REACTIONS OF FLOUR PROTEIN SULFHYDRYL WITH N-ETHYLMALEIMIDE AND IODATE[1]

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ABSTRACT

Chemical evidence is presented that the identity of flour sulfhydryl (–SH) is due to cysteine-containing proteins. Approximately one-half of the flour –SH reacts readily with N-ethylmaleimide (NEMI), the remainder only in the presence of 8*M* urea. Flour –SH is involved in the improver reaction, since there was no –SH available for reaction with NEMI in sufficiently mixed doughs previously treated with iodate.

With L-cysteine, NEMI forms two products or isomers, which are slowly hydrolyzed to S-succinyl-L-cysteine and ethylamine in 6*N* hydrochloric acid at 120°C., the complete hydrolysis requiring 60 hr. With GSH, NEMI forms one product plus ethylamine. The GSH-NEMI product on a 22-hr. hydrolysis yielded S-succinyl-L-cysteine, glycine, and glutamic acid. Wheat proteins treated with NEMI behaved analogously to GSH.

There has been much evidence that sulfhydryl (–SH) groups play an important role in influencing the properties of flour dough. However, evidence for the identity of the actual entity involved and the formation of the corresponding products formed has never been presented. As evidence of the latter is needed to understand eventually the fundamental structure and properties of dough, the present investigation was undertaken in an attempt to obtain some of this information. This was done with the use of N-ethylmaleimide-1-C^{14} (NEMI-1-C^{14}) as a radioactive tracer and the aid of automatic ion-exchange techniques, coupled with simultaneous and continuous radioactivity measurements. The use of radioactive NEMI-1-C^{14} was necessary in the present work because the small amounts of –SH present in flour make it difficult to follow the reactions of flour –SH by using ordinary chemical techniques.

Materials and Methods

A straight-grade, improver-free, unbleached commercial sample of flour milled from No. 2 Manitoba Northern wheat was used in this study. It had a moisture and protein content of 13.2 and 14.8%. Albumins were isolated from the flour by the method described by Pence and Elder (13). NEMI-1-C^{14} (sp. act. 6.1 μc. per mg. — lot No. 6105) and glutathione, reduced, C.P. grade, lot 6105, were obtained from Schwarz

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Bio-Research, Inc. L-Cysteine·HCl·H_2O, lot 7639, was obtained from Nutritional Biochemical Corp. Bovine serum albumin, twice crystallized, lot B6, was obtained from Pentex, Inc.

Dough. The –SH content was determined in flour-water doughs of 60% adsorption containing 1.00 g. flour and various amounts of NEMI-1-C^{14}. The doughs were hand-mixed for 3 min. in air, allowed to stand for 3 hr., made into a water slurry and dialyzed for 24 hr., and then freeze-dried. Inactive glutathione-NEMI (GSH-NEMI) carrier (~2 μm.) was added to homogeneous portions (~100 mg.) of the freeze-dried material; the mixture was hydrolyzed, and eventually analyzed for both all the ninhydrin-positive, and also the radioactive constituents, that were separated by the ion exchange columns.

Hydrolyses were carried out with 6*N* redistilled hydrochloric acid at 120°C. for 22 hr. in sealed Pyrex glass tubes previously evacuated to approximately 50 μ. Usually, 50 to 100 mg. of flour were hydrolyzed with 6 to 8 ml. of 6*N* hydrochloric acid. The hydrochloric acid was removed from the hydrolysate mixture by placing the frozen mixture into a desiccator containing sodium hydroxide pellets, and evacuating. Citrate buffer, 0.2*N* Na^+, pH 2.2, was added to the residue, and the humin removed by filtering through a fine-porosity, sintered-glass funnel. The filtrate was accurately made up to volume with the pH 2.2 buffer, and aliquots were analyzed for their amino acid content by the method of Spackman, Stein, and Moore (17), with a Beckman/Spinco Model 120 amino acid analyzer. The amino acid nitrogen recovery was 98 ± 3% when compared with the Kjeldahl nitrogen value.

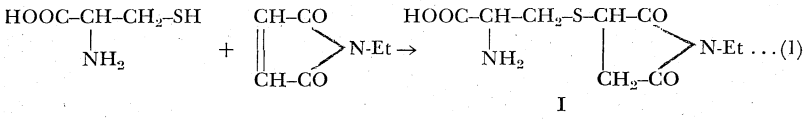
Radioactivity measurements were carried out simultaneously with amino acid analyses by continuous recording of the radioactivity of the effluents from the ion-exchange columns on the amino acid analyzer with a scintillation detector (19). Since the scintillation detector lies before the recording spectrophotometer in the amino acid analyzer in the elution scheme used, the position of the radioactive peak on the scintillation recorder lies 11 ml. before the corresponding position on the amino acid analyzer recorder. Thus in the descriptions given in the present work, 11 ml. are added to the elution positions of the radioactive peaks, as indicated on the scintillation recorder, to give values which may be compared with those indicated by the amino acid analyzer elution positions.

Results and Discussion

Reaction of NEMI with Model Sulfhydryl Compounds. Initially, the reactions of L-cysteine (CySH) and GSH with NEMI were studied at pH 5.5 (sodium acetate buffer). The ion-exchange elution pattern

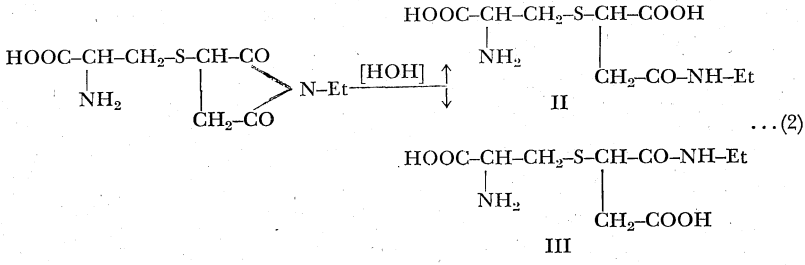
of the CySH-NEMI reaction mixture is shown in Fig. 1 A, and it is seen that two peaks are obtained at the 191- and 231-ml. elution positions of the 150-cm. column. When the CySH-NEMI reaction product was hydrolyzed by 6*N* hydrochloric acid at 120°C., chromatographic analyses showed that the two 191- and 231-ml. peaks slowly and equally disappear with the corresponding emergence of two new peaks. One of the new peaks appears at the 106-ml. elution position on the short column, as is illustrated in Fig. 1 B, and therefore should be a basic compound (17), and the other new peak appears at 102 ml. on the long column, which indicates that it should be an acidic compound (17).

These results may be explained with the aid of the following equations, in which case the two peaks may either be due to asymmetric isomers of S-(N-ethylsuccinimido)-L-cysteine (I), the latter compound being the reported structure for the reaction of NEMI with CySH (6,14):

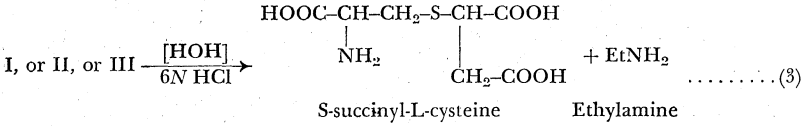


I

or to the formation of the two possible hydroxamic acids II and III resulting from the possible partial hydrolysis of I:



Any of the products illustrated in equations 1 or 2 then can on hydrolysis yield S-succinyl-L-cysteine and ethylamine, as is illustrated in equation 3:



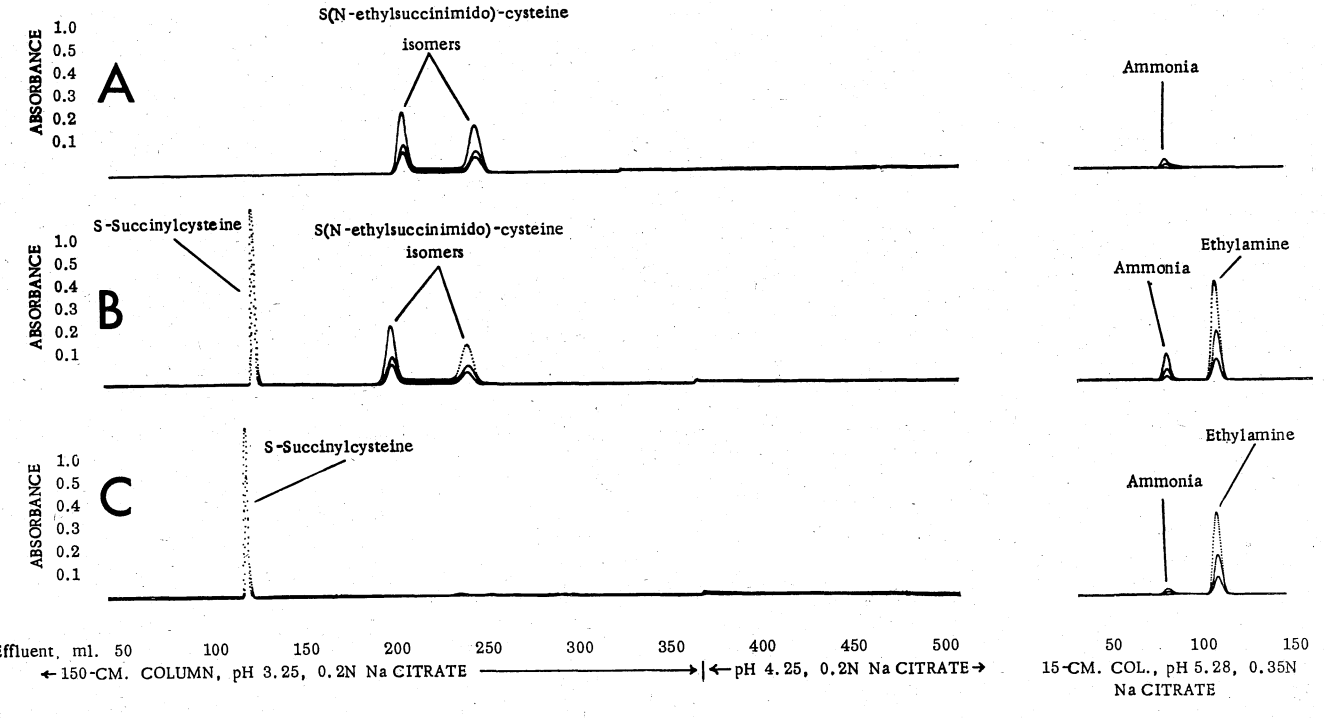
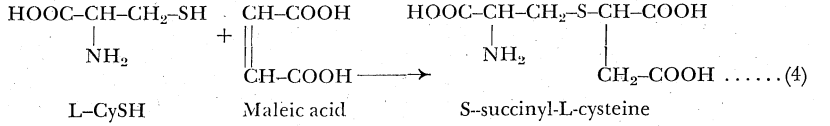


Fig. 1. Chromatographic analysis showing the absorbance at 570 and 440 mμ of the color formed between ninhydrin and: A, products obtained by reacting 2 μm. NEMI with 1.0 μm. L-cysteine. B, CySH-NEMI adduct(s) equivalent to 2.26 μm. hydrolyzed for 22 hr. C, CySH-NEMI adduct(s) equivalent to 1.0 μm. hydrolyzed for 60 hr. in 6*N* hydrochloric acid at 120°C., with the formation of S-succinyl-L-cysteine and ethylamine. The ammonia peak is essentially due to ammonia-contaminated buffers used for the chromatography and a small amount arising through decomposition of the materials during hydrolysis.

That in fact, ethylamine and S-succinyl-L-cysteine are the final hydrolysis products was verified by observing the elution behavior and the adsorption characteristics of authentic ethylamine and S-succinyl-L-cysteine on the amino acid analyzer. Authentic S-succinyl-L-cysteine was synthesized from maleic acid and CySH as described by Morgan and Friedmann (12):



(4)

It was found, as is illustrated in Fig. 1, B, that a hydrolysis period of 22 hr. at 120°C. with 6*N* hydrochloric acid, which was normally used for the hydrolysis of proteins, caused an approximate 70% hydrolysis of the two isomers, the complete hydrolysis being accomplished in 60 hr., as is illustrated in Fig. 1, C.

The reaction of NEMI with GSH at pH 5.5, however, is not analogous to that with CySH, since chromatographic analysis indicated only one peak at 120 ml. on the long column and one peak at 106 ml. on the short column of the amino acid analyzer. The single peak on the long column represented a quantitative chemical (on a molar basis) and radioactive recovery of the adduct analyzed. The peak on the short column was identified as ethylamine, since both its elution and adsorption characteristics were identical to that of authentic ethylamine. The size of the peak on the short column represented a quantitative recovery (on a molar basis) of the GSH-NEMI adduct analyzed. These findings indicate that under these conditions NEMI forms only one addition compound with GSH with a corresponding very fast, if not immediate, release of ethylamine. Hydrolysis of the glutathione-NEMI reaction solution with 6*N* hydrochloric acid at 120°C. for 22 hr. showed, when analyzed on the amino acid analyzer, that complete hydrolysis had now occurred, since S-succinyl-L-cysteine, ethylamine, glycine, and glutamic acid were found in amounts equivalent to the amount of GSH-NEMI reaction product hydrolyzed.

While the product from NEMI and CySH has previously been described as being S-(N-ethylsuccinimido)-L-cysteine (6,14), it is considered that the products may really be partially hydrolyzed derivatives of S-(N-ethylsuccinimido)-L-cysteine, as was illustrated in equation 1. One reason for postulating that the CySH-NEMI adduct(s) may not have the imide form is the good separation of the adduct product(s) on the ion exchange column, a separation which is not

obtained for any isomers of diastereomeric origin, such as has been observed for *meso*-cystine or allo forms of hydroxylysine or isoleucine (11). However, the best evidence for the partial hydrolysis of the imide ring comes from the clear demonstration of the release of ethylamine from the reaction of NEMI with GSH. The two products obtained for the reaction of CySH with NEMI in the present work cannot be a mixture of S-(N-ethylsuccinimido)-L-cysteine and the thiazane derivative, which can be obtained by exposing S-(N-ethylsuccinimide)-L-cysteine to basic conditions, as described by Smyth, Nagamatsu, and Fruton (14), since the thiazane derivative is ninhydrin-negative (14).

The reaction of NEMI-1-C^{14} was also checked with bovine serum albumin (BSA) as a model –SH protein compound. After a solution of BSA was treated with excess NEMI-1-C^{14} in water at pH 5.5, it was dialyzed and then hydrolyzed. Analysis of the hydrolysate showed the presence of only one radioactive product, S-succinyl(-1-C^{14})-L-cysteine, and the inactive constituent amino acids. The amount of S-succinyl-(-1-C^{14})-L-cysteine found corresponded to 0.21 –SH per molecule protein. While this is less than the theoretical amount of 0.68 –SH per molecule BSA, it should be noted that the highest amounts of –SH are found in BSA only when the analysis is done in the presence of denaturing reagents, and in samples of BSA from which all traces of metals have been removed (4,5).

It was noted in the work with model –SH compounds, that in analysis of solutions containing excess NEMI-1-C^{14}, additional radioactive peaks were usually obtained on the long column. These were due to the emergence of unreacted NEMI-1-C^{14} at 144 ml. in unhydrolyzed samples and to maleic-1-C^{14} acid at 77 ml. in the hydrolyzed samples.

Recoveries of S-Succinyl-L-Cysteine. To obtain a quantitative estimate of the –SH content in flour, it was necessary to know the amount of S-succinyl-L-cysteine which is decomposed during the hydrolysis of flour. This loss was estimated by hydrolyzing the adduct which NEMI-1-C^{14} forms with GSH, alone and also in the presence of flour. The products in aliquots of the original GSH-NEMI solution, and of the two hydrolysates, were then analyzed on the amino acid analyzer with simultaneous radioactivity analysis. The radioactivities measured were of the GSH-NEMI-1-C^{14} in the unhydrolyzed solution, and S-succinyl(-1-C^{14})-L-cysteine in each of the hydrolysates. The results are given in Table I, and it is observed that good recovery of S-succinyl-L-cysteine from flour results only if some inactive GSH-NEMI carrier is added to the NEMI-1-C^{14}-treated flour previous to hydrolysis. Some inactive GSH-NEMI carrier was thus added to NEMI-1-C^{14}-treated

flour prior to hydrolysis, in order to recover S-succinyl-L-cysteine more completely.

Reaction of NEMI with Dough. The analysis of NEMI-1-C^{14}-treated doughs hydrolyzed for 22 hr. revealed the presence of only one radioactive peak eluting out at 102 ml., as illustrated in Fig. 2, A and B. This peak accounted for all of the radioactivity present in the

TABLE I

RECOVERIES OF S-SUCCINYL (-C^{14})-L-CYSTEINE

COMPOUNDS ANALYZED	RELATIVE ACTIVITY	
	Without Carrier	With Carrier[a]
0.01 μm. GSH-NEMI-1-C^{14}	100%[b]	100%[b]
0.01 μm. GSH-NEMI-1-C^{14}, hydrolyzed for 22 hr. at 120°C. in 6*N* HCl	72.5[c]	95.7[c]
0.01 μm. GSH-NEMI-1-C^{14}, hydrolyzed for 22 hr. at 120°C. in 6*N* HCl, in the presence of 100 mg. flour	61.5[c]	91.2[c]

[a] 2 μm. GSH-NEMI added previous to hydrolysis.
[b] GSH-NEMI-1-C^{14} counted. All values listed are averages of at least two analyses.
[c] S-succinyl (-1-C^{14})-cysteine counted.

hydrolyzed, filtered dough sample, and its elution position corresponded exactly to that of authentic S-succinyl-L-cysteine. This peak cannot be mistaken for those corresponding to the L-cysteine-NEMI isomers which elute out at very different positions on the chromatogram at 191 and 231 ml., as illustrated in Fig. 1, A. Thus, flour behaves analogously to GSH, in that a 22-hr. hydrolysis is sufficient to liberate all of the activity present in the form of one symmetrical peak corresponding to S-succinyl-L-cysteine.

Other experiments were done, where NEMI-1-C^{14}-treated doughs were dialyzed as slurries for 3 days against approximately 10 changes of water. Analysis indicated a 4.3% loss of S-succinyl-L-cysteine in the dialyzed dough, when compared to the amount of S-succinyl-L-cysteine present in undialyzed doughs. When doughs were not dialyzed before hydrolysis, it was observed sometimes that a minor radioactive peak preceded S-succinyl-L-cysteine along with a smaller neighboring one. It was considered that the larger one of these peaks at 77 ml. was due to the presence of hydrolyzed, unreacted NEMI-1-C^{14}, which was not removed during the freeze-drying step. This view was substantiated by observation of the chromatographic behavior of NEMI-1-C^{14} hydrolyzed with 6*N* hydrochloric acid at 120°C. for 22 hr., which showed a peak at 77 ml. (probably maleic(-1-C^{14}) acid). The major peak represented 95% of the total activity chromatographed. It was interesting to note that in doughs from which excess NEMI-1-C^{14}

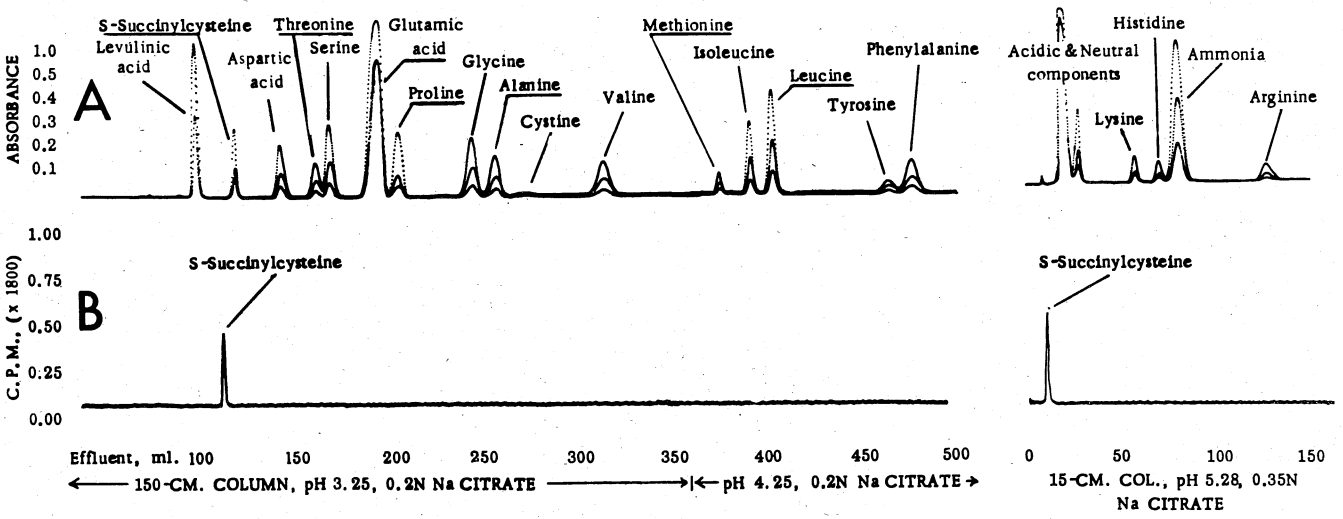


Fig. 2. A, chromatographic analysis of amino acids and S-succinyl (-1-C^{14})-L-cysteine obtained by a 22-hr. hydrolysis in 6*N* hydrochloric acid at 120°C. of NEMI-1-C^{14}-treated dough. The dough contained some inactive GSH-NEMI as carrier. The curves show the absorbance of the color formed between amino acids and related substances, and ninhydrin at 570 mμ using two different cell depths and at 440 mμ for the determination of proline. It is observed that S-succinyl (-1-C^{14})-L-cysteine is distinctly separated from the amino acids. B, radioactivity record obtained simultaneously while the curve A was being obtained, showing the presence of a single radioactive peak due to S-succinyl (-1-C^{14})-L-cysteine. The scintillation counter was operating at 29.9% efficiency for C^{14} and had a detector volume of 1.01 ml.

was not removed either by freeze-drying or dialysis, analysis showed the same recovery of S-succinyl-L-cysteine as in doughs from which the excess NEMI-1-C^{14} was removed. However, the interpretation of results obtained from analysis of doughs containing excess NEMI-1-C^{14} was slightly more complicated, because of the presence of several peaks probably due to hydrolyzed excess NEMI-1-C^{14} and also possibly those due to NEMI-1-C^{14} reaction products occurring with proteins or amino acids under the influence of 6*N* hydrochloric acid at 120°C.

A summary of some representative results on analysis of NEMI-1-C^{14}-treated doughs and proteins is given in Table II. Some experiments with doughs mixed with iodate are included also. The iodate doughs before treatment with NEMI were extensively dialyzed against deaerated water so that traces of iodate would not be present during acid hydrolysis.

TABLE II

SULFHYDRYL CONTENT OF DOUGHS

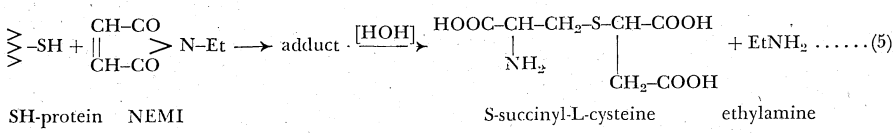
SAMPLE	–SH
	μm./g. protein
Dough (8*M* urea)	11.1[a]
Dough	4.85
Dough (dialyzed)	4.64
Dough (2-min. mix; 75.8 μeq. KIO_3 per g. protein)	2.71
Dough (3-min. mix; 7.58 μeq. KIO_3 per g. protein)	0.92
Dough (15-min. mix; 22.7 μeq. KIO_3 per g. protein)	0
Dough (15-min. mix; 22.7 μeq. KIO_3 per g. protein; then further mixed for 15 min. after addition of 13.2 μeq. NEMI per g. protein)	0
Wheat albumins	6.91

[a] Averages of at least two separate analyses. Example –SH calculation: A 9.703-mg. flour sample (1.281 mg. protein) gave on analysis a S-succinyl (-1-C^{14})-L-cysteine peak, the size of which was determined by tracing it onto bond paper and weighing the resulting excised peak. This weight was 76.07 mg. As by previously analyzing aliquots of CySH-NEMI-1-C^{14} it was found that 1 mg. weight of a similarly traced peak corresponded to 1.70×10^{-4} μm. –SH, then in the present instance, 76.07 mg. represents 0.0129 μm. –SH, which is equivalent to 10.1 μm. –SH per g. protein. Correction for the 8.8% destruction occurring during hydrolysis gives the final value of 11.1 μm. –SH per g. protein. It is recognized that the accuracy of the presented results are dependent on the accuracy of the sp. activity of the NEMI-1-C^{14} as stated by the supplier of the NEMI-1-C^{14}. While there is no simple method to check the sp. activity of a small amount of a dilute solution of the volatile NEMI-1-C^{14}, there is no reason to believe that the rated sp. activity of the NEMI-1-C^{14} is not correct, since the activity of an aliquot of a CySH-NEMI-1-C^{14} solution showed it to possess 105% of the rated activity. This CySH-NEMI-1-C^{14} solution was prepared by reacting NEMI-1-C^{14} with an excess of L-cysteine, and 93% of the NEMI-1-C^{14} had reacted to form the expected isomers. The 7% which had not reacted seemed to be due to the presence of hydrolyzed NEMI-1-C^{14}.

Concluding Remarks

Sulfhydryl-NEMI Chemistry. In the present work it has been found that NEMI reacts with CySH to form two products or isomers. The product from NEMI and CySH has previously been described as being S- (N-ethylsuccinimido)-L-cysteine (6,14). Both of these products, slowly and at equal rates, hydrolyze to give the same product, S-succinyl-L-

cysteine, the complete hydrolysis in 6*N* hydrochloric acid at 120°C. requiring 60 hr. With GSH, however, NEMI was found to give one adduct, which required only a 22-hr. hydrolysis to completely yield the same NEMI-derivative as in the case with cysteine, S-succinyl-L-cysteine. Wheat proteins were found to behave analogously to GSH, where a normal hydrolysis period of 22 hr. gave a quantitative yield of S-succinyl-L-cysteine. Thus, when NEMI is added to CySH, or a CySH-containing peptide or protein, the isolation of S-succinyl-L-cysteine from the hydrolysate mixture is a direct measure of the SH reacted:



The results obtained by reacting NEMI with various –SH compounds, as described in the previous paragraph, are consistent with most of the published views on the chemistry of the reaction of N-substituted maleimides with –SH compounds. Thus, S-succinyl-L-cysteine was separated after hydrolysis when pepsin was treated with NEMI (2); when wool was treated either with N-phenylmaleimide, or N,N′-(1,2-phenylene)-bis-maleamic acid, or N,N′-(1,3-phenylene)-bis-maleimide (10); when human or bovine serum albumin was treated with N-(4-dimethylamino-3,5-dinitrophenyl) maleimide (21). Our results are also in agreement with the report describing the loss of CySH in the presence of NEMI, which resulted in the formulation of two new products (18). These two products were attributed to NEMI derivatives of CySH, the ion-exchange elution behavior of which is in accord with the results presented in the present work.

A differing view on the chemistry of the NEMI-CySH reaction is, however, expressed in a note and two communications by Lee and Samuels (6,7,8). These authors report that the adduct formed from NEMI and CySH, (S-(N-ethylsuccinimido)-L-cysteine), is stable to hydrolysis with 6*N* hydrochloric acid at 100°–103°C., and that the same CySH-NEMI adduct is obtained from cysteine, or GSH or wheat gluten after hydrolysis.

The different hydrolysis method used by Lee and Samuels, who refluxed their samples in 6*N* hydrochloric acid for 18 hr., cannot account for the differences, since a repetition of their hydrolysis method yielded essentially the same results as reported in the present paper, and since under these conditions 50% of the CySH-NEMI

products were found to be hydrolyzed. The hydrolysis method used by Blumenfeld and Perlmann, who hydrolyzed their samples at 110°C. (instead of 120°C. as used in the present work) in a sealed, previously evacuated glass tube (2), was also repeated in this laboratory. Again, results as reported in the present paper were obtained, since the yield of S-succinyl-L-cysteine and ethylamine was approximately 61% during a hydrolysis with 6*N* hydrochloric acid for 24 hr. at 110°C.

NEMI-1-C^{14} Analytical Method for –SH. It is believed that the present method of –SH analysis is capable of giving reliable, accurate, and precise results. This is possible even in analysis of the –SH content of materials containing a low –SH content of 1.0×10^{-5} μm. –SH per mg. protein. Even lower –SH content could easily be measured if NEMI of a higher specific activity could be obtained than that used in the present work. The present method of analysis, however, is not suitable for work involving numerous routine measurements, because of the lengthy and detailed procedures that are necessary.

Dough Sulfhydryl. The recovery of S-succinyl-L-cysteine as the sole undialyzable radioactive product which is formed between flour and NEMI indicates that the source of flour sulfhydryl is essentially that due to CySH-containing proteins. Support for this view was the observation that S-succinyl-L-cysteine was the sole radioactive product from NEMI-treated flour albumins, the albumins being a good representation of a flour protein, as they had a protein content of 94.6%.

The reaction of approximately 4.9 μeq. –SH per g. flour protein with NEMI alone appears to be the reaction of an accessible type of flour –SH. The amount of accessible (or reactive) –SH in flour may well be the result of "exposure" of –SH due to denaturation of flour proteins by mixing, since the results in Table II indicate that it is the extent of mixing and not the amount of iodate present which determines the amount of –SH that is left for reaction with NEMI. Further support for this view was the analysis of 11.1 μeq. –SH per g. protein when the flour was dispersed in the denaturant 8*M* urea. These results are in agreement with the findings that the largest amount of iodate is reduced in ordinarily mixed doughs dispersed in 2*M* or 3*M* guanidine (3), and the use of denaturants like urea, guanidine, or detergents in order to obtain the highest values for flour –SH whenever amperometric analytical techniques are used (1,15,20).

It is interesting to compare the total flour –SH content as obtained by various analytical techniques. These are illustrated in Table III, and it is seen that the NEMI-1-C^{14} method (8*M* urea) gives virtually the same result as does the accessible iodate method in the instance

where the dough mixing was prolonged to 15 min. (3). The same flour was used in both of these studies. The accessible iodate method when used with doughs dispersed in 2*M* or 3*M* guanidine also gives comparable values with the NEMI-1-C^{14} method, but here, however, values are compared for very similar but not identical samples of flour. It is observed that values obtained by the amperometric method vary a bit more. Strangely enough, an amperometric result of the identical flour used in the present study gave the most differing result (20).

TABLE III
TOTAL SULFHYDRYL CONTENT OF HARD RED SPRING FLOUR BY VARIOUS METHODS

METHOD	–SH
	μeq./g. protein
NEMI-1-C^{14} (8*M* urea)	11.1[a]
Accessible iodate, prolonged mixing (3)	10.8[a]
Accessible iodate, 2*M* or 3*M* guanidine (3)	9.4[b]
Amperometric (Tsen and Hlynka, ref. 20)	7.16[a]
Amperometric (Sokol, Mecham, and Pence, ref. 15)	8.1[c]
Amperometric (Matsumoto and Hlynka, ref. 9)	13.8[c]

[a] Identical flour.
[b] Flour very similar to that indicated by footnote a.
[c] Different lots of flour.

The present, and other published work (1,3,16,20) illustrates that the amount of flour –SH which reacts is very dependent on the experimental conditions used. This same type of reactivity then must exist in doughs used for breadmaking, where the amount of –SH reacted would depend on the amount and kind of improver used, duration and type of mixing, physical nature of both the flour and dough, amount of air and salts incorporated, etc. Since the reactive –SH content of flour may well be an important characteristic in assessing the properties of quality of a particular flour, it is thus obvious that in this assessment one will be able to define that reactive portion out of the total –SH content only after some difficulty. Analogously, the same argument could apply to the disulfide (S–S) content of flour, since there is now some indirect evidence that –SH/S–S interchange plays an important role in dough properties. Thus, perhaps before one will be able to relate some properties of flour to the amount of –SH and S–S content present, a careful basic study of the flour –SH and S–S properties will be necessary.

Acknowledgment

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