STUDIES WITH RADIOACTIVE TRACERS

IX. The Fate of Sucrose-^{14}C during Breadmaking[1]

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ABSTRACT

Bread was made with 5.00 g. of sucrose-^{14}C per 100 g. flour as one of the ingredients in the baking formula. About 42% of the activity remained in the finished bread and nearly 20% of the activity was recovered in the oven vapor condensate. Most of the residual activity in the crumb and crust consisted of neutral compounds, but some active basic and acidic components, which may include products from the early stages of the Maillard type of browning reaction, were also found. Paper-chromatographic studies of the neutral fractions from 70% alcohol extracts of crumb and crust showed the presence of glucose and fructose besides some unidentified components, the ratio of active glucose to fructose being approximately 1:3.5. Nearly all of the activity in the oven vapor condensate was found in the alcoholic fraction. The carbonyl and acidic fractions each contained only 0.5% or less of the activity in the condensate. Paper chromatograms of the 2,4-dinitrophenylhydrazones of the carbonyl compounds in the condensate showed that only 5-hydroxymethylfurfural was appreciably radioactive.

There is considerable current interest in the volatile components of bread (1,2) and in the nature of the reactions leading to browning products during baking (3). The application of radioactive isotopes as label in these areas of investigation should be of advantage in providing the means of tracing the history of a labeled ingredient and in giving a direct indication of the origin of any radioactive final product.

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The present paper reports the results from a study on the fate of uniformly labeled sucrose-^{14}C in breadmaking. The most recent studies on the fate of sugars during baking were the work of Geddes and coworkers (4,5), who analyzed various sugars by means of paper chromatography. The general conclusion on sucrose is that it is rapidly hydrolyzed to glucose and fructose and that glucose is fermented more rapidly than fructose. It is expected that the present work should confirm these earlier findings and may also provide further data on other chemical changes that sucrose may undergo during the making of bread.

Materials and Methods

A commercial "baker's grade" flour, milled from Western Canadian hard red spring wheat, was used in the experiments. It contained 14.1% protein and 0.41% ash on 14% moisture basis. The sucrose-^{14}C, uniformly labeled in all carbons, was obtained from Atomic Energy of Canada, Limited. The baking formula consisted of 100 g. flour, 5.00 g. recrystalized sucrose-^{14}C containing about 0.15 mc. of activity, 3.00 g. yeast, 3.00 g. shortening, 1.75 g. sodium chloride, 4.00 g. milk powder, 0.30 g. malt, 0.10 g. ammonium dihydrogen phosphate, and 63.5 ml. water.

Fermentation. A modified 5-liter vacuum desiccator immersed in a water bath at 30°C. served as fermentation chamber. The cover of the desiccator has a rubber stopper which was fitted with a glass rod and plunger for punching the dough, and inlet and outlet tubes, the latter leading to a series of two Dry Ice-acetone traps and two gas washing bottles containing 20% carbonate-free sodium hydroxide solution. The mixed dough in a bowl was placed in the desiccator, the bottom of which contained a layer of water to provide humidity. Fermentation was allowed to proceed for 3 hr. while a water aspirator was used to draw the volatiles through the traps and gas washing bottles. The dough was then removed for panning and then replaced into the desiccator for a 50-min. proofing.

Baking. Duplicate loaves were baked individually in a vacuum oven at 220°–235°C. for 25 min. The outlet at the top of the oven was connected to a series of traps and gas washing bottles as described under "Fermentation." The oven vapors were drawn through the traps by a water aspirator. The finished bread was placed in a desiccator and chilled. Some liquid condensate was formed in the desiccator and it was collected and added to the oven vapor condensate.

Fractionation of the Bread. Loaf I was separated into crust and crumb, frozen in a freezer, and then pulverized in a Waring Blendor.

Portions of the pulverized crust and crumb were subjected to distillation at 60°–70°C. and 20 mm. of pressure for 4 hr., the distillates being collected in two Dry Ice-acetone traps and one liquid air trap.

A 10-g. aliquot of the crust or crumb residue from the vacuum distillation was extracted with 200 ml. of water for 30 min. in a Waring Blendor. The slurry was centrifuged and filtered through a layer of Filter-cel. The aqueous extract was separated into basic, acidic, and neutral fractions by treatment with ion-exchange resins (6a,7). The basic components were first adsorbed by passage through a column of the cation-exchange resin Dowex 50W × 8 (H^+ form). The effluent was then passed through a column of the anion-exchange resin Dowex 1 × 8 ($CO_3^=$ form), which adsorbed the acidic components. The effluent contained the neutral materials.

The cation-exchange resin was washed with water and the washings were discarded. The adsorbed basic components were then recovered by washing with 5*N* ammonium hydroxide. Similarly, the anion-exchange resin was washed with water and then the adsorbed acidic components were recovered by washing with 1*N* sulfuric acid. The excess sulfuric acid was destroyed by treatment with barium carbonate, the barium sulfate was removed by filtration, and the filtrate was passed through a column of Amberlite 120 cation-exchange resin to remove the barium ions (7). Each fraction containing the basic, acidic, or neutral components was concentrated under reduced pressure at 50°C. to about 10 ml.

Paper Chromatography on the Neutral Extracts of Crust and Crumb. A third loaf of bread was baked and aliquots of the crust or crumb (without having been subjected to vacuum distillation) were extracted with 70% ethanol (4). The extract was passed through the cation and anion exchange resins and the neutral effluent concentrated as described above. The resulting solution was spotted on Whatman No. 3MM paper and the chromatogram developed by the descending technique. Two solvent systems (6a) were used and these were 1-propanol, benzyl alcohol, water, 85% formic acid (PBWF) at the ratio of 50:72:20:20 (v./v.), and 1-butanol, ethanol, water (BEW) at the ratio of 10:1:2 (v./v.). Development with the PBWF and BEW systems was effected in 72 and 92 hr., respectively. The activity distributions in the dried chromatograms were recorded by a chromatogram scanner (Nuclear-Chicago Actigraph II). The paper was sprayed with 3% *p*-anisidine hydrochloride and then heated at 100°–110°C. for 5 min. in an oven which gave colored spots indicating the locations of mono- and disaccharides (6a,8).

Studies on the Volatile Condensates. The activities of the alcohols,

carbonyls, and acids in the oven vapor condensates and in the crumb distillates were estimated by an isotope dilution technique. Since ethanol, acetaldehyde, and acetic acid were found to be the main compounds in oven vapors (9), these were used as carriers. To aliquots of the volatile condensates were added known amounts of inactive ethanol, acetaldehyde, or acetic acid. Each sample was then converted to the appropriate solid derivative. Thus the alcohols, carbonyls, and acids were converted, respectively, to the 3,5-dinitrobenzoates, 2,4-dinitrophenylhydrazones, and *p*-bromophenacyl esters. Although in each case only one inactive compound (ethanol, acetaldehyde, or acetic acid) was used as carrier, it is expected that solid derivatives would have resulted from all compounds having the same functional group. Thus, for example, the recrystalized 2,4-dinitrophenylhydrazones would be free of contamination by active components which did not possess the carbonyl group.

Paper Chromatography of the 2,4-Dinitrophenylhydrazones (6b,10). A 10-g. aliquot of the oven vapor condensate, without any inactive carrier, was treated with 2,4-dinitrophenylhydrazine to give about 3 mg. recrystalized 2,4-dinitrophenylhydrazones. A chloroform solution of these hydrazones was spotted on Whatman No. 1 paper. The paper was dipped, without wetting the spot, in a 50:50 (v./v.) mixture of N,N-dimethylformamide and ether and then air-dried to provide the stationary phase. The chromatogram was then developed by the descending method for 36 hr. with cyclohexane as the moving phase. The dried chromatogram showed nine yellow spots besides the original spot. This unresolved original spot was cut out and subjected to descending development in the second dimension; a solution of cyclohexane saturated with N,N-dimethylformamide, instead of pure cyclohexane, was used as solvent.

Activity Determinations. Appropriate aliquots of the various fractions obtained in the present studies were wet-oxidized by the Van Slyke-Folch reagent (11). The resulting carbon dioxide was converted to barium carbonate and counted as "infinitely thick" samples of constant geometry in a windowless gas flow Geiger counter. The observed activity per "infinitely thick" sample was corrected by multiplication with the total weight (in g.) of barium carbonate that would have been obtained from oxidation of the entire fraction; that is, corrected activity = observed activity × g. of $BaCO_3$ obtained × (wt. of fraction/wt. of aliquot oxidized). For each fraction assayed, at least duplicate and often triplicate determinations were carried out and the average of the corrected activities was used as a measure of the total activity of the fraction.

In the determination of the activities of the alcohols, carbonyls, and acids in the volatile condensates, to an appropriate aliquot of the condensate (0.5–5.0 g.) a known amount (0.2–0.5 g.) of ethanol, acetaldehyde, or acetic acid was added as carrier. The resulting solutions were converted to the appropriate solid derivatives which were used for conversion to barium carbonate for counting. Since the condensates were largely water, the actual weights of active alcohols, carbonyls, or acids in the aliquots used were negligible compared to the weights of inactive carriers. Thus in each case the expected yield of barium carbonate could be calculated from the theoretical yield of solid derivative based on the weight of the carrier added; hence, corrected activity = observed activity × calculated wt. of $BaCO_3$ × (wt. of condensate/wt. of aliquot used). Again, at least duplicate runs were carried out for each determination.

As a check on the validity of this method of activity determination, selected samples were oxidized and counted as carbon dioxide gas in a vibrating-reed electrometer. The relative activities so obtained for several samples, expressed on a percentage basis, were found to be essentially the same as the relative activities based on the corrected activities obtained by counting "infinitely thick" barium carbonate.

Results and Discussion

Activity Distribution in Various Fractions. The activities recovered in the various fractions during the making of bread with sucrose-^{14}C as one of the baking ingredients are shown in Table I. It is to be noted

TABLE I

ACTIVITY DISTRIBUTION IN BREADMAKING WITH SUCROSE-^{14}C

FRACTION	CORRECTED ACTIVITY		PERCENT	
	Loaf I	Loaf II	Loaf I	Loaf II
	c.p.m.	*c.p.m.*	%	%
Sucrose-^{14}C	384,000	366,000	100.0	100.0
Fermentation condensate	900	340	0.2	0.1
Fermentation CO_2	16,900	14,100	4.4	3.9
Oven vapor condensate	75,200	68,900	19.6	18.8
Oven vapor CO_2	8,190	4,430	2.1	1.2
Bread[a]	161,000	155,000	42.4	42.3
Total recovery			68.7	66.3

[a] Sum of crumb and crust fractions.

that total recoveries in all the fractions amounted to only 68.7 and 66.3% for the duplicate loaves. Under the conditions of the present

experiments, complete recovery is not possible. The loss is presumably due to untrapped carbon dioxide and other volatile materials, since certain manipulations, including mixing, panning, the loading of the dough into the oven, and the unloading of the bread, were carried out in the open hood. Much of the loss is believed to be due to leakage during the actual baking, as the vacuum oven used was not under sufficiently reduced pressure to ensure an air-tight system.

It is of interest to note that about 42% of the originally added sucrose-^{14}C activity remained in the finished bread. The fraction with the second largest amount of activity is the oven vapor condensate, which weighed about 25 g. and contained almost 20% of the original activity. These two fractions, the bread and oven vapor condensate, were subjected to further investigations.

Fractionation of the Bread. When the crumb and crust were subjected to vacuum distillation and the residue further extracted with water, results were obtained which are summarized in Table II. Only

TABLE II

ACTIVITY DATA FROM FRACTIONATION OF LOAF I

	CRUMB				CRUST			
	Total	Distillate	Residue		Total	Distillate	Residue	
			Total	Water Extr.			Total	Water Extr.
Correct activity (c.p.m.)	101,000	7,100	93,900	75,600	60,100	630	56,900	42,700
Relative distribution (%)	100.0	7.0	93.0	75.0	100.0	1.0	94.7	71.0
Percent based on sucrose-^{14}C	26.3	1.9	24.4	19.7	15.6	0.2	14.8	11.1

a relatively small portion of the activity in the crumb or crust was of sufficient volatility to permit its removal by vacuum distillation. Of the active materials remaining after the distillation, a large proportion could be extracted by water, as one might expect since the original, labeled material was a water-soluble sugar. Of interest is the fact that part of the original sucrose-^{14}C has been converted to water-insoluble material in the bread, and these amounted to about 18 and 24%, respectively, of the nonvolatile activities in the crumb and crust. The somewhat higher amount of insoluble active materials in the crust is consistent with expectation, as formation of polymeric browning substances would be more pronounced in the crust.

When the aqueous extract of the crumb or crust was separated into basic, acidic, and neutral components by ion-exchange resins, active materials were found in all three fractions (Table III). The neutral

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

FRACTION	CRUMB EXTRACT			CRUST EXTRACT		
	Basic	Acidic	Neutral	Basic	Acidic	Neutral
Corrected activity (c.p.m.)	1,690	2,280	46,400	1,400	1,200	27,200
Relative distribution[a], %	3.4	4.5	92.1	4.7	4.0	91.3

[a] About 30% of the activity in the original aqueous extract was not recovered after the treatment with ion-exchange resins.

fraction, which should include all labeled carbohydrates, accounted for more than 90% of the recovered activity. The presence of ^{14}C activity as basic and acidic components, though small (about 4%), is definite and the observation may be regarded as consistent with the occurrence of the Maillard type of browning processes (3,12). The initial stages of these browning reactions involve sugar-amine condensations to give N-substituted glycosylamines, and such condensation products from ^{14}C-labeled glucose and fructose would be included in the active basic components. Cole, Hale, and Pence (13) have found that when dough and bread were made from pre-ferments with different levels of sucrose and yeast, greater total amounts of organic acids were formed if the pre-ferment contained more sugar and yeast. The present results indicate that at least some organic acids are derived directly from the sucrose originally used in the baking formula.

The activities in the paper chromatograms of the neutral fractions from the 70% alcohol extracts of the crumb and crust are shown in Figs. 1 and 2. Active glucose and fructose are definitely identified, and the relative amount of glucose:fructose is approximately 1:3.5. These findings are in general agreement with those of Koch, Smith, and Geddes (4), who have also found a greater amount of fructose than glucose in bread. Apparently glucose and fructose are not the only active neutral components, and this is indicated by the additional peaks in Figs. 1 and 2. Further studies on the nature of the unknown components in the neutral extracts are in progress. It may also be pointed out that from the color intensities of the developed chromatogram spots, maltose is noted to be the major carbohydrate in these extracts. However, as expected, the location of the maltose spot did not correspond to a region of significant radioactivity.

The Volatile Condensates. The activities of the alcohols, carbonyls,

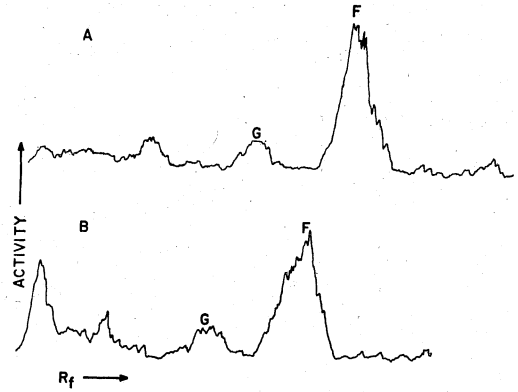

Fig. 1. Activity of paper chromatograms of the neutral fraction from the 70% alcohol extract of crumb: A, development by the PBWF solvent system; B, development by the BEW solvent system; G, glucose; F, fructose.

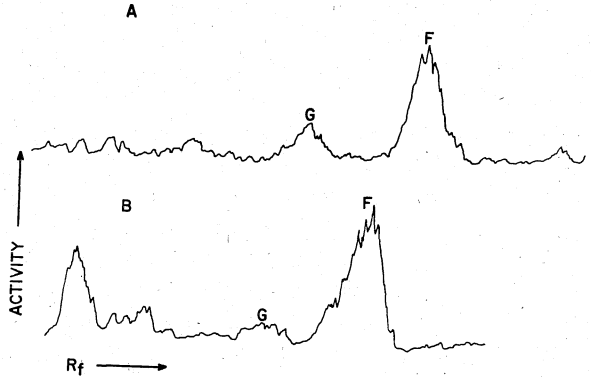

Fig. 2. Activity of paper chromatograms of the neutral fraction from the 70% alcohol extract of crust: A, development by the PBWF solvent system; B, development by the BEW solvent system; G, glucose; F, fructose.

and acids in the oven vapor condensate and crumb distillate are shown in Table IV. Although the alcoholic components accounted for nearly all of the activity, small amounts of carbonyl and acidic compounds were present. The somewhat higher activities of the acids found in the crumb distillate is in general accord with the lesser volatility of organic acids. Carbonyl compounds formed in baking have been extensively studied in relation to bread flavors (1,2). The present results showed that only very minor amounts of volatile carbonyl compounds could have arisen directly from the sucrose in the baking formula. To show that the 0.3–0.4% of the activity in the oven vapor was

TABLE IV
ACTIVITY DISTRIBUTIONS IN VOLATILE CONDENSATES

Loaf	Total Condensate		Alcohols		Carbonyls		Acids	
	Corrected Activity		Corrected Activity		Corrected Activity		Corrected Activity	
	c.p.m.	%	*c.p.m.*	%	*c.p.m.*	%	*c.p.m.*	%
	Oven vapor condensate							
I	75,200	100.0	75,400	100.2	217	0.3	373	0.5
II	68,900	100.0	68,000	98.6	251	0.4	356	0.5
	Crumb distillate							
I	7,100	100.0	6,960	98.0	32	0.5	239	3.4
III	2,520	100.0	2,490	98.9	16	0.6	81	3.2

actually due to carbonyl compounds, a sample of the active 2,4-dinitrophenylhydrazones (DNPH) was treated with alpha-ketoglutaric acid (14) and the regenerated carbonyl compounds were again converted to DNPH. No appreciable decrease in specific activity was observed, indicating that the regenerated carbonyl compounds were actually radioactive.

The distribution of activity in the pertinent parts of the paper chromatograms of the DNPH from the oven vapor condensate is shown in Fig. 3. When cyclohexane was used as solvent for developing the chromatogram, essentially all the activity remained with the original spot (Fig. 3A), even though nine additional well-resolved colored spots were observed. The migrations of these nine spots, measured relative to the DNPH of methyl ethyl ketone (R_{MEK}), were consistent with the DNPH of furfural, diacetyl, acetaldehyde, crotonaldehyde, acetone, isobutyraldehyde, isovaleraldehyde, n-hexaldehyde, and 2-hexanone. These identifications are only tentative since they were based solely on their R_{MEK}. None of these compounds, however, showed appreciable radioactivity. The active spot in Fig. 3 proved to be due to the DNPH of 5-hydroxymethylfurfural (HMF). When cyclohexane saturated with N,N-dimethylformamide was used as solvent, the paper chromatogram (Fig. 3B) showed this DNPH to have a R_{MEK} of 0.56. The active spot was eluted with ethanol, and its UV spectrum in ethanol, with λ_{max} at 385 mμ, was the same as that of an authentic sample of HMF-DNPH.

It is well known that HMF may be formed readily from hexoses. HMF is also generally regarded as being implicated in browning reactions (12); for example, it is one of the more than two dozen compounds observed in Maillard-type reactions between ^{14}C-labeled glucose and glycine (15). The present finding that HMF is formed from the sucrose-^{14}C in the baking formula is, therefore, not surprising. Figure 3 also indicates that any other carbonyl compound with more than

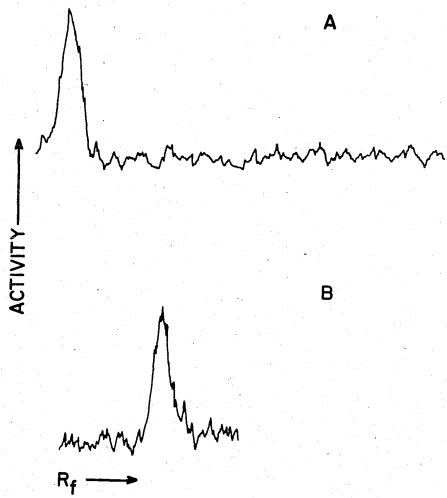

Fig. 3. Activity of paper chromatograms of 2,4-dinitrophenylhydrazones of carbonyl compounds in the oven vapor condensate: A, development by cyclohexane; B, development by cyclohexane saturated with N,N-dimethylformamide.

one-tenth of the activity of HMF would have been detectable. Considering that the carbonyl compounds constituted less than 0.5% of the activity in the oven vapor condensate and that this condensate contained about 20% of the original total activity, it may be estimated that no more than about 0.01% of the sucrose-^{14}C in the present baking experiments could have been converted to any volatile carbonyl compound other than HMF. Rooney and co-workers (16,17) very recently reported that carbonyl compounds arise by way of the Maillard reaction via Strecker degradation of the amino compounds, and this is in accord with the present finding that volatile carbonyls other than HMF are not derived from sucrose.

Acknowledgment

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