Mixograph Studies. III. Reaction of Fumaric Acid with Gluten Proteins During Dough Mixing[1]

J. S. SIDHU, P. NORDIN, and R. C. HOSENEY[2]

ABSTRACT

The effect of fumaric acid on the mixograph curve is similar to that of N-ethylmaleimide. After the peak, extensive dough breakdown results in a narrow mixogram tail. In this study, about 2% of 50 μCi of ^{14}C-fumaric acid was incorporated into gluten proteins during 9 min of dough mixing. Most of the added fumaric acid was found in the water-soluble fraction as free fumaric acid. Gel filtration on Sephadex G-200 showed that most of the bound ^{14}C-fumaric acid was associated with the higher molecular weight protein fraction. Although most of the free sulfhydryl groups in flour are found in the water-soluble fraction, only a small amount of the ^{14}C-fumaric acid reacted with water-soluble proteins. Hydrolysis of gluten proteins containing bound ^{14}C-fumaric acid, using pronase or 3*N* *p*-toluenesulfonic acid, gave a single radioactive compound. This compound was shown to be *S*-succinyl-L-cysteine by paper chromatography (two solvent systems), paper electrophoresis, and column chromatography with an amino acid analyzer. Free radicals are known to be created during dough mixing, and the effect of fumaric acid can be stopped by free radical scavengers. Thus a logical assumption is that fumaric acid reacts by a free radical mechanism. The finding that fumaric acid reacts with gluten protein more readily than with soluble proteins to form *S*-succinyl protein derivatives suggests the disulfide bonds as the site of the free radical formation. Further supporting the hypothesis, potassium iodate, which is known to react with free sulfhydryl groups, did not materially reduce the incorporation of ^{14}C-fumaric acid into gluten proteins. Thus fumaric acid reacts with a free radical formed by the cleavage of a disulfide bond in the gluten proteins.

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The use of fumaric acid and unsaturated alcohols to reduce the mixing time in conventional bread production has been patented by Conn and Kickline (1971a, 1971b). The effect of fumaric acid on the mixogram curve (Weak et al 1976) is similar to that caused by N-ethylmaleimide (NEMI). NEMI has been shown to react readily with approximately one half of the flour sulfhydryls (—SH) and with the remainder in the presence of 8*M* urea (Tkachuk and Hlynka 1963). Meredith and Bushuk (1962) have speculated that, in addition to reacting with —SH groups, NEMI reacts with disulfide bonds that were cleaved during dough mixing.

The action of fumaric acid and related compounds is unknown. These compounds, however, reportedly react with —SH groups under certain conditions (Jocelyn 1972). The objective of this study was to determine the mechanism by which fumaric acid reacts with gluten proteins during dough mixing and to explain the rapid rate of dough breakdown caused by fumaric acid.

MATERIALS AND METHODS

A composite hard winter wheat flour with protein content of 12.7% (N × 5.7) and ash of 0.38% (14% moisture basis) was used for this study. All chemicals used were of analytic grade. Fumaric acid (1-^{14}C), 3.03 mCi/mM, was obtained from ICN Pharmaceuticals, Inc.

A 10-g mixograph was used to mix dough using Finney and Shogren's procedure (1972). Samples were run at their optimum water absorption level, and ^{14}C-fumaric acid was added to the water. The dough was lyophilized, and gluten was isolated as shown in Fig. 1.

Measurement of Radioactivity

An aqueous sample (1 ml) was placed in a scintillation vial and mixed with 5 ml of toluene/triton X-100 (2:1) containing 0.4% of 2,5-diphenyloxazole (w/v), and counted in a Beckman LS-200B scintillation spectrophotometer (Turner 1968).

Gluten protein content was determined by a micro-Kjeldahl procedure (AACC 1976). *S*-Succinyl-L-cysteine was synthesized by two different methods (Morgan and Friedman 1938, Pine and Peacock 1955).

[1]Contribution 79-335-j, Department of Grain Science and Industry and Department of Biochemistry, Kansas Agricultural Experiment Station, Kansas State University, Manhattan 66506.

[2]Graduate research assistant, Department of Grain Science and Industry, professor, Department of Biochemistry, and professor, Department of Grain Science and Industry, respectively.

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Depolymerization of Gluten Proteins

Gluten proteins were hydrolyzed by two methods as follows: 1) by Liu and Chang's method (1971), with 3*N* *p*-toluenesulfonic acid, and 2) by use of pronase in tricine buffer (pH 7.5) at 37°C for 72 hr (Narahashi 1970).

Gel Filtration Studies

Sephadex G-10, G-5, and G-200 columns were prepared in 0.1*N* acetic acid according to the Pharmacia Handbook (Anonymous 1975). Gluten proteins (10–20 mg, depending upon the type of gel) in 0.1*N* acetic acid solution were loaded on the column, and 1-ml fractions were collected by eluting with 0.1*N* acetic acid.

Paper Chromatography of Hydrolyzed Gluten Proteins

Samples were applied to Whatman 3-mm paper and developed using descending flow with two different solvent systems: 1) *n*-butanol/acetone/diethylamine/water (10:10:2:5) and 2) isopropanol/97% formic acid/water (20:1:5) (Arx and Neher 1963). The samples, after hydrolysis with 3*N* *p*-toluenesulfonic acid, were also chromatographed using the above solvent systems.

Paper Electrophoresis of Samples and Succinyl Cysteine

Strips (2 × 50 cm) of acid-washed Whatman No. 1 paper were used. A buffer system consisting of pyridine/acetic acid/water, pH 5.2 (pyridine, AR grade, 20 g; acetic acid, 99.6%, 9.5 g, and water to make 1 L) was employed as an electrolyte (Atfield and Morris 1961). Electrophoresis was also conducted in 0.03*N* Na acetate, pH 5.2, as described by Markham and Smith (1951). At end of the experiment, one-half of the air-dried strip was sprayed with ninhydrin reagent and the other half was cut into 1-cm segments and counted for radioactivity.

Amino Acid Analyzer

Both the sample and authentic succinyl cysteine were hydrolyzed with 3*N* *p*-toluenesulfonic acid at 100°C for 31 hr. After neutralizing *p*-toluenesulfonic acid with 6*N* sodium hydroxide, a suitable quantity of sample was loaded on each of the short and long columns of the Beckman (model 120) amino acid analyzer. After the amino acid profile was recorded, the effluent from the spectrophotometer was used for counting radioactivity. Corrections were made for quenching.

RESULTS AND DISCUSSION

Incorporation of ^{14}C-Fumaric Acid in Gluten Proteins

Earlier work has shown that fumaric acid would affect the mixing time and the shape of mixograms (Schroeder and Hoseney

1978). To determine if the effect of the fumaric acid on the mixograms was the result of fumaric acid binding to the gluten proteins, ^{14}C-fumaric acid was mixed with flour in the mixograph. The resultant dough was lyophilized and fractionated into water solubles, starch, and gluten. The soluble gluten was exhaustively dialyzed against 0.1 *N* acetic acid. A small but significant fraction of the ^{14}C-fumaric acid remained with the dialyzed gluten fraction.

As the mixing time was increased from 1.5 to 9 min, the incorporation of ^{14}C-fumaric acid increased from 48.98 to 171.94 counts per minute per milligram of dialyzed gluten proteins (Fig. 2). Hand-mixing of dough incorporated much less ^{14}C-fumaric acid than was obtained with the mixograph (Table I). Schroeder and Hoseney (1978) had shown that the reaction of fumaric acid with flour appeared to be by a free radical mechanism. The energy spent on the dough system by the mixograph would be much greater than that attained by hand-mixing and could create free radicals on the gluten proteins, thus increasing the incorporation of fumaric acid. Increasing the quantity of ^{14}C-fumaric acid mixed with flour from 2.5 μCi to 50 μCi proportionately increased the amount of fumaric acid retained with the dialyzed gluten (Table II).

TABLE I
Effect of Mixing by Hand and by Mixograph on the Incorporation of ^{14}C-Fumaric Acid[a] in Dough

	Retention (%)	
	Hand Mixing	Mixograph
Water solubles	68.83	69.01
Starch	10.56	10.65
Soluble gluten	20.61	20.34
Dialyzed gluten	0.225[b]	0.57[c]

[a] 2.5 μCi of ^{14}C-fumaric acid and 500 ppm of unlabeled fumaric acid per 10 g of flour.
[b] 19.61 counts per minute per milligram of protein.
[c] 52.29 counts per minute per milligram of protein.

TABLE II
Effect of Quantity of ^{14}C-Fumaric Acid[a] on its Incorporation in the Dough During 9 Min of Mixing in a Mixograph

	Retention (%) of ^{14}C-Fumaric Acid, at Addition Level	
	2.5 μCi	50 μCi
Water solubles	72.48	66.64
Starch	7.30	10.86
Soluble gluten	20.22	22.50
Dialyzed gluten	1.56[b]	2.08[c]

[a] No unlabeled fumaric acid added.
[b] 123.6 counts per minute per milligram of protein.
[c] 2,660.0 counts per minute per milligram of protein.

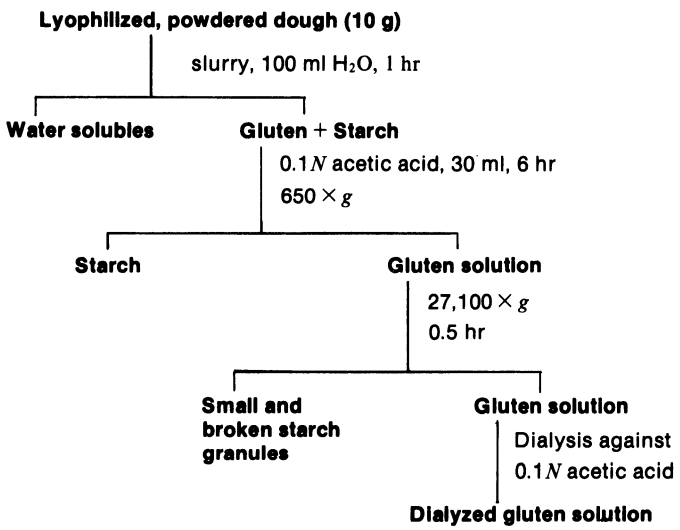

Fig. 1. Fractionation scheme to obtain dialyzed gluten solution.

Gel Filtration Studies

After fractionating dough into water solubles, starch, and gluten, the radioactivity of each fraction was counted. The radioactivity among water solubles, starch, and gluten fractions was distributed as 66.9, 10.9, and 22.5%, respectively. A good separation of the gluten proteins (at the void volume) from unreacted fumaric acid (at the total volume) was obtained on Sephadex G-25 (Fig. 3). Only about 10% of the ^{14}C-fumaric acid in the gluten fraction was strongly bonded to the gluten proteins. The remainder was dialyzable and separable by gel filtration (Table II, Fig. 3). The dialyzed gluten protein was loaded on a Sephadex G-200 column (Fig. 4). About 62% of the ^{14}C-fumaric acid was clearly associated with the higher molecular weight fraction, the remainder with lower molecular weight proteins.

When the water-soluble fraction of flour was loaded on a Sephadex G-25 column (Fig. 5), only a negligible amount (compare Figs. 3 and 5) of the ^{14}C-fumaric acid was associated with the water-soluble proteins (at the void volume). Because most of the free —SH groups in flour are found in the water-soluble fraction (Lee and Lai 1967), the small amount of fumaric acid associated with the water-soluble protein tends to confirm the conclusion of Schroeder and Hoseney (1978) that fumaric acid does not react with free —SH groups. On the other hand, the high percentage of ^{14}C-fumaric acid incorporated into the gluten proteins indicates the possible involvement of cleaved disulfide bonds. The labile nature of the disulfide bond during dough mixing has been shown by MacRitchie (1975).

Chromatography and Paper Electrophoresis of Gluten Proteins Hydrolysate

After determining that a significant amount of ^{14}C-fumaric acid

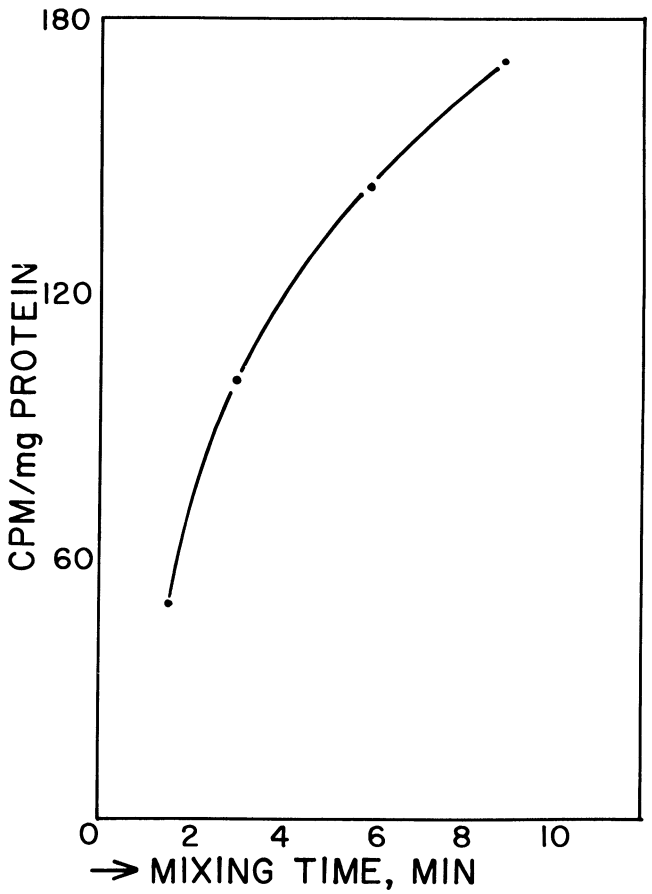

Fig. 2. Effect of mixing on the incorporation of ^{14}C-fumaric acid into dialyzed gluten. 2.5 μCi of ^{14}C-fumaric acid was mixed with 10 g of flour in a mixograph.

was associated with the gluten proteins, the next step was to determine the nature of the binding and, if possible, to identify what amino acid residue(s) were involved. The dialyzed gluten protein isolated from dough mixed with ^{14}C-fumaric acid was hydrolyzed with pronase and separated on a Sephadex G-25 column. The activity was eluted as a single peak incompletely separated from amino acids. As expected, the pronase enzyme system did not completely hydrolyze the gluten proteins. When the pronase hydrolysate was placed on an amino acid analyzer, the results showed the presence of several small peptides. Further hydrolysis of the radioactive peak from Sephadex G-25 with 3*N* *p*-toluenesulfonic acid and subsequent amino acid analysis showed the loss of those peptides.

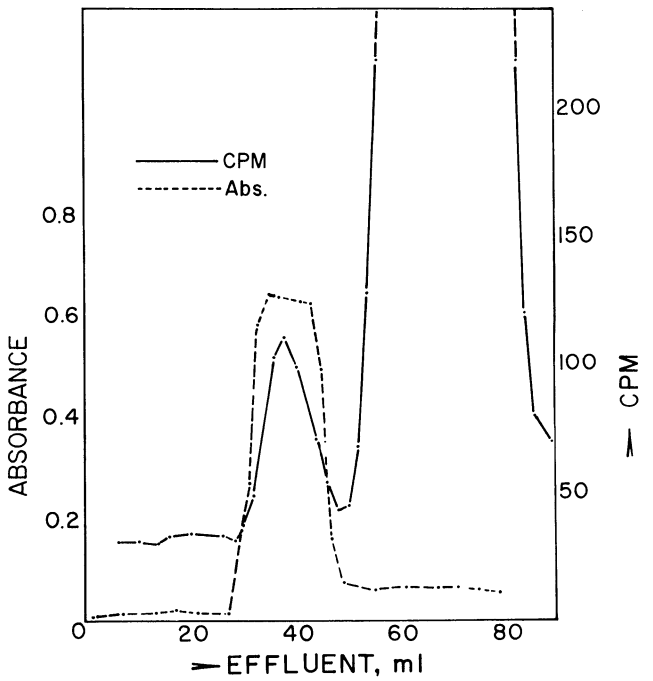

Fig. 3. Elution profile of gluten proteins from Sephadex G-25 column (1.6 × 40 cm), eluted with 0.1*N* acetic acid at a flow rate of 0.5 ml/min. Fractions (1 ml) were collected; protein monitored (280 nm) in odd-numbered tubes, and even-numbered tubes were counted in a scintillation counter.

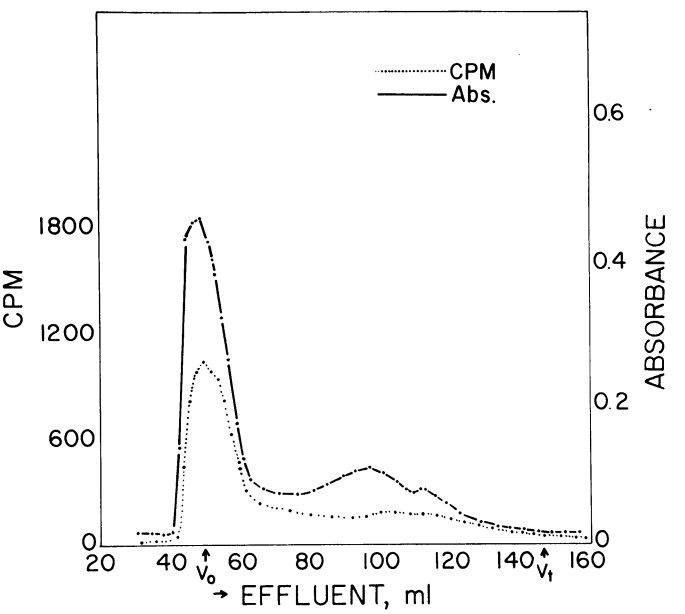

Fig. 4. Elution pattern of dialyzed gluten on Sephadex G-200 (2.6 × 40 cm), eluted with 0.1*N* acetic acid at a flow rate of 0.2 ml/min.

The pronase hydrolysate of the radioactive peak from Sephadex G-25 was chromatographed on Whatman 3-mm paper, using a basic solvent system of *n*-butanol/acetone/diethylamine/water (10:10:2:5) and an acidic solvent system of isopropanol/97% formic acid/water (20:1:5). Most of the radioactivity traveled the same distance as that of authentic succinyl cysteine. After hydrolysis with 3*N* *p*-toluenesulfonic acid, the sample was again chromatographed on Whatman 3-mm paper, using both solvent systems. The sample gave more unlabeled amino acids, but the radioactive spot had a retention value the same as that for the succinyl cysteine standard.

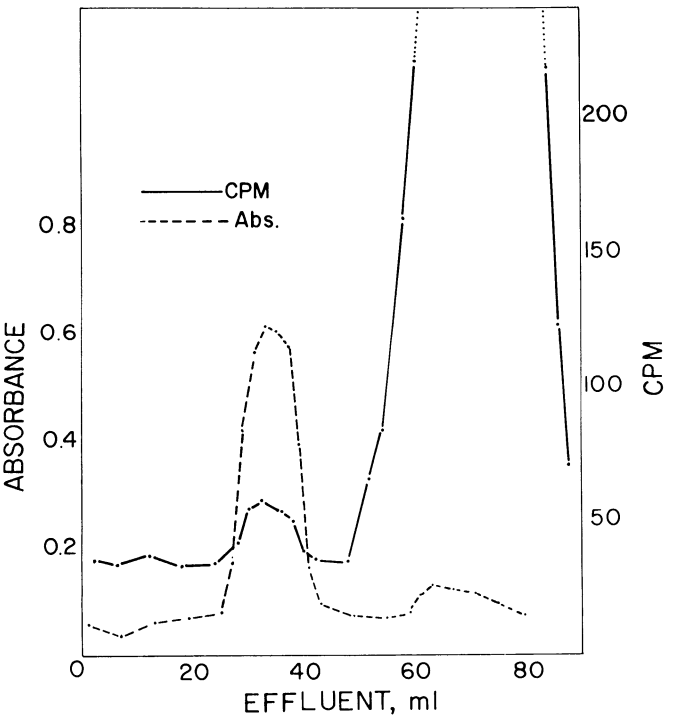

Fig. 5. Elution profile of the water-soluble fraction from a Sephadex G-25 column (1.6 × 40 cm), eluted with 0.1*N* acetic acid at a flow rate of 0.5 ml/min.

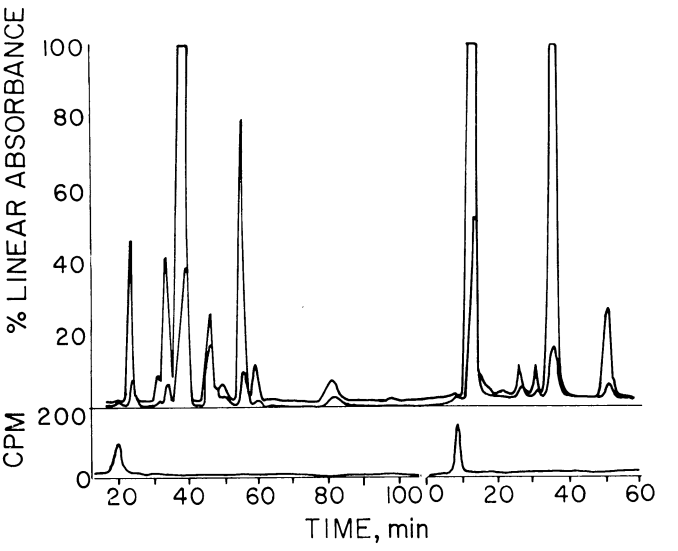

Fig. 6. Amino acid analysis of protein isolated from ^{14}C-fumaric acid-treated dough. The sample was hydrolyzed with 3*N* *p*-toluenesulfonic acid at 100°C for 31 hr and spiked with authentic succinyl cysteine (unlabeled). Spiking was used because the level of succinyl cysteine in the hydrolysate was too low to detect with ninhydrin. **Top,** monitored by ninhydrin; **bottom,** monitored by scintillation counting, showing the presence of a single radioactive peak due to ^{14}C-succinyl-L-cysteine.

Thus it appeared that ^{14}C-fumaric acid had reacted with cysteine in the gluten proteins to form succinyl cysteine. The succinyl cysteine was stable to hydrolysis with 3*N* *p*-toluenesulfonic acid.

To confirm the results obtained with paper chromatography, the samples were subjected to paper electrophoresis at pH 5.2. The radioactivity migrated as two closely spaced bands, the slower of which migrated the same distance as did the succinyl cysteine standards made by reacting cysteine with fumaric acid and maleic acid. The faster-moving band gave a yellow color and the slower-moving band a purple color with ninhydrin. However, after hydrolysis of the sample with *p*-toluenesulfonic acid, the yellow band disappeared and all the radioactivity was in the band that gave a purple color with ninhydrin. The electrophoretic behavior of that band was the same as that of the two succinyl cysteine standards. We think the yellow band was an isomer formed by the dehydration of the amino group and one of the carboxyl groups of the succinyl cysteine, producing an internal amide. After hydrolysis, the amino group was freed and the compound gave a purple color with ninhydrin. An addition compound of NEMI and cysteine, having structural similarity to succinyl cysteine, has been reported (Smyth et al 1960) to undergo internal amide formation at pH 7.6 within 36 hr at room temperature.

Succinyl cysteine in the sample could probably undergo a similar rearrangement while the gluten proteins were being hydrolyzed by pronase (pH 7.4) at 37°C for 72 hr.

Interestingly, the radioactive band on the electrophoresis strip gave a strong fluorescence under UV light (340 nm). When 0.03*N* sodium citrate (pH 5.2) was used, one radioactive band was again obtained but no fluorescence was observed. Thus the pyridine salt of succinyl cysteine appears to fluoresce under UV light.

The radioactive sample from the Sephadex G-25 column was hydrolyzed with 3*N* *p*-toluenesulfonic acid at 100°C for 31 hr and spotted on a paper electrophoresis strip. Standard succinyl cysteine was placed on a second strip, and a mixture of succinyl cysteine and the hydrolysate on a third strip. All the radioactivity migrated as one peak to the same distance as that of the succinyl cysteine standard. However, after hydrolysis, more unlabeled ninhydrin-positive material—presumably amino acids liberated from peptides—was produced. Again the results confirmed that although pronase hydrolyzed tha labeled gluten incompletely, the fumaric acid derivatives of cysteine were totally freed.

To further confirm that the ^{14}C-fumaric acid was bonded to cysteine residues in gluten proteins, the samples were analyzed on a Beckman amino acid analyzer. The amino acid analysis of gluten isolated from ^{14}C-fumaric acid-treated dough hydrolyzed with pronase and subsequently with 3*N* *p*-toluenesulfonic acid gave a single radioactive peak (Fig. 6). The elution position corresponded to that of authentic succinyl cysteine and is essentially the same as that reported by Tkachuk and Hlynka (1963). Succinyl cysteine eluted about 3 min ahead of aspartic acid on the long column. On the short column it eluted about 1 min ahead of the acidic and neutral amino acids. Apparently no residual radioactivity stayed on either the long or the short column, because 0.2*N* sodium hydroxide strippings of the columns were devoid of activity.

The incorporation of ^{14}C-fumaric acid into the gluten proteins was also studied in the presence of certain rheologically active reagents (Table III).

As might be expected, addition of NEMI (50 ppm) drastically reduced the incorporation of fumaric acid. The reagents have similar structures and would be expected to compete for reaction sites. Surprisingly, addition of ascorbic acid (50 ppm) increased the incorporation of ^{14}C-fumaric acid by almost 25%.

Addition of potassium iodate (30 ppm) reduced the incorporation of fumaric acid by only 15%. Because potassium iodate is known to react with free —SH groups, this is further evidence that the fumaric acid does not react with free —SH groups. If both reagents were competing for the same groups, then the reduction in the incorporation of fumaric acid should be more pronounced.

Meredith and Bushuk (1962) have shown that NEMI, in addition to reacting with —SH groups, reacts with another functional group (the disulfide group) that does not react with iodate. Because iodate does not drastically reduce the incorporation of ^{14}C-fumaric acid into gluten proteins and NEMI reduces it by 75%, a logical assumption is that fumaric acid competes with NEMI for a reaction site that does not react with iodate. The common site at which NEMI and fumaric acid react may be a free radical created by the cleavage of certain disulfide linkages during dough mixing. We calculated, based on the disulfide content of flour (Schroeder and Hoseney 1978), that when 50 μCi of ^{14}C-fumaric acid was mixed with 10 g of flour for 9 min in a mixograph, 0.24% of the disulfide bonds reacted with the fumaric acid.

TABLE III
Effect of Various Additives on the Incorporation of ^{14}C-Fumaric Acid[a] in Dough

With Additive	^{14}C-Fumaric Acid in Dialyzed Gluten Protein[b] (CPM/mg)[c]	Percent of the Control
N-ethylmaleimide, 500 ppm	27.19	27.30
KIO_3, 30 ppm	84.34	84.72
Ascorbic acid, 50 ppm	117.17	117.64
Isoascorbic acid, 50 ppm	75.27	75.57
Yeast, 2%	76.14	76.45
Control	99.60	...

[a] 2.5 μCi of ^{14}C-fumaric acid per 10 g of flour was used, along with other additives listed in the first column.
[b] All values differ significantly ($P < 0.005$).
[c] Counts per minute per milligram.

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