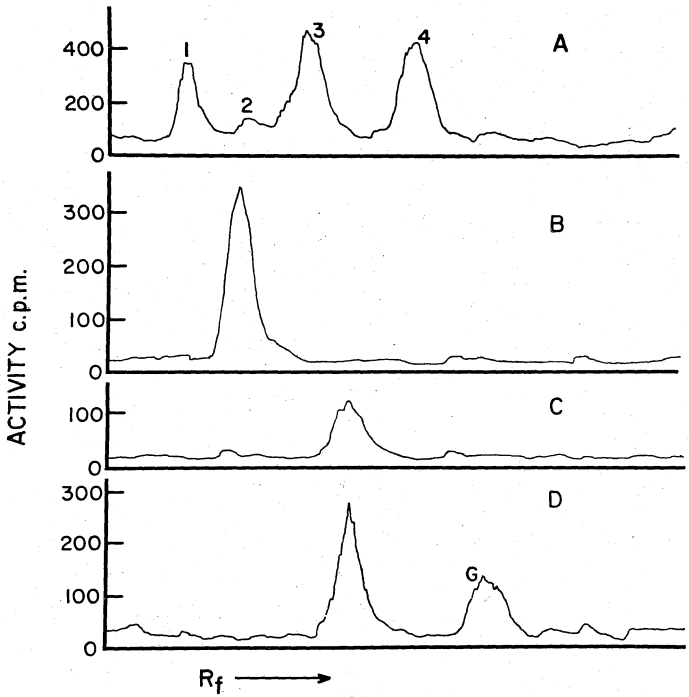

Fig. 3. Activity distributions on chromatograms developed by the 1-butanol-acetic acid-water solvent system. A, subfraction Ct-B-2; B, C, and D, component 2 of Ct-B-2 before hydrolysis, after hydrolysis for 30 min., and after hydrolysis for 1 hr., respectively; G, glycine-1-^{14}C.

which show the activity distributions on the chromatograms of subfractions Cb-B-2 and Cb-B-5, respectively, developed by the BAW system four times for Cb-B-2 and only once for Cb-B-5. In this way, besides identifying Ct-B-3 and Cb-B-3 as glycine-1-^{14}C, at least 10 and 20 other well-resolved peaks were observed for Ct-B and Cb-B, respectively. These findings indicate the presence of more than 10 and 20 amino compounds, respectively, in the crust and crumb extracts.

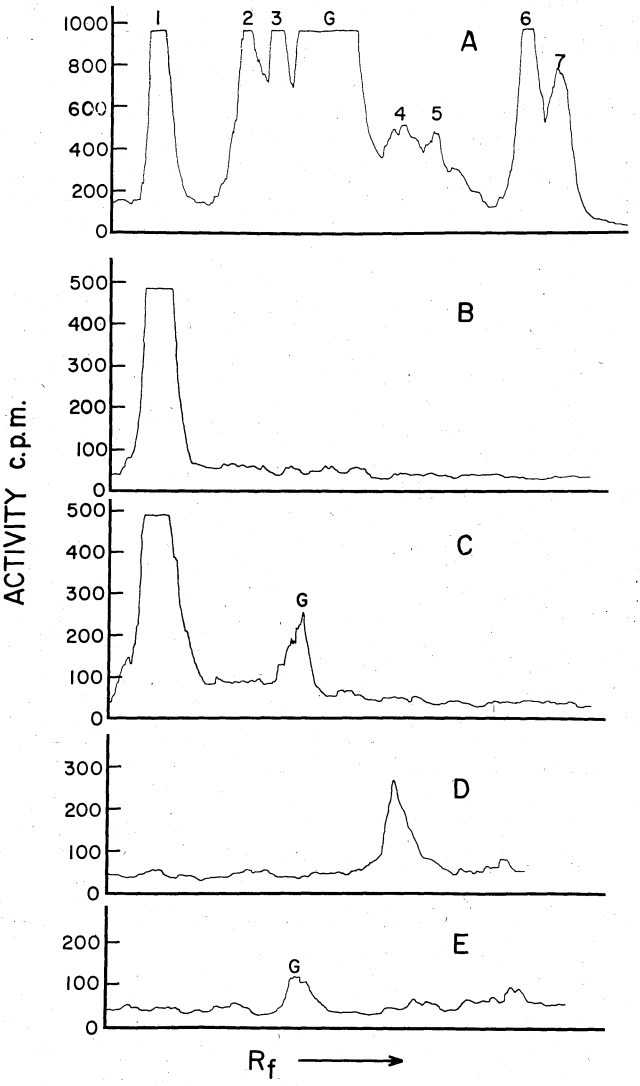

Fig. 4. Activity distributions on chromatograms developed by the 1-butanol-acetic acid-water solvent system. A, subfraction Cb-B-2; B and C, component 1 of Cb-B-2 before and after hydrolysis for 1 hr.; D and E, component 4 of Cb-B-2 before and after hydrolysis for 1 hr.; G, glycine-1-^{14}C.

Again, larger quantities of some of the active components were obtained by chromatographing the appropriate subfraction on Whatman No. 3MM paper followed by elution with water. The eluates were hydrolyzed in 5% hydrochloric acid by heating, in most cases for 1 hr.,

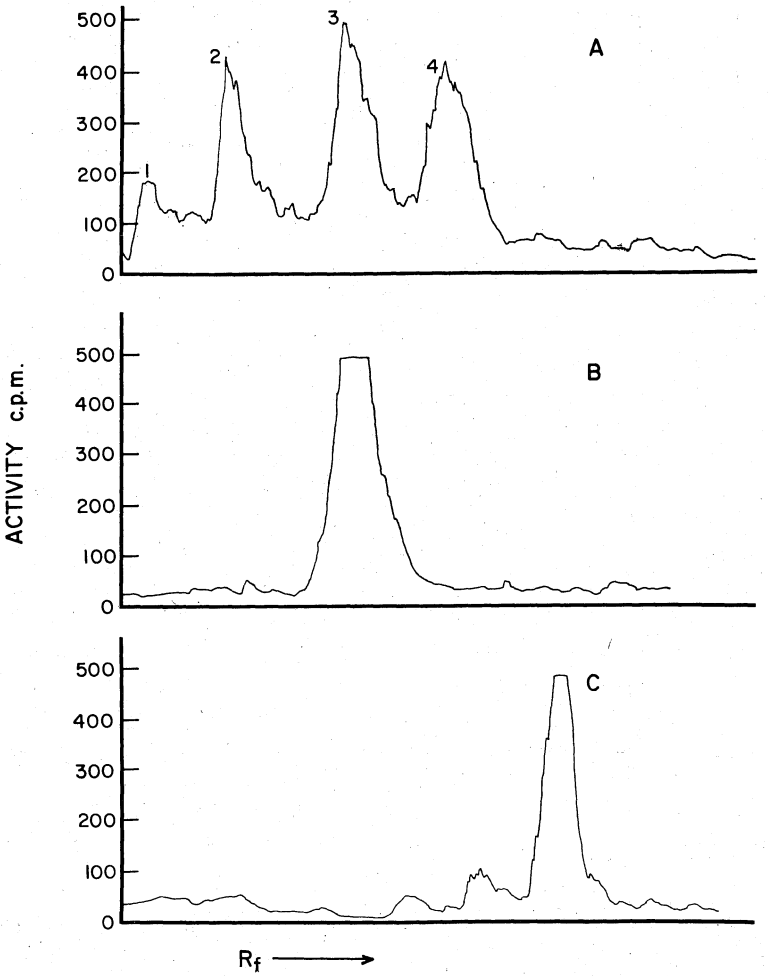

Fig. 5. Activity distributions on chromatograms developed by the 1-butanol-acetic acid-water solvent system. A, subfraction Cb-B-5; B and C, component 3 of Cb-B-5 before and after hydrolysis for 1 hr.

on a steam bath; and the materials before and after hydrolysis were chromatographed. Some active components showed no apparent change on hydrolysis and others gave indefinite results, possibly because of breakdown and formation of hydrolysis products with undetectable amounts of activity. However, most of the active components investigated gave definite results, and the active hydrolysis products may or may not be glycine-1-^{14}C. Some examples are given in Figs. 3B–D, 4B–E, and 5B–C. While components 1 and 3 of Ct-B-2 showed only

indefinite results after hydrolysis, component 2 gave a new product after a 30-min. hydrolysis, and the same new product as well as glycine was obtained when the hydrolysis was carried out for 1 hr. (Fig. 3-B, C, and D). Component 1 of Cb-B-2 gave some glycine after 1 hr. of hydrolysis, but much of the original material still remained unchanged, indicating that the hydrolysis was incomplete (Fig. 4-B and C). On the other hand, component 4 of Cb-B-2 was completely hydrolyzed after 1 hr. and glycine was found as a product (Fig. 4-D and E). Another illustration is given by component 3 of Cb-B-5. This component was completely hydrolyzed in 1 hr. to give new products which are not glycine.

Considering the results from the hydrolysis studies, it may be concluded that some of the labeled glycine originally present in the baking formula has definitely undergone condensation reactions to give a variety of products. Hydrolysis of some of these products could regenerate the glycine or give new products. The observations are certainly in accord with the occurrence of the Maillard type of browning reactions which initially involve condensations between amino acids and reducing sugars (1). In model browning reactions using ^{14}C-label glycine and glucose, Chichester and co-workers (9) have observed, among more than two dozen products, an active compound derived from both glycine and glucose, the hydrolysis of which also liberated glycine. The present results, however, demonstrated that such condensations could actually occur during breadmaking. These results thus constitute strong evidence in support of the Maillard type of browning reactions taking place during the baking of bread.

Acknowledgment

The technical assistance of J. Dyck of the Prairie Regional Laboratory of the National Research Council in carrying out the activity measurements and the financial supports given by the National Research Council of Canada and the Saskatchewan Agricultural Research Foundation are gratefully acknowledged.

Literature Cited

1. Hodge, J. E. Chemistry of browning reactions in model system. J. Agr. Food Chem. **1:** 928–943 (1953).
2. Johnson, J. A., and Miller, B. S. Browning of baked products. Baker's Dig. **35** (10): 52–59 (1961).
3. Horvat, R. J., Ng, H., and Pence, J. Amino acids in fermented bread doughs. J. Food Sci. **27:** 583–586 (1962).
4. Kretovich, V. L., and Ponomareva, A. N. Amino acid participation in melanoidin formation in bread making. Biokhimiya **62:** 237–242 (1961).
5. Linko, Y-Y., and Johnson, J. A. Changes in amino acids and formation of carbonyl compounds during baking. J. Agr. Food Chem. **11:** 150–152 (1963).
6. Rubenthaler, G., Pomeranz, Y., and Finney, K. F. Effects of sugars and certain free amino acids on bread characteristics. Cereal Chem. **40:** 658–665 (1963).

7. LEE, C. C., and CHEN, C-H. Studies with radioactive tracers. IX. The fate of sucrose-^{14}C during breadmaking. Cereal Chem. **43:** 695–705 (1966).
8. BLOCK, R. J., DURRUM, E. L., and ZWEIG, G. A manual of paper chromatography and paper electrophoresis (2nd ed.), pp. 113, 173, 228, 229. Academic Press: New York (1958).
9. CHICHESTER, C. O., STADTMAN, F. H., and MACKINNEY, G. J. Am. Chem. Soc. **74:** 3418–3420 (1952).