# SOME STUDIES WITH MALEIMIDE-TYPE SULFHYDRYL-BLOCKING AGENTS[1]

C. C. LEE AND L. M. REYNOLDS[2]

## ABSTRACT

Maleimide (MI), N-ethylmalemide (NEMI), and N-phenylmaleimide (NPMI) all react with L-cysteine to give the expected adducts. Similar effects are exerted by each of these three reagents on mixogram characteristics, on gassing power of yeast, or on loaf volume. Such similarities indicate that sulfhydryl groups must be involved in the mixing of dough and the baking of bread. NPMI exerts its sulfhydryl-blocking influence less effectively than MI or NEMI. The possible relationships between the over-all size of the reagent and its effectiveness in approaching and reacting with sulfhydryls of macromolecules such as proteins are discussed.

The use of N-ethylmaleimide (NEMI) as a sulfhydryl-blocking agent in flour and dough systems has received considerable attention in recent years (2,3,4,10,11,12,14,15). Other maleimides have not been investigated by the cereal chemist, although it is known that maleimide (MI) itself as well as N-phenylmaleimide (NPMI) can react with thiols (5,9). Some comparative studies on the effects of MI, NEMI, and NPMI on systems related to cereal chemistry may, therefore, be of interest.

## Materials and Methods

*Sulfhydryl-Blocking Agents.* MI and NEMI were obtained commercially. NPMI was prepared from N-phenylmaleamic acid according to the method of Marrian (9).

*Reaction of Sulfhydryl-Blocking Agents with L-Cysteine.* Although NEMI has been used as a sulfhydryl-blocking reagent since 1949, the reaction product between NEMI and L-cysteine was only recently isolated and characterized as S-(N-ethylsuccinimido)-L-cysteine (8,13). The addition products between MI and NPMI with L-cysteine have not been reported. These adducts were therefore synthesized, using the procedure described by Lee and Samuels (8) for the preparation of the NEMI-cysteine adduct.

*Flour.* A single, thoroughly mixed batch of commercial "Baker's Grade" flour milled from Western Canadian hard red spring wheat was used in all the experiments. It had not been subjected to bleach-

[1]Manuscript received March 9, 1962. Contribution from Department of Chemistry, University of Saskatchewan, Saskatoon, Sask.
[2]Holder of the Consolidated Mining and Smelting Company of Canada Fellowship at the University of Saskatchewan, 1959–1961.

ing or improver treatments. Its crude protein and ash contents (14% moisture basis) were 14.8 and 0.46%, respectively.

*Mixograph Studies.* In view of the reported pronounced modification of dough mixing behavior by NEMI (11,12), a study on the effects of MI, NEMI, and NPMI on mixogram characteristics was carried out. A Swanson-Working recording dough mixer was used. Thirty-five grams of flour were mixed with 3.5 ml. of 25% ethanol containing the appropriate amounts of MI, NEMI, or NPMI (NPMI is insoluble in water), together with enough water to give the correct absorption. The levels of sulfhydryl-blocking agents employed ranged from zero to 0.7 $\mu$mols. per g. of flour. Each curve was automatically recorded over a period of 440 seconds.

*Gassing Power of Yeast.* The production of gas by yeast is, of course, intimately involved in breadmaking. In conjunction with a study on the effects of MI, NEMI, and NPMI on the loaf volumes of bread, the effects of these reagents on the gassing power of yeast were investigated. The pressuremeter procedure described in *Cereal Laboratory Methods* (1) was used. Because the sulfhydryl groups of flour might interfere with the effects of MI, NEMI, and NPMI on the yeast, ordinary sucrose instead of flour was employed as the substrate. The gas pressures from mixtures of 3.0 g. yeast, 10.0 g. sucrose, and 10.0 ml. of 25% ethanol containing 0–100 $\mu$mols. of MI, NEMI, or NPMI were read at intervals of 2 and 3 hours. In these experiments, the amounts of yeast and sulfhydryl-blocking agents were the same as those used in the baking studies described below.

*Baking Studies.* Loaves of bread were baked using the following formula: 100 g. flour, 3.0 g. yeast, 5.0 g. sucrose, 1.8 g. sodium chloride, 3.0 g. shortening, 4.0 g. nonfat dry milk, 0.3 g. nondiastatic malt, 0.1 g. ammonium dihydrogen phosphate, water or 25% ethanol containing 0–120 $\mu$mols. of MI, NEMI, or NPMI, plus additional water to give the correct absorption of 60.5%.

## Results

*Adducts from Reaction of MI and NPMI with L-cysteine.* From MI and L-cysteine, S-succinimido-L-cysteine, m.p. 191°–193°C., decomp., was obtained in 83% yield. It gave a positive ninhydrin reaction and a negative nitroprusside test.

Anal. calcd. for $C_7H_{10}O_4N_2S$: C, 38.53; H, 4.59; N, 12.84; S, 14.68. Found: C, 38.38; H, 4.61; N, 12.63; S, 14.84.

From NPMI and L-cysteine, S-(N-phenylsuccinimido)-L-cysteine, m.p. 182°–183°C., decomp., was obtained in 75% yield. It also gave a positive ninhydrin reaction and a negative nitroprusside test. This adduct was very hydroscopic. A great deal of care was taken to exclude moisture during its analysis.

Anal. calcd. for $C_{13}H_{14}O_4N_2S$: C, 53.06; H, 4.76; N, 9.52; S, 10.88. Found: C, 52.71; H, 5.00; N, 9.33; S, 10.92.

As mentioned previously, the characterization and analysis of the adduct from NEMI and L-cysteine have already been reported (8,13).

*Effects on Mixogram, Gassing Power, and Loaf Volume.* The effects of different concentrations of MI, NEMI, or NPMI on the mixograms are shown in Figs. 1, 2, and 3. Some characteristics measured from these mixing curves are given in Table I.

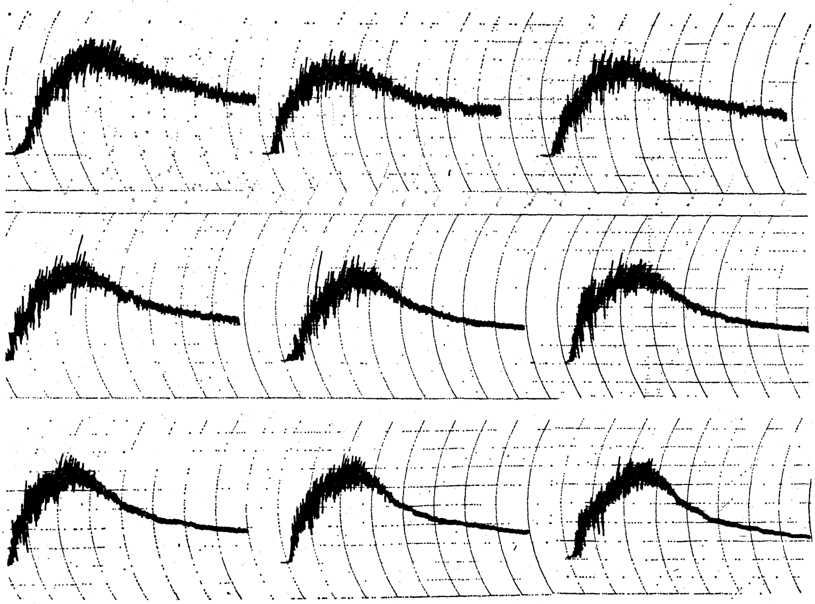

Fig. 1. Mixograms showing effects of various amounts of maleimide. Curves from left to right for first row, second row, then third row represent blank (35.0 g. flour and 21.2 ml. water) and MI concentrations of 0, 10, 20, 30, 35, 40, 50, 70 $\mu$mols. per 100 g. flour in 3.5 ml. of 25% ethanol replacing 3.5 ml. of the water in the blank. Each division of the abscissa corresponds to a mixing time of 55 seconds.

Any one of the three sulfhydryl-blocking agents studied can depress gas production by yeast acting on sucrose. A set of typical results is shown graphically in Fig. 4.

Typical results from studies on the effects of various concentrations of MI, NEMI, or NPMI on loaf volume are given in Table II and Fig. 5.

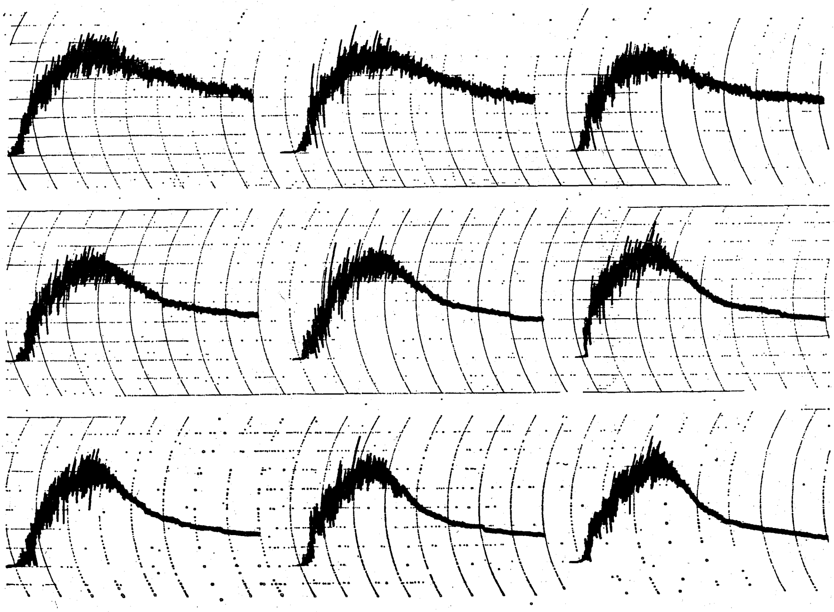

Fig. 2. Mixograms showing effects of various amounts of N-ethylmaleimide. Curves from left to right for first row, second row, then third row represent blank (35.0 g. flour and 21.2 ml. water) and NEMI concentrations of 0, 10, 20, 30, 35, 40, 50, 70 $\mu$mols. per 100 g. flour in 3.5 ml. of 25% ethanol replacing 3.5 ml. of the water in the blank. Each division of the abscissa corresponds to a mixing time of 55 seconds.

TABLE I
MIXOGRAM MEASUREMENTS

| Blocking Agent Added | Time to Maximum | | | Maximum | | | 220 Seconds after Maximum | | |
|---|---|---|---|---|---|---|---|---|---|
| | MI | NEMI | NPMI | MI | NEMI | NPMI | MI | NEMI | NPMI |
| *µmols/100 g flour* | *sec* | *sec* | *sec* | *mm* | *mm* | *mm* | *mm* | *mm* | *mm* |
| Blank | 149 | 154 | 151 | 52 | 53 | 52 | 32 | 34 | 35 |
| 0 | 143 | 154 | 143 | 46 | 49 | 46 | 26 | 30 | 30 |
| 10 | 143 | 138 | 146 | 45 | 47 | 44 | 25 | 28 | 28 |
| 20 | 146 | 149 | 157 | 46 | 48 | 46 | 25 | 25 | 28 |
| 30 | 140 | 151 | 146 | 46 | 50 | 47 | 21 | 23 | 28 |
| 35 | 140 | 135 | 151 | 46 | 54 | 50 | 20 | 22 | 27 |
| 40 | 135 | 143 | 143 | 48 | 50 | 50 | 20 | 20 | 27 |
| 50 | 129 | 138 | 138 | 48 | 50 | 49 | 19 | 17 | 25 |
| 70 | 138 | 138 | 143 | 47 | 50 | 48 | 15 | 15 | 19 |

## Discussion

*Effects on Mixogram Characteristics.* All three maleimide-type reagents, MI, NEMI, and NPMI, exert similar effects on the mixograph curves. In agreement with the reported influence of NEMI

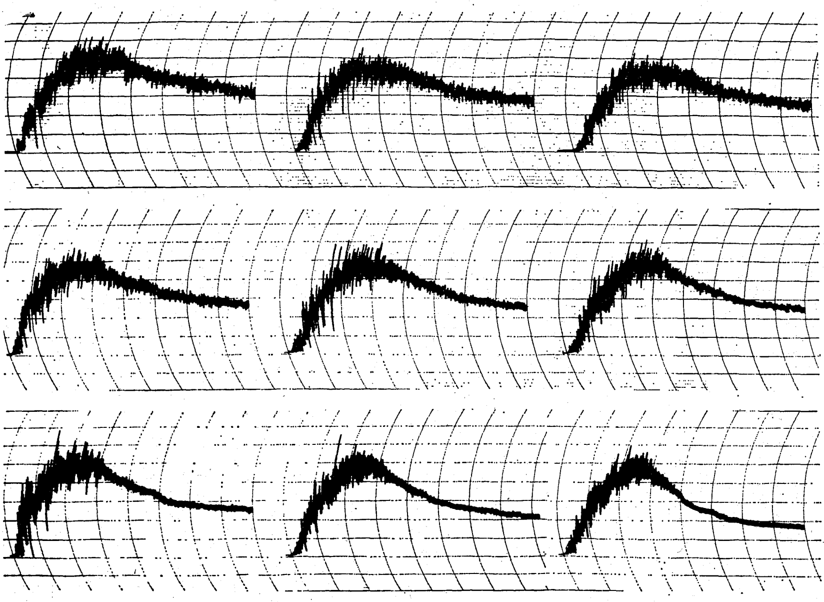

Fig. 3. Mixograms showing effects of various amounts of N-phenylmaleimide. Curves from left to right for first row, second row, then third row represent blank (35.0 g. flour and 21.2 ml. water) and NPMI concentrations of 0, 10, 20, 30, 35, 40, 50, 70 $\mu$mols. per 100 g. flour in 3.5 ml. of 25% ethanol replacing 3.5 ml. of the water in the blank. Each division of the abscissa corresponds to a mixing time of 55 seconds.

on mixing behavior (11,12), the observed trend shows that with increasing amounts of the blocking agents, the mixing time required to reach maximum resistance decreases and the rate of breakdown beyond the maximum increases (Table I). Of the three reagents, MI and NEMI are roughly equal in effectiveness in causing these changes; NPMI is somewhat less efficient.

The characteristic narrow band beyond the maximum, first reported by Mecham, Sokol, and Pence (12), is observed when sufficient amounts of any one of the three reagents are present. Close inspection of Figs. 1, 2, and 3 indicates that this narrow band appears to be definitely established at a concentration of about 30 $\mu$mols. per 100 g. flour for MI and NEMI. With NPMI, this effect again seems to be more gradual, the narrow band being definitely established at a concentration of about 40 $\mu$mols. per 100 g. flour.

The accessible sulfhydryl content of the flour used in the present experiments, as determined by the iodate oxidation method (2), is 80 $\mu$mols. per 100 g. flour. It is of interest to note that a relatively small amount of about 30 $\mu$mols. per 100 g. flour of sulfhydryl-blocking

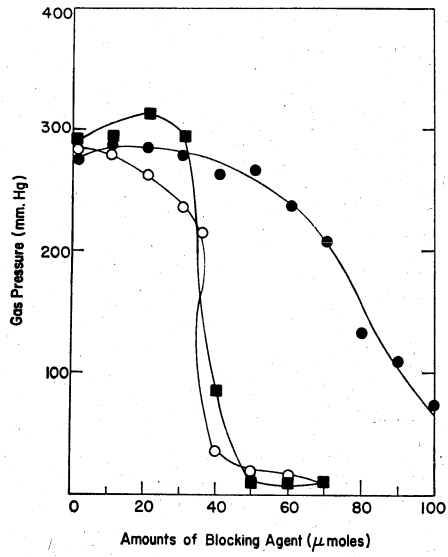


Fig. 4. Effects of sulfhydryl-blocking agents on gassing power of yeast on sugar. Pressure readings taken at 3 hours. Open circles, maleimide; closed squares, N-ethylmaleimide; closed circles, N-phenylmaleimide.

TABLE II

EFFECTS OF SULFHYDRYL-BLOCKING AGENTS ON LOAF VOLUMES

| BLOCKING AGENT ADDED | LOAF VOLUME[a] | | | | |
|---|---|---|---|---|---|
| | MI | | NEMI | | NPMI |
| | In Water | In 25% Ethanol[b] | In Water | In 25% Ethanol[b] | In 25% Ethanol[b] |
| *μmols/100 g flour* | *ml* | *ml* | *ml* | *ml* | *ml* |
| 0 | 840 | 775 | 838 | 780 | 770 |
| 10 | 855 | 765 | 850 | 790 | 775 |
| 20 | 870 | 815 | 855 | 788 | 790 |
| 30 | 905 | 785 | 838 | 708 | 755 |
| 35 | | | | 640 | 750 |
| 40 | 900 | 785 | 640 | 600 | 745 |
| 50 | 895 | 735 | 460 | 450 | 698 |
| 60 | 870 | 700 | 400 | 380 | 673 |
| 70 | 820 | 620 | 350 | 350 | 610 |
| 80 | 715 | 500 | 345 | 335 | 525 |
| 90 | 515 | 440 | 365 | 350 | 490 |
| 100 | 420 | 400 | 360 | 340 | 480 |
| 120 | 350 | 300[c] | | | 380[c] |
| 140 | 250 | 200[d] | | | 330[d] |

[a] Each value is the average of duplicate loaves.
[b] For the experiments with 0–100 μmols. of blocking agent per 100 g. flour, 10 ml. of 25% ethanol containing the appropriate amount of blocking agent were added in each case.
[c] The blocking agent was dissolved in 12 ml. of 25% ethanol.
[d] The blocking agent was dissolved in 14 ml. of 25% ethanol.

agent is sufficient to cause the characteristic narrowing of the breakdown portion of the mixograph curves. In view of the finding of

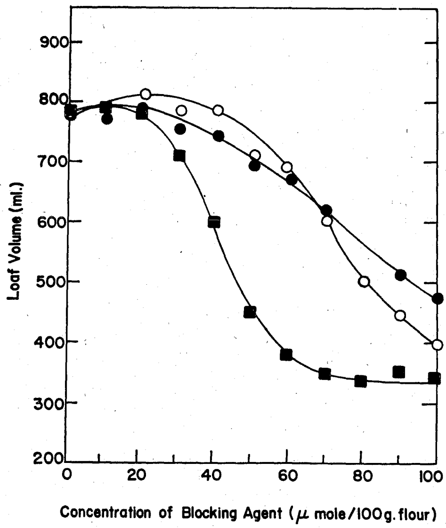


Fig. 5. Effects of sulfhydryl-blocking agents on loaf volume. Open circles, maleimide; closed squares, N-ethylmaleimide; closed circles, N-phenylmaleimide.

Mecham and co-workers (12) that the action of NEMI on the less soluble gluten residue containing only a fraction of the total sulfhydryl groups is responsible for the changes in dough-mixing behavior, it is not surprising that the amount of blocking agent needed to cause these changes in the mixing curves is much less than an equivalent of all accessible sulfhydryl groups in the entire flour.

*Effects on Gassing Power of Yeast.* The three sulfhydryl-blocking agents, MI, NEMI, and NPMI, all exhibit an inhibiting effect on gas production by yeast acting on sucrose. This effect probably may be a result of the blocking of sulfhydryl groups of the enzyme systems in the yeast. As observed in the studies on the mixograms, NPMI exerts its influence more gradually than MI or NEMI. Under the conditions of the present experiments, with 3.0 g. of yeast and 10.0 g. of sucrose at 30°C., 50 μmols. of MI or NEMI are sufficient to inhibit gas production completely.

*Effects on Loaf Volume.* The results shown in Table II and Fig. 5 indicate once again that all three maleimide-type reagents exert a similar influence. With increasing concentrations of sulfhydryl-blocking agent, there is initially a very small rise followed by more pronounced decreases in loaf volume. This type of behavior was also observed by Sullivan, Dahle, and Nelson (15) in their baking studies with *p*-chloromercuribenzoate.

Considering the increased rate of breakdown during mixing and flour sulfhydryl groups, thus leaving behind a lesser amount of MI to deactivate the yeast. Gas production can, therefore, continue even though the initial amount of MI added is greater than 50 $\mu$mols., the quantity required to deactivate completely the yeast in the absence of flour. The net result in the baking test with added MI would then be a gradual decrease in loaf volume with increasing MI concentration, because the yeast is not completely deactivated even at relatively high initial concentrations of MI.

**Acknowledgment**

The authors wish to thank W. Bushuk, Grain Research Laboratory, Winnipeg, Manitoba, for measuring the accessible sulfhydryl content of the flour. The financial support given by the National Research Council of Canada and by the Saskatchewan Agricultural Research Foundation is gratefully acknowledged.

**Literature Cited**

1. American Association of Cereal Chemists. Cereal laboratory methods (6th ed.). The Association: St. Paul, Minnesota (1957).
2. Bushuk, W. Accessible sulfhydryl groups in dough. Cereal Chem. **38:** 438–448 (1961).
3. Bushuk, W., and Hlynka, I. The bromate reaction in dough. II. Inhibition and activation studies. Cereal Chem. **37:** 343–351 (1960).
4. Frater, R., Hird, F. J. R., Moss, H. J., and Yates, J. R. A role for thiol and disulphide groups in determining the rheological properties of dough made from wheaten flour. Nature **186:** 451–454 (1960).
5. Friedmann, E., Marrian, D. H., and Simon-Reuss, I. Antimitotic action of maleimide and related substances. Brit. J. Pharmacol. **4:** 105–108 (1949).
6. Godfrey, J. C. Accurate molecular models. J. Chem. Educ. **36:** 140–143 (1959).
7. Goldstein, S. Sulfhydryl- und Disulfidgruppen der Klebereiweisse und ihre Beziehung zur Backfähigkeit der Brotmehle. Mitt. Lebensm. Hyg. Bern **48:** 87–93 (1957).
8. Lee, C. C., and Samuels, E. R. Adducts from the reaction of N-ethylmalemide with L-cysteine and with glutathione. Can. J. Chem. **39:** 1152–1154 (1961).
9. Marrian, D. H. The reaction of substituted maleimides with thiols. J. Chem. Soc. **1949:** 1515–1516.
10. Matsumoto, H., and Hlynka, I. Some aspects of the sulfhydryl-disulfide system in flour and dough. Cereal Chem. **36:** 513–521 (1959).
11. Mecham, D. K. Effects of sulfhydryl-blocking reagents on the mixing characteristics of doughs. Cereal Chem. **36:** 134–145 (1959).
12. Mecham, D. K., Sokol, H. A., and Pence, J. W. The dough-mixing behavior of gluten and other flour fractions treated with N-ethylmalemide. Cereal Chem. **37:** 129–141 (1960).
13. Smyth, D. G., Nagamatsu, A., and Fruton, J. S. Some reactions of N-ethylmaleimide. J. Am. Chem. Soc. **82:** 4600–4604 (1960).
14. Sokol, H. A., Mecham, D. K., and Pence, J. W. Observations on the reactivity of sulfhydryl groups in wheat flour. Cereal Chem. **37:** 151–158 (1960).
15. Sullivan, Betty, Dahle, L., and Nelson, O. R. The oxidation of wheat flour. II. Effect of sulfhydryl-blocking agents. Cereal Chem. **38:** 281–291 (1961).