KINETICS OF THE REACTION OF N-ETHYLMALEMIDE WITH CYSTEINE AND SOME CONGENERS

By

PETAR A. MARTIĆ

Bachelor of Science

University of Belgrade

Belgrade, Yugoslavia

1962

Submitted to the Faculty of the Graduate School of
the Oklahoma State University
in partial fulfillment of the requirements
for the degree of
MASTER OF SCIENCE
May, 1966

KINETICS OF THE REACTION OF N-ETHYLMALEIMIDE WITH CYSTEINE AND SOME CONGENERS

Thesis Approved:

George Gorin
Thesis Adviser

Ernest M. Hodnett

J. A. Boggs
Dean of the Graduate School

ACKNOWLEDGMENTS (1)

I want to express my gratitude to Professor George Gorin for his advice and supervision throughout my studies. I also want to thank the National Institutes of Health for financial help and support in the form of a research assistantship.

My gratitude is extended to my colleague Mr. Gavin Doughty who performed the least-square analysis with an IBM 1410 computer.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER I

INTRODUCTION

The present thesis deals with the kinetics of the reaction of N-ethylmaleimide (NEM) with cysteine and with some other mercaptans. The reaction of NEM with mercapto groups in proteins is of interest because the chemical modification of these groups can greatly alter the behavior of the protein. This reaction was first observed by Friedman, Marrian, and Simon-Reuss (2) in 1949. These investigators studied the reaction with glutathione spectrophotometrically by measuring the rate of disappearance of the NEM peak in the region of 205 - 240 mμ. In 1955, Gregory (3) again called attention to this reaction. Since NEM has a spectrum in the UV with a peak at 300 mμ, which disappears upon reaction with mercaptans, it should be possible to employ this reagent for the determination of mercapto groups. He also pointed out an important limitation; NEM is not stable in alkaline media because the imide linkage is hydrolysed. The utility of NEM for quantitative determination of cysteine and glutathione was demonstrated in 1958 by Roberts and Rouser (4) and by Alexander (5).

It was natural that reaction with NEM should also be applied to the determination of mercapto groups in proteins. The first such application seems to have been made by Tsao and Bailey in 1953 (6); mercapto groups were estimated by measuring the disappearance of the nitroprusside color upon titration with NEM. A bibliography of the applications of NEM is

presented in Chapter II.

The reaction of NEM with mercaptans is an addition to the double bond and may be usefully compared with other reactions of the same type. Some pertinent literature reports are discussed in Chapter II. Chapter III presents the results of the present work, written in a manner suitable for publication in a journal. Because of the limitations applied by journals, many details have been omitted from this chapter; such details are presented in Chapter IV.

CHAPTER II

REVIEW OF PERTINENT LITERATURE

The subject of this chapter is subdivided into several sections. First, the role of mercapto groups in proteins and enzymes is considered; this section does not contain a comprehensive literature survey but serves to provide some background material. Secondly, a bibliography of the reactions of NEM with mercaptans and proteins is given. Thirdly, the literature relating to the addition of mercaptans to double bonds is reviewed.

Mercapto Groups in Proteins and Enzymes

The first comprehensive study on the role of mercapto groups in the biological activity of enzymes was made by Barron and Singer in 1943 (7). They treated various enzymes with iodoacetamide, chloromercuribenzoic acid and several organic mercurials, and this resulted in the loss of catalytic activity. The activity could usually be recovered after reaction with glutathione; this suggested the idea that mercapto groups are essential for the enzyme activity.

A great deal of work on this problem has been done since that time. A result which emerged from this work is that there are two kinds of mercapto groups in proteins, reactive and unreactive. Reactive mercapto groups react with ordinary mercaptan reagents when the proteins are in their native state. In the early work nitroprusside was often employed

for detection of the mercapto groups. More recently N-ethylmaleimide has come to be widely used. Unreactive mercapto groups can be detected only after the protein has been denatured.

Owing to this difference in reactivity, enzymes do not show the same behavior towards -SH group reagents. Some of the enzymes for carbohydrate metabolism (such as pyruvate oxidase and myosin or adenosinetriphosphatase), for fat metabolism, and for alcohol oxidation - to mention a few - have reactive -SH groups and are inhibited by -SH reagents. Other enzymes are not affected by these reagents. Aldolase is an example containing both kinds of mercapto groups. Swenson and Boyer (8) studied aldolase and found that it contains 29 mercapto groups; some of them react very fast; others react slower; and some do not react at all. The unreactive groups are often called "sluggish" or "masked" because they may be hidden in the molecule. It is important to mention that the groups are divided on a purely experimental basis and there are no sharp distinction between them.

There are a number of hypotheses concerning the reactivity of mercapto groups (9). One possibility is that the -SH groups may be hydrogen-bonded, which would diminish their reactivity; or, they may be shielded by charged groups - such as a carboxyl - close to them. Evidence for this neighboring group effect will be provided by the rates of reaction of N-ethylmaleimide with cysteine and 2-aminoethanethiol, reported in Chapter III.

There is much evidence that the tertiary structure of proteins - which is the result of hydrogen bonding between the peptide groups and of the "hydrophobic" interactions between the chains of the amino acids - influences the reactivity of mercapto groups. As mentioned above, the

unreactive -SH groups become detectable after denaturation, which is a change in configuration of the protein. There are several ways in which denaturation can be brought about. One may heat the protein, in which case the hydrogen bonds between the peptide chains are broken. Also a number of reagents, such as urea, some guanidinium salts, detergents, etc., can cause a change in the structure of the native protein. Probably, the urea can form hydrogen bonds with the peptide linkages and therefore compete with the hydrogen bonds originally present within the protein.

One of the characteristics of the mercapto groups in proteins is their reducing nature. They may be detected by reaction with some oxidizing agent, for example, the dye porphyrindin, which undergoes a color change on reduction. The biological activity of mercapto enzymes may therefore be regulated by oxidation-reduction reactions. There are indications that mercaptan-disulfide interconversion is important in maintaining the proper intracellular oxidation-reduction potential (10). More experiments are necessary to confirm this idea.

Bibliography of applications of the reactions between N-ethylmaleimide (NEM) and mercapto groups in proteins and peptides

For the purpose of this chemical literature survey, the indices to Chemical Abstracts were consulted. The articles are arranged according to year of publication and cover the five year period starting from 1964 and including 1960. Two articles which had not been indexed by Chemical Abstracts have been found by cross references; they are denoted by an asterisk (*). For the articles appearing before 1960 one can consult reviews by Boyer (11) and by Cecil (9).

1964

CA, 61, 10933g: Effect of NEM on the starch-liquefying enzyme from soy flour; Pomeranz, Y., and Mamaril, F. P., Nature 203, 863 (1964)--Incubation with NEM affects the starch-liquefying activity of β-amylase from soy-bean seeds; there is no effect of α-amylase. This confirms the presence of essential SH groups in β-amylase.

6205f: NEM inhibition of horseshoe crab hemocyte agglutination; Bryan, F. T., et al., Science 144, 1147 (1964)--The inhibitory action of NEM is neutralized by adding cysteine, indicating involvement of SH groups.

60, 13482h: The characterization of modified human hemoglobin; Guidotti, G., and Konisberg, W., J. Biol. Chem. 239, 1474 (1964)--NEM reacts with the SH groups of carbon monoxide-hemoglobin. It may also react to some extent with the NH_2 terminal group of the α-chain.

8643b: ^{14}C-labeled NEM for the estimation of sulfhydryl groups; Lee, C. C., and Samuels, E. R., Can. J. Chem. 42, 164 (1964)--N-ethylmaleimide-1-^{14}C is employed for the estimation of SH groups.

7078e: The enzymatic properties of NEM modified myosin; Sekine, T., and Kielley, W. W., Biochim. Biophys. Acta 81, 336 (1964)--Comparison is made of the catalytic properties of native myosin in which one SH group per subunit chain is blocked by NEM.

6920e: The kinetics of reaction between cysteine and some maleimides; Lee, C. C., and Samuels, E. R., Can. J. Chem. 42, 168 (1964)--Reaction rates are determined in aqueous and ethanolic solution at pH 1.0 to 3.4.

3221c: Alloxan. III. Effect on biosynthesis of uridine nucleotides from uracil; Younathan, E. S., Stone, J. E., and Harris, T. S., J. Biol. Chem. 239, 290 (1964)--NEM inhibits the formation of uridine triphosphate from uracil-2-^{14}C.

1963

CA 60, 12301f: Effect of adenosinetriphosphate (ATP) on the binding of NEM to SH groups in the active site of myosin ATPase; Sekine, T., and Yamaguchi, M., J. Biochem. (Tokyo) 54, 196 (1963)--In the presence of ATP, the Ca activated ATPase is almost completely inhibited after incubated with NEM.

9842c: Structure and activity of molluscicides; phenacyl halides, a group of potentially useful molluscicides; Villiers de, J. P., and MacKenzie, J. G., Bull. World Health Organ. 29, 424 (1963)--NEM influences the activity of molluscicides.

8553h: Reactions of flour protein sulfhydryl with NEM and iodate; Tkachuk, R., and Hlynka, I., Cereal Chem. 40, 704 (1963)--NEM is used to determine SH groups in flour.

8372g: The role of active cellular sulfhydryl groups in virus reproduction; Zemla, J., Life Sci. 2, 579 (1963)--NEM inhibits the growth of type 2 poliovirus and tick-borne encephalitis virus in trypsinized chick embryo cells.

6969f: The supercontraction of chemically modified wool fibers in 9 M LiCl at 94°; Weigmann, H. D., and Rebenfeld, L., Textile Res. J. 33, 985 (1963)--The supercontraction of wool fibers is influenced by SH and SS groups.

6082b: Inhibition of the glucose permeability of human erythrocytes by NEM; Dawson, E. C., and Widdas, W. F., J. Physiol. (London) 168, 644 (1963)--Different reactivity of SH groups is demonstrated.

4562g: The dissociation of normal adult human hemoglobin; Guidotti, G., Konisberg, W., and Craig, L. C., Proc. Natl. Acad. Sci. U.S. 50, 774 (1963)--NEM reacts with carbon monoxide-hemoglobin. The presence of two SH groups is indicated.

3418b: The oxidation of wheat flour. IV. Labile and nonlabile sulfhydryl groups; Sullivan, B., Dahle, L. K., and Schipka, J. H., Cereal Chem. 40, 515 (1963)--The loss of SH groups in doughs is influenced by different concentrations of NEM.

3254d: Effect of chemical modifiers on inactivation and mutation induction by γ-radiation in Escherichia coli; Bridges, B.A., J. Gen. Microbiol. 31, 405 (1963)--Inactivating and mutation-inducing actions of radiations are affected by NEM, which acts as a sensitizing agent.

385f: A note on the rates of reaction of cysteine with certain maleimides; Lee, C. C., and Samuels, E. R., Cereal Chem. 40, 563 (1963)--Reaction rates in ethanolic and aqueous solutions at low pH are compared.

59, 14275g: The radiation-sensitizing effects of NEM and iodoacetic acid on a radiation resistant micrococcus; Lee, J. S., Anderson, A. W., and Elliker, P. R., Radiation Res. 19, 593 (1963)--NEM shows protective characteristics whereas iodoacetic acid does not.

14249f: Sulfhydryl groups in the reactivation of heat-inactivated milk alkaline phosphatase; Kresheck, G. C., Acta Chem. Scand. 17, Suppl. 1, S295 (1963)--SH-containing proteins promote reactivation of milk alkaline phosphatase, NEM inhibits the reaction.

10360h: The solubility of hemoglobin treated with NEM; Sheenan, W. W., Taketa, F., and Morell, S. A., Marquette Med. Rev. 29, 41 (1963)--NEM affects deoxygenation or oxygenation of hemoglobin. Possible mechanisms for the different uptake of NEM are reviewed.

7806a: Effects of NEM and insulin upon adipose tissue; Ott, F. T., and Recant, L., Proc. Soc. Exptl. Biol. Med. 112, 880 (1963)--NEM inhibits the response of adipose tissue of rat epididymal fat pad to insulin in vitro. The inhibitory mechanism is not competitive.

6860g: Effect of pretreatment of pancreas slices with NEM on insulin secretion in vitro; R-Candela, J. L., Martin-Hernandez, D., and Castilla-Cortazar, T., Proc. Soc. Exptl. Biol. Med. 112, 898 (1963)--Insulin secretion is diminished after incubation with NEM.

6860e: Inhibition of insulin-binding to tissues by p-hydroxy-mercuribenzoate and NEM; Whitney, J. E., Cutler, O. E., and Wright, F. E., Metab., Clin. Exptl. 12, 352 (1963)--Insulin may be bound to tissue by mixed-disulfide interchanges.

5690g: Modification of flour proteins by dough mixing: effects of sulfhydryl-blocking and oxidizing agents; Mecham, D, K., Cole, E. G., and Sokol, H. A., Cereal Chem. 40, 1 (1963)--Part of the protein in wheat flour cannot be extracted, but can be converted to an extractable form by dough mixing. The rate and extent of this conversion are increased by adding NEM to doughs.

2091c: Alterations of transmissions in symphatetic ganglia treated with NEM; Komalahiranya, A., and Volle, R. L., J. Pharmacol. Exptl. Therap. 139, 204 (1963)--The effect of NEM on the responses of the exposed superior cervical ganglia of anestetized cats to stimulation of the preganglionic nerve is discussed.

1008f: Binding of γ-aminobutyric acid by mouse brain preparations; Sano, K., and Roberts, E., Biochem. Pharmacol. 12, 489 (1963)--The binding capacity of γ-aminobutyric acid may be associated with proteins containing reactive SH groups.

889f: Effect of oxygen and NEM on the inactivation of ribonuclease by γ-radiation; Bridges, B. A., Biochim. Biophys. Acta 67, 331 (1963)--NEM prevents inactivation of ribonuclease under anoxic conditions.

58, 10447f: Glutathione reductase from germinated peas; Mapson, L. W., and Isherwood, F. A., Biochem. J. 86, 173 (1963)--Glutathione reductase in the reduced state is readily inactivated by NEM.

1962

60, 9517e: Interaction of 2,4-dichlorophenoxyacetic acid, ethylene-chlorohydrin, and glutathione in the stability of the microsomal ribonucleic acid of excised cotton cotyledons; Bassler, E., Proc. Okla. Acad. Sci. 43, 35 (1962)--Loss of ribonucleic acid (RNA) in the soluble protein is reversed by chloroacetic acid but not by NEM and glutathione.

58, 11698b: Studies on the alginase of Agarbacterium alginicum; Williams, A. K., and Eagon, R. G., Can. J. Microbiol. 8, 649 (1962)--Optimum conditions for hydrolysis of sodium alginate by cell-free bacterial preparations are investigated. NEM shows inhibitory properties.

9553d: The effect of iodate, NEM, and oxygen on the mixing tolerance of doughs; Meredith, P., and Bushuk, W., Cereal Chem. 39, 411 (1962)--The breakdown of doughs under extended mixing in nitrogen, oxygen or air is studied.

9547e: Maleimide-type sulfhydryl blocking agents; Lee, C. C., and Reynolds, L. M., Cereal Chem. 39, 427 (1962)--Determination of SH groups in flour proteins is described and discussed.

6080c: Cation content and output of isolated guinea pig auricles after experimental injury; Foerster, W., Acta Biol. Med. Ger. 9, 211 (1962)--NEM reduces the cardiac activity, decreasing the frequency of the beat.

1688a: Disulfide-sulfhydryl interchange as a mechanism of insulin action; Carlin, H., and Hechter, O., J. Biol. Chem. 237, 1371 (1962)--When a cell is treated with NEM before reacting with insulin, the activity of the latter is inhibited. It is therefore suggested that insulin acts by reacting with SH groups in the cell.

57, 17301e: Inhibition of the action of insulin on rat epididymal adipose tissue by SH-blocking agents; Mirsky, A., and Perisutti, G., Biochim. Biophys. Acta 62, 490 (1962)--The action of insulin and also the spontaneous oxidation of glucose is inhibited by pretreatment of the adipose tissue with NEM.

15463d: Effect of pretreatment with NO and NEM on the level of sulfhydryl compounds in bacteria and on their sensitivity to x-irradiation under anoxia; Lynch, J. P., and Howard-Flanders, P., Nature 194, 1247 (1962)--NEM increases the sensitivity of anoxic bacteria to x-rays due to reduction in the level of intracellular SH compounds after treatment with NEM.

12898c: The active site of myosin adenosinetriphosphatase. I. Localization of one of the sulfhydryl groups; Sekine, T., Barnett, L. M. and Kielley, W. W., J. Biol. Chem. 237, 2769 (1962)--Reaction with NEM is used in locating the cysteine residue involved in the activation of adenosinetriphosphatase (ATPase) by ethylenediaminotetraacetate (EDTA).

12851e: Reversible changes in the NEM-reactive SH groups of hemoglobin on oxygenation-deoxygenation; Morell, S. A., et al., Proc. Natl. Acad. Sci. U.S. 48, 1057 (1962)--The number of SH groups of hemoglobin is increased by one half upon deoxygenation by nitrogen gas, and increased again by reoxygenation.

11789b: Effects of sulfhydryl inhibition on red blood cells. II. Studies in vivo; Jacob, H. S., and Jandl, J. H., J. Clin. Invest. 41, 1514 (1962)--Loss of viability of certain red blood cells upon reaction with NEM is related to the inhibition of membrane SH groups. A quantative discussion is given.

10168c: Role of sulfhydryl groups in the functioning of actomyosin; Blum, J. J., Arch. Biochem. Biophys. 97, 321 (1962)--The possible reaction of NEM with SH groups of actomyosin is considered.

6326e: Relation between glutathione and protein sulfhydryl groups in germinating pea seeds; Spragg, S. P., Lievesley, P. M., and Wilson, K. M.,

Biochem. J. 83, 314 (1962)--Concentration of the glutathione in protein is reduced by contact with NEM.

4992d: Chemical sensitization of Pseudomonas species to ionizing radiation; Bridges, B. A., Radiation Res. 16, 232 (1962)--Several derivatives of maleic acid and its imides are compared with respect to their sensitizing ability.

3903d: Influence of maleate and NEM on the synthesis of amino acids from α-ketoglutarate and ammonia in liver and the kidney of rats; Rogulsky, J., et al., Acta Biochim. Polon. 9, 27 (1962)--Amino acid synthesis is inhibited by NEM, but it is partly reversed by sulfhydryl compounds. Mechanism of the role of NEM is considered.

3731i: Determination of mercapto groups in proteins with NEM; Leslie, J., Williams, D. L., and Gorin, G., Anal. Biochem. 3, 257 (1962)--NEM is applied to SH-group determination of ovalbumin, β-lactoglobulin and bovine serum albumin.

58, 1662d: Leslie, J., Butler, L. G., and Gorin, G., Mercapto group content and denaturation of β-lactoglobulin; Arch. Biochem. Biophys. 99, 86 (1962)--Different denaturing agents are used to determine SH group content.

56, 9324i: Study of the pathogenesis of fever. X. The effect of certain enzyme inhibitors on the production and activity of leukocyte pyrogen; Kaiser, H. K., and Wood, Jr., W. B., J. Exptl. Med. 115, 37 (1962)--Formation of pyrogen is blocked by NEM which has no effect on a preformed pyrogen.

1961

60, 4503e: Studies on the antimicrobial activity of N-substituted maleimides. II. Further examination of N-phenylmaleimide and related compounds; Ferenczy, L., Matkovicz, B., and Zsolt, J., Acta Univ. Szeged., Acta Biol. 7, 75 (1961)--Procedure for determining the inhibitory action of NEM is described.

56, 14591f: The effect of NEM on the radiation sensitivity of bacteria; Bridges, B. A., J. Gen. Microbiol. 26, 467 (1961)--NEM increases the sensitivity of several kinds of bacteria to γ-radiation.

10848f: The action of NEM on the cation permeability of human erythrocytes; Giebel, O., and Passow, H., Naturwissenschaften 48, 721 (1961)--NEM reacts with cells and causes the weakening of their walls in 90% of the cases.

9345h: Inhibition of the insulin effect on sugar transport by NEM; Cadenas, E., et al., J. Biol. Chem. 236, PC63 (1961)--L-Arabinose transport in the presence of insulin is reduced by NEM. It is suggested that insulin is bound to tissues by a SS bond.

9105h: The mercapto groups of ficin; Linener, I. E., Biochim. Biophys. Acta 53, 332 (1961)--Ficin, a proteolytic enzyme that occurs in the latex of the fig tree, reacts with one mole of NEM with loss of activity.

7672a: The identity of myosin subunits; Kielley, W. W., and Barnett, L. M., Biochim. Biophys. Acta 51, 589 (1961)--Polypeptide chains of myosin have identical amino acid sequences.

840g: Relation between water permeability and integrity of sulfhydryl groups in malignant and normal cells; Belkin, M., and Hardy, W. G., J. Biophys. Biochem. Cytol. 9, 733 (1961)--Several cultures of animal tumor cells formed scallop blebs when treated with NEM. This indicates that the cellular permeability to water is changed by the action of the SH reagent.

55, 27119h: Adducts from the reaction of NEM with cysteine and glutathione; Lee, C. C., and Samuels, E. R., Can. J. Chem. 39, 1152 (1961)--The product of reaction of NEM with S^{35}-labeled proteins is S^{35}-(N-ethylsuccinimido)-L-cysteine.

24873i: Binding of NEM by human hemoglobin and its effect on the oxygen equilibrium; Riggs, A., J. Biol. Chem. 236, 1948 (1961)--NEM increases the oxygen affinity of hemoglobin and reacts with one available SH group in each of the β-chains of human carboxyhemoglobin.

22692d: Screening tests of hydrazides, pyridazine derivatives, and related compounds for possible influence on planth growth and development; Greulach, V. A., J. Elisha Mitchell Sci. Soc. 77, 65 (1961)--The effect of NEM on the growth of bean and tomato plants and cucumber seedlings is studied.

21404f: The oxidation of wheat flour. II. Effect of sulfhydryl-blocking agents; Sullivan, B., and Dahle, L., and Nelson, O. R., Cereal Chem. 38, 281 (1961)--NEM shortens the mixing time of a flour, but also eliminates the effect of maturing agents such as $KBrO_3$.

19055h: Role of disulfide exchange reactions in the relaxations of strains introduced in doughs; Frater, R., Hird, F. J. R., and Moss, H. J., J. Sci. Food Agr. 12, 269 (1961)--NEM retards the relaxation of strains in doughs, which occurs through disulfide exchange reactions.

17929c: Vasopressin-like effects of adenosine-3',5'-phosphate and theophylline in the toad bladder; Orloff, J., and Handler, J. S., Biochem. Biophys. Research Communs. 5, 63 (1961)-NEM blocks the effects of adenosine-3',5-phosphate and vasopressin on water movement in the toad bladder.

16651f: Ninhydrin assay in the presence of thiol compounds; Whitaker, J. R., Nature 189, 622 (1961)--The SH groups of cysteine are blocked by NEM and exhibit a color reaction with ninhydrin.

12505g: Chemistry of the Bohr effect. Reaction of NEM with oxygen-linked acid groups of hemoglobin; Benesch, R., and Benesch, R. E., J. Biol. Chem. 236, 405 (1961)--Mechanism of reaction of hemoglobin with NEM is considered.

8868c: Supercontraction of wool in aqueous LiBr, inhibition by free Br, and its relation to mercapto-disulfide interchange; Crewther, W. G., and Dowling, L. M., Textile Research J. 31, 31 (1961)--NEM is used to decrease the SH content in wool, which then exhibits super-contraction to a much smaller extent.

(*): Reaction of NEM with sweet potato β-amylase; Speck, J. C., Riehm, J. P., and Evard, R., Federation Proc. 20, 220 (1961)--Active sites of this enzyme are studied.

1960

55, 23861h: Bromate reaction in dough. II. Inhibition and activation studies; Bushuk, W., and Hlynka, I., Cereal Chem. 37, 343 (1960)--The bromate reaction in doughs is inhibited by NEM. It is therefore suggested that SH is the main group involved in the reaction.

15750g: The effect of NEM on the level of SH groups in some rat organs in acute intoxications; Cernoch, M., and Nemeckova, A., Casopis lekaru ceskyh 99, 1419 (1960)--Different organs of rats are intoxicated with NEM and the level of SH groups is studied polarographically.

15615e: Enzyme systems in microbacteria. IX. Reductive acetylation of lipoic acid; Goldman, D. S., Biochim. Biophys. Acta 45, 279 (1960)--NEM inhibits the reductive acetylation of lipoic acid.

9528h: Cytochemical studies with the electron microscope. II. Sulfhydryl groups of rat spermatozoa; Nelson, L., J. Ultrastruct. Res 4, 182 (1960)--NEM is used to locate SH groups in specimens of lyophilized rat sperm.

7538g: Sensitization of Escherichia coli to γ-radiation by NEM; Bridges, B. A., Nature 188, 415 (1960)--NEM causes a pronounced sensitization of bacteria.

7395e: Reactions of NEM; Smyth, D. G., Nagamatsu, A., and Fruton, J. S., J. Am. Chem. Soc. 82, 4600 (1960)--Chromatographic studies of the reaction of NEM with imidazole, histidine derivatives, amino groups, and cysteine are described.

4653f: The effect of enzyme inhibitors on Vibrio fetus, Proteus vulgaris, and Pseudomonas aeruginosa; Zemjanis, R., Hoyt, H. H., Am. J. Vet. Research 21, 1066 (1960)--NEM is compared with several other enzyme inhibitors with respect to its effect on the growth of microorganisms.

3743c: Xylem regeneration in stem wound response in Colens; Roberts, L. W., Botan. Gaz. 121, 201 (1960)--NEM induces subsurface cell division and wound-xylem formation.

54, 25272b: Inhibition of mast cell disruption and histamine release in rat anaphylaxis in vitro. Comparison with compound 48/80; Mota, I., and Ishii, T., Brit. J. Pharmacol. 15, 82 (1960)--The effect of histamine release and mast cell disruption is abolished by reaction with NEM.

21522e: A role of thiol and disulfide groups in determining the rheological properties of dough made from wheat flour; Frater, R., et al., Nature 186, 451 (1960)--The mixing properties of doughs are altered by NEM; the resistance to mixing is increased.

13478e: The reactivity of mercapto groups in wheat flour; Sokol, H. A., Mecham, D. K., and Pence, J. W., Cereal Chem. 37, 151 (1960)--Reactions of SH groups in flours with NEM and several other reagents are compared and discussed.

13478g: The dough-mixing behavior of gluten and other flour fractions treated with NEM; Mecham, D. K., Sokol, H. A., and Pence, J. W., Cereal Chem. 37, 129 (1960)--The modification of dough-mixing behavior by NEM seems to result from the reaction with SH groups in the less soluble portions of gluten.

8954c: The sulfhydryl content of β-lactoglobulin; Habeeb, A. F. S. A., Can. J. Biochem. Physiol. 38, 269 (1960)--The NEM method is applied in determining mercapto groups in this protein.

(*): The identification of the oxygenation-linked acid groups with the β-chain of human hemoglobin; Riggs, A. F., and Wells, M., Federation Proc. 19, 78 (1960)--Labeled NEM is used to show that it reacts only with β-chains of human hemoglobin.

Additions of Mercaptans to Double Bonds

It is a well established fact that sulfur compounds can react by addition with compounds containing double bonds (12). Hydrogen sulfide adds to olefins in accordance to Markovnikov's rule ("normal addition"), but most of the reactions reported for mercaptans and thiophenols occur in the manner contrary to this rule (13,14). Addition in the manner opposite to that expected from Markovnikov's rule is catalyzed by peroxides (15,16), light (17), and phosphoric acid (18). Reports on the mode of addition which appear in the older literature are uncertain because the factors influencing the addition were not completely understood.

In acidic media mercaptans and hydrogen sulfide add to double bonds in accordance to Markovnikov's rule (19). The double bond is protonated and the carbonium ion formed undergoes nucleophilic attack by a sulfur atom forming a sulfonium ion, according to following scheme:

$$H_2C = CH_2 + H^+ \rightleftarrows H_2\overset{+}{C} - CH_3$$

$$H_2\overset{+}{C} - CH_3 + RSH \rightleftarrows H_2C(\overset{+}{RSH}) - CH_3 \rightleftarrows H_2C(SR) - CH_3 + H^+$$

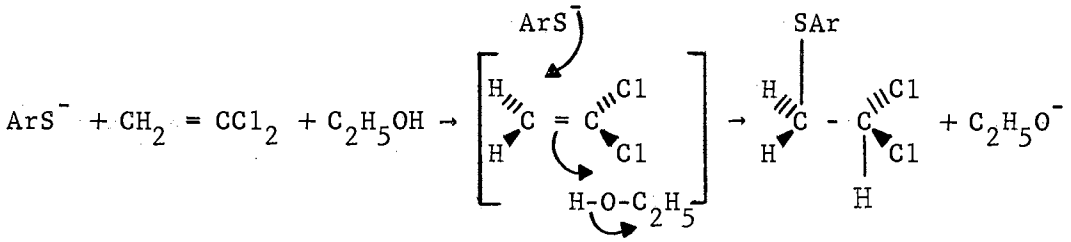
The principle involved in these reactions is similar to the hydration of olefins. The difference is that in the latter case the oxonium ion is involved instead of sulfonium ion.

α,β-Unsaturated systems react with mercaptans and such additions are catalyzed by both acids and bases. However, acid is preferred because in this way base-catalyzed side reactions can be avoided (20). The reaction of thiophenol with methyl acrylate is an example:

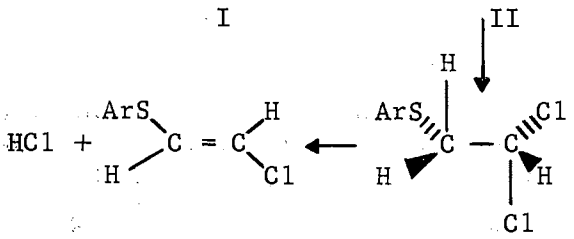
$$C_6H_5SH \underset{}{\overset{NaOCH_3\,(trace)}{\rightleftharpoons}} C_6H_5\bar{S} \xrightarrow{CH_2 = CH\text{-}\overset{O}{\overset{\|}{C}}\text{-}OCH_3} [C_6H_5SCH_2\text{-}\bar{C}H\text{-}\overset{O}{\overset{//}{C}}\text{-}OCH_3 \leftrightarrow C_6H_5SCH_2CH = C(\bar{O})\text{-}OCH_3]$$

$$[C_6H_5SCH_2CH = C(\bar{O})\text{-}OCH_3 \xleftarrow{C_6H_5SH} C_6H_5SCH_2CH_2\overset{O}{\overset{\|}{C}}\text{-}OCH_3 + C_6H_5\bar{S}]$$

Base-catalyzed additions of mercaptide ion usually involve stereospecific <u>trans</u> addition. For example, the reaction of thiophenol with vinylidene chloride in ethanolic solution proceeds according to the following scheme (19):



I



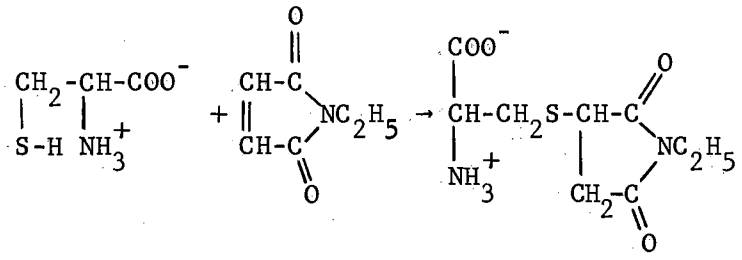
III

The ethoxide-catalyzed addition of p-tolyl mercaptan to cis-dichloroethylene is exceptional in that it gives a cis product. Kinetics of such reactions will be considered later.

In 1938 it was observed (21) that maleic acid reacts with various mercaptans. When the mercaptan used was cysteine, the product was isolated easily; with glutathione and thiolacetic acid a reaction did not occur, because maleic acid was converted to fumaric acid which does not undergo addition by mercaptans. The advantages of derivatives of maleic acid, particularly of N-ethylmaleimide, for reaction with mercapto groups of proteins, were first pointed out by Friedman and coworkers in 1949 (2). NEM is quite specific toward protein SH groups, although it reacts with other amino acids when they are in high concentrations (22, 23). The rate of reaction is much slower than with mercapto groups, which was demonstrated by Benesch and Benesch (24). They investigated the reaction of NEM with human oxyhemoglobin. When the mercapto groups of this protein were blocked by treatment with

iodoacetamide, the product was allowed to react with excess NEM, 94% of the NEM was recovered from the filtrate after precipitation of the protein. This indicates that the reaction of NEM with other amino acids will not interfere the reaction with mercaptans.

The reaction with cysteine proceeds according to the equation:



The addition product S-(N-ethylsuccinimido)-L-cysteine was first isolated in 1960 by Smyth and coworkers, who succeeded in obtaining the product in 84% yield from an aqueous reaction mixture (initially of pH 6) of 0.2 M cysteine and 0.2 M NEM. The product is soluble in water (pH of aqueous solution 6.0) and gives a positive ninhydrin reaction but a negative nitroprusside test. The presence of the imide group in this compound is indicated by the fact that at pH 9.0 it readily reacts with hydroxylamine forming hydroxamic acid. Recent work on N-ethylmaleimide shows that it may polymerize at room temperature and pH 7.4 and that cysteine enhances this process (22), but the mechanism is still unclear.

The usefulness of NEM in determining the mercapto groups of ovalbumin, β-lactoglobulin and bovine serum albumin has been pointed out by Leslie, Williams, and Gorin (25) and by Habeeb (26). The mercapto groups in ovalbumin and β-lactoglobulin are unreactive and these proteins have to be denatured by 8 M urea, 5.8 M guanidine hydrochloride or other

denaturing agents. After denaturation they readily react with NEM consuming 3.8 and 1.9 moles of NEM/mole of protein, respectively, which is in agreement with the results obtained with other methods.

The products of the reaction of mercaptans with NEM give a red color in alkaline solution. Since the color disappears in acidic media and appears again when in alkali, these colored products could serve as acid-base indicators (27). The strongest color is obtained with an excess of NEM. When the NEM reacts with alkali before reacting with mercaptan, no color product is observed; this must be due to the fact that the imide linkage is hydrolyzed in alkaline media. The colored product is more stable in ethanol and isopropanol than in water; this would indicate that the color is due to the imide linkage. The colored product is not stable in methanol, however, and the reason is not yet understood. Disulfide compounds do not give a color test. Therefore, the above reaction can be used as a specific one for mercapto groups. This has been successfully applied in the chromatographic separations of mercapto-containing amino acids and peptides from other peptides with same R_f value.

A more sensitive technique for the paper chromatography of mercaptans is made possible by use of N-(4-hydroxy-1-naphthyl)isomaleimide. With this compound, the colored product can be detected with as low as 3×10^{-10} M of mercaptan. This method is of much importance in the histochemical determination of protein mercapto groups in plant tissues (28).

Labeled C^{14}-N-ethylmaleimide has been used in the study of human hemoglobin. It is known (29) that human hemoglobin contains two chains, α and β, which can be separated. Riggs and Wells (23) showed that the radioactivity of the α-chain was negligible compared to β-chain; this

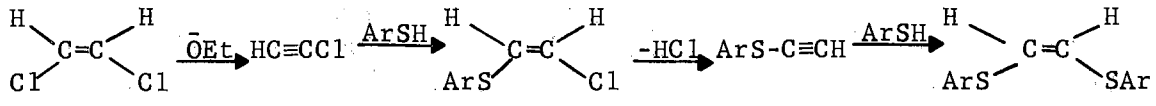
indicates that NEM reacts only with the β-chain.

Mercaptans react with aldehydes and ketones in the same manner as alcohols; the products are mercaptals and mercaptols, respectively. With benzoquinones three reactions may take place: oxidation of the mercaptan to disulfide with concomitant reduction of the quinone to hydroquinone; addition to the double bonds; or mercaptol formation (30). These reactions have found no wide applicability.

Kinetics of Addition Reactions of Mercaptans to Carbon-Carbon Double Bonds

The kinetics of additions of mercaptans to C=C double bond is not simple. Some mercaptans add according to the first-order rate law; some follow second-order kinetics and some fit the third-order rate equation. Examples of each will be briefly discussed.

The reaction of cis-dichloroethylene with mercaptans has been investigated by Truce et al. (31). Three important observations have been made: (a) the trans-isomer does not react; (b) sodium ethoxide is necessary for reaction, and (c) the final product has exclusively the cis structure. The rate of reaction was found to be proportional to the concentrations of both haloplefin and ethoxide but was independent of aryl thiolate concentration (eq. 2). The following sequence of reactions was proposed:



I II III IV V

It was postulated that the first step of the mechanism is

$$I + NaOEt \rightarrow II + NaCl + EtOH \quad (1)$$

and that this reaction is rate-determining. Thus:

$$-d[I]/dt = k_1[I][EtO^-] \quad (2)$$

If all other steps are fast compared to (1), sodium ethoxide will be regenerated as fast as it is consumed and

$$k_1[EtO^-] = k$$

Equation (2) simplifies to

$$-d[I]/dt = k[I] \quad (3)$$

which is the expression for a first-order reaction. The interpretation of equation (3) is that the formation of chloroacetylene (II) is the rate-determining step in the over-all conversion.

The reaction of 1-butanethiol (32) with maleic anhydride in the presence of triethylenediamine in xylene solution follows third-order kinetics, first order each in mercaptan, maleic anhydride and triethylenediamine. The following sequence of reactions is suggested:

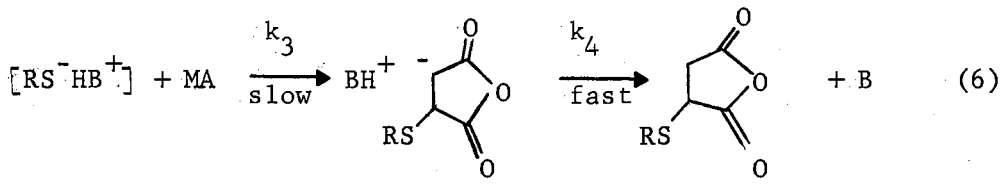
$$RSH + B \underset{k_2}{\overset{k_1}{\rightleftarrows}} [RS^-HB^+] \quad (4)$$

$$[RS^-HB^+] + MA \overset{k_3}{\rightleftarrows} \text{products} \quad (5)$$

where RSH is mercaptan, B triethylenediamine, and MA maleic anhydride. The complex $[RS^-HB^+]$ is shown by spectroscopic measurements to be an ion pair with no hydrogen bonding. Spectroscopic studies also show that the mercaptan reacts with a base but not with the maleic anhydride. It was assumed that $k_2 >> k_3$ [MA] with k (experimental) = $k_1k_3/k_2 = k_3K$ where

$$K = k_1/k_2.$$

The most likely rate-limiting step is the addition of the thiolate portion of the ion pair to maleic anhydride, followed by a rapid transfer of a proton from the protonated amine to the adduct anion:



(6)

The reaction of cysteine with N-ethylmaleimide and its derivatives in water solutions was studied by Lee and Samuels and found to follow second-order kinetics (33). These investigators studied the reaction in aqueous solution in the pH range 1.0 to 3.4. The solvent used was water, which was adjusted to the desired pH by adding hydrochloric acid or sodium hydroxide solution. Equal concentrations of mercaptan and NEM were used; the rate constant was computed by the equation:

$$1/(\underline{a} - \underline{x}) = \underline{k}_2\underline{t} + \text{const.}$$

where $(\underline{a} - \underline{x})$ is the concentration of each reactant at time $\underline{t}$. The reciprocal concentration of NEM at time $\underline{t}$ was plotted against time, and the specific rate constant obtained from the slope of the straight line.

CHAPTER III*

*As stated in the Introduction, this Chapter consists of a manuscript which reports the results of the thesis for publication in a journal.

KINETICS OF THE REACTION OF N-ETHYLMALEIMIDE WITH CYSTEINE AND SOME CONGENERS

(SUMMARY)

The kinetics of the reaction between N-ethylmaleimide and cysteine are of second order; the specific rate constant at 25° in 0.018 M acetic -0.11 M sodium chloride buffer of pH 4.92 is 13.1 liter mole^{-1} sec^{-1}. The specific rate constants in the pH range to pH 3.0 are given by the equation:

$$k = 0.20 + (1.53 \times 10^{-4})/[H^+]$$

From this it is estimated that the half-reaction time at pH 7.0 and 10^{-3} M concentration would be 0.7 sec. Rate measurements also are reported for some related compounds. Qualitatively, the rate of reaction is: 2-hydroxymethyl-2-(2'-mercaptoethylamino)-1,3-propanediol > cysteine ~ glutathione > 1-amino-2-propanethiol > 1,1-dimethyl-2-aminoethanethiol.

N-ethylmaleimide (NEM) has recently found considerable use in the determination (25,34) and modification of mercapto groups in proteins (24). Accordingly, it seems desirable to obtain some quantitative data on the rate of its reaction with cysteine and related compounds. Lee and Samuels (33) have reported rates for the reaction in the pH range

1.0 - 3.4, which was achieved by adding small amount of strong acid or base to aqueous solutions of NEM. Since it will be seen that the reaction is strongly dependent on the pH, the measurements reported in this paper were made in buffered media of nearly constant ionic strength and the pH range was extended to higher pH values, which are of greater interest to biochemists. Measurements were also made with some compounds related to cysteine. The results afford some indication of the effect of structural changes on the rate of the reaction.

MATERIALS AND METHODS

Materials

Cysteine hydrochloride hydrate, A Grade (sample I), and B Grade (sample II), and NEM (sample III) were obtained from the California Corp. for Biochemical Research, Los Angeles 63. NEM (samples I and II) and glutathione were obtained from Schwarz Bioresearch, Inc., Mount Vernon, N. Y. 2-Mercaptoethylamine and all its derivatives were obtained from the Walter Reed Army Institute of Research, Washington, D.C. (1). Disodium ethylenedinitrilotetraacetate (EDTA) and all buffer salts were of A.C.S.-reagent grade.

As will be seen in the next section, the rate of reaction of cysteine in the vicinity of pH 5.0 increases 3% per 0.01 pH unit. Controlling the pH of the buffer solutions with an ordinary pH meter, which has an estimated accuracy of about ± 0.05 units, was accordingly inadequate. The buffers were mixed from accurately standardized reagents, as specified below: the acetate buffers were prepared by mixing 0.2 N acetic acid and 0.2 N sodium acetate, each 0.11 M in sodium chloride: 300 ml to 700 ml, pH 4.92; 400 to 600, pH 4.78; 600 to 400, pH 4.45; 800

to 200, pH 4.05. Citrate buffer, pH 3.00, was prepared from 205.5 ml of 0.2 M Na_2HPO_4 and 794.5 ml of 0.1 M citric acid in 0.11 M sodium chloride. Each buffer contained 0.3723 g of EDTA per liter of solution. The pH value quoted for the buffers are the averages of several measurements made on the resulting reaction mixture.

Apparatus

Absorbance measurements were made with a Beckman DU spectrophotometer in silica cells of 1-cm path length. Measurements of pH were made with a Beckman Model G or an Expandomatic pH meter, which had been calibrated with commercial buffers of pH 4.00.

Procedure

NEM was dissolved in 0.11 M NaCl solution, the cysteine or other mercaptan was dissolved in buffer, and the solutions were placed in a thermostat. As soon as temperature equilibrium had been established (only freshly prepared solutions were used), 3 ml of mercaptan solution was mixed with 30 ml of NEM solution and absorbance measurements were made as a function of time, against water as a blank. The absorbance at zero time was determined by mixing the NEM solution with buffer in the same proportions (the absorbance of all mercaptans was negligible). In the experiments at pH 4.95 to 4.78, the absorbance at infinite time was determined directly after the absorbance value had remained constant for ten minutes. Experiments at lower pH were done in conjunction with a pH 4.9 experiment and the final absorbance obtained in the latter case was used for the former as well. In most experiments, the initial concentrations of NEM and of mercaptan, were 1.0×10^{-3} M and 7.0×10^{-4} M,

respectively; the solutions were 0.018 M in acetate and 0.11 M in sodium chloride. Some experiments were done at lower concentrations of NEM and cysteine. The value of the final absorbance could be utilized to calculate the purity of mercaptan, taking 620 as the molar absorbance of NEM; the percentage of the nominal purity, calculated from the weight taken, is calculated from the following expression:

$$\% \text{ purity} = \frac{(A_o - A_\infty)(\text{M.W. of RSH})(\text{volume of soln. in ml})}{620\ (\text{wt of RSH in mg})}$$

Calculations

For a reaction of second order, A + B→C, the specific reaction-rate constant is given by:

$$k = [2.303/(a - b)t][\log b(a - x)/a(b - x)] \qquad (1)$$

where a and b are the initial concentrations of the reactants and x the concentration consumed by time t (35). If a represents the initial concentration of NEM taken in excess, and b the concentration of cysteine, the following relations can be written in terms of the molar absorbancy of NEM, ε, and the initial, final, and intermediate absorbancies, respectively, A_o, A_∞, and A_t:

$$a = A_o/\varepsilon$$

$$b = (A_o - A_\infty)/\varepsilon$$

$$(a - x) = A_t/\varepsilon$$

$$(b - x) = (A_t - A_\infty)/\varepsilon.$$

Substitution of these expressions into equation (1) gives the equation:

$$k = (2.303\ \varepsilon/A_\infty \cdot t)\ \log\ [A_t\ (A_o - A_\infty)/A_o\ (A_t - A_\infty)] \qquad (2)$$

The values of k given in Table I were calculated from this equation. Also, the data were subjected to a least-square analysis and k was calculated from the slope of the line which would be obtained by plotting the logarithmic term *vs.* time t:

$$\text{slope} = A_\infty \times k/2.303\ \varepsilon.$$

The specific rate constants given in Table II are the averages of the values obtained in this way from the indicated number of experiments.

RESULTS AND DISCUSSION

Table I gives some data for the reaction of cysteine (cySH) and NEM at pH 4.78. It can be seen that the second-order kinetic equation (2) gives consistent values of k to about 80% reaction, i.e. more than two half-life periods. Furthermore, the same value of the specific rate constant is obtained when the concentration of reagents is halved, which considerably decreases the rate.

In determining the effect of pH on the rate of reaction it is desirable to maintain the medium as nearly the same as possible. For this reason, 0.11 *M* sodium chloride was added to all the reaction media. It is then a fairly good approximation to consider the activity coefficient of hydrogen ion as constant. Table II gives the values of the constants obtained with cysteine at various pH values, and Fig. 1 shows a plot of the rate constant against $1/a_H$, the reciprocal hydrogen ion "activity", a_H = antilog (-pH). It can be seen that the data conform to a straight line, and the equation giving the least-square fit is:

TABLE I

KINETICS OF REACTION OF CYSTEINE WITH NEM, pH 4.78

Time, sec	A_t	k, liter mole^{-1} sec^{-1}
	1.0×10^{-3} M NEM and 7.0×10^{-4} M cySH	
0	.636	—
90	.450	8.43
105	.432	8.46
120	.418	8.35
135	.402	8.50
150	.390	8.48
180	.368	8.58
∞	.245	Avg. 8.47[a]
	5.0×10^{-4} M NEM and 3.5×10^{-4} M cySH	
0	.323	—
75	.270	7.78
105	.252	(8.10)
120	.245	8.09
135	.238	8.15
165	.225	8.32
180	.220	8.27
240	.200	8.52
300	.184	8.76
∞	.103	Avg. 8.32[b]

[a] value from least-square fit, 8.43

[b] value from least-square fit, 8.55

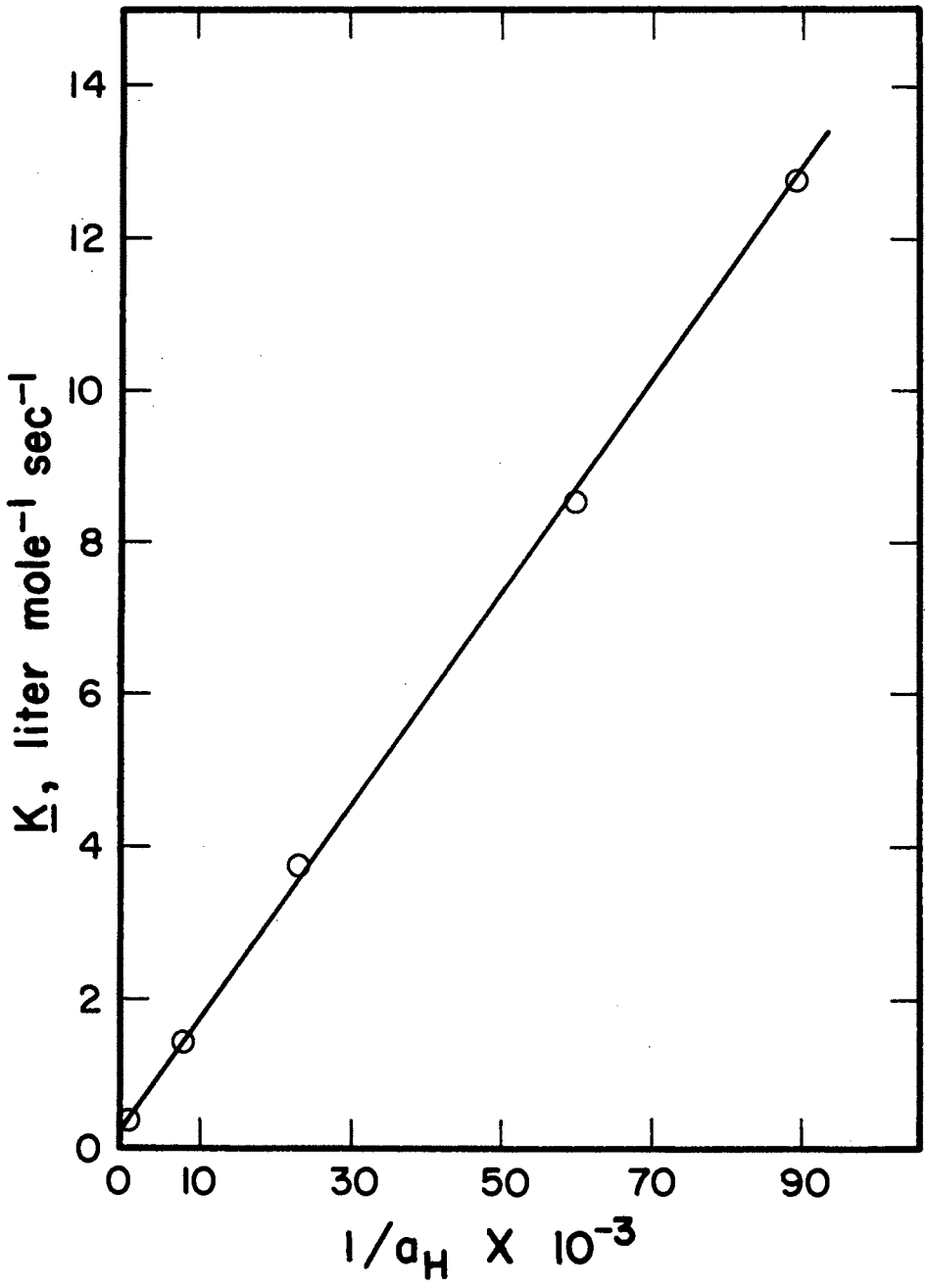


Fig. 1 Dependence of rate constant on pH

TABLE II

RATE CONSTANTS FOR REACTION WITH CYSTEINE[a] AT VARYING pH

pH	number of detns.	k, liter mole^{-1} sec^{-1} exptl.	k, liter mole^{-1} sec^{-1} given by eq. (3)
4.95[b]	8[b]	14.1 ± .9[b]	13.84
4.92	4	13.1 ± .8[c]	12.82
4.78	2	8.5 ± .1	9.42
4.38	2	3.73 ± .12	3.67
3.94	3	1.59 ± .18	1.53
3.00	2	.346 ± .023	0.35

[a] Sample II, except as noted, assay 94 ± 2%; NEM samples II and III.

[b] Sample I, assay 97 ± 1%, NEM sample I.

[c] k, adjusted to pH 4.95 by eq. (3) = 13.9; overall average at pH 4.95, 14.0.

$$\underline{k} = 0.20 + 1.53 \times 10^{-4}/\underline{a}_H \qquad (3)$$

These data indicate that the reaction rate expression contains two terms, one pH dependent and the other pH independent.

It may reasonably be deduced that the pH-independent term corresponds to reaction of NEM with cySH and the pH-dependent term to reaction with cyS^-. One can then write the expression:

$$-d(\underline{NEM})/d\underline{t} = \underline{k}_o\ (\underline{NEM})(\underline{cySH}) + \underline{k}_H\ (\underline{NEM})(\underline{cyS^-})$$

$(\underline{cyS^-})$ is related to $(\underline{cySH})$ by the ionization constant expression:

$$(\underline{cyS^-}) = \underline{K}_i\ (\underline{cySH})/\underline{a}_H.$$

In this equation, it is intended that $(\underline{cySH})$ and $(\underline{cyS^-})$ represent concentrations rather than activities and $\underline{K}_i$ cannot therefore be considered a truly invariant thermodynamic constant; it may, however, be considered constant to a fairly good degree of approximation. Then:

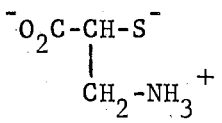
$$-d(\underline{NEM})/d\underline{t} = (\underline{NEM})(\underline{cySH})(\underline{k}_o + \underline{k}_H\underline{K}_i/\underline{a}_H).$$

Integration of this expression gives a result which corresponds to the experimental equation (3); $\underline{k}_o = 0.22_9$ and $\underline{K}_i\ \underline{k}_H = 1.53 \times 10^{-4}$. If one takes $\underline{K}_i = 10^{-8.30}$ (at $\mu = 0.1$) (36,37,38,39), $\underline{k}_H = 3.06 \times 10^4$ liter $mole^{-1}$ sec^{-1}. The magnitude of $\underline{k}_H$ is noteworthy; it is responsible for the rapid increase of the rate with pH. It can be estimated that at pH 7.0 the rate constant would be 1.53×10^3 liter $mole^{-1}$ sec^{-1}; this would mean that in a reaction mixture $10^{-3}\underline{M}$ in NEM and mercaptan the half-life period would be 0.7 sec, i.e. the reaction would be very fast.

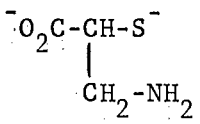
The value found at pH 3.0, 0.346, is of the same order of magnitude as that reported by Lee and Samuels (33); since the medium used in that investigation was not buffered and was of much lower ionic strength, a closer comparison would be pointless. Lee and Samuels' data at pH 3.0

- 3.4 cover too small a range to serve as a test of the pH dependence but do show qualitatively that the rate increases sharply with pH. It is not possible to make a significant comparison between the value of $\underline{k}_o$ in equation (3) and the data found by Lee and Samuels at pH 2 and 1 because at these pH values a substantial amount of cysteine is converted to its cation ($cySH_2^+$); $\underline{k}_o = 0.23$ therefore does not correspond to any actual rate. Insofar as the data can be compared (i.e., neglecting possible medium effects and errors), one would conclude that the rate of reaction of NEM with $(cySH_2)^+$ is slower than that with cysteine, and might be zero. The present work was not extended into this pH region because it is not of so much interest.

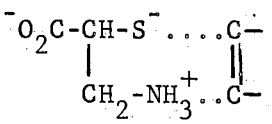
Rate measurements were made on glutathione (glSH), partly in the hope that it would react much more slowly than cysteine. Were this the case, it might be possible to analyse a mixture of glutathione and cysteine by differential kinetic methods (40). It was therefore disappointing to find (Table III) that the rates differ by a factor of only 1.4; this does not completely preclude application of differential kinetic analysis but the prospective precision is not attractive. The comparison of cysteine and glutathione is of interest, however, from the mechanistic point of view. From the ionization constant of glutathione, (41), $10^{-8.75}$ one calculates for the reaction of (glS^-) with NEM a value of $\underline{k}_H = 7.92 \times 10^4$, somewhat greater than for (cyS^-). The anion (cyS^-) exists in the tautomeric forms I and II, and when the large value of the rate constant for



I



II



III

TABLE III

RATE CONSTANTS FOR SOME MERCAPTANS

Compound	pH	% theor. titre	$\underline{k}$ liter mole^{-1} sec^{-1}	$\underline{k}$ for cySH at given pH
$HS\text{-}CH_2CH_2NC(CH_2OH)_3$	4.78	96 ± 1	24.2	8.5
$HS\text{-}CH_2CH_2\text{-}NH_2$	4.78	98 ± 1	14.6	8.5
$HS\text{-}CH(CH_3)CH_2NH_2$	4.78	95 ± 1	5.26	8.5
Glutathione	4.95	95 ± 2	10.6	14.1
Glutathione	3.94	95 ± 2	1.17	1.59
$HS\text{-}C(CH_3)_2CH_2NH_2$	6.98	96 ± 2	13.2	1.46×10^3

(cyS^-) was first established it was thought that the $-NH_3^+$ group of I might facilitate the reaction by helping to polarize the double bond, as depicted in III. The fact that (glS^-) reacts even faster than (cyS^-) even though it does not have an appropriately placed NH_3^+ group argues against this hypothesis, however. It would seem, therefore, that the rather high rate of reaction must be ascribed to the combination of a comparatively high polarity of the bond in NEM and to the known high nucleophilic reactivity of sulfide anions.

The other data given in Table III do not warrant much comment, but the considerable difference in the values of $\underline{k}$ are worthy of note. It is because of the magnitude of these differences that the determinations were not all done at the same pH. In order to facilitate comparison, the last column lists values of the rate constant, calculated from equation (3), which would obtain for cysteine at the pH of the measurement. It is thus seen that 1-amino-2-methylpropane-2-thiol, a tertiary mercaptan, reacts 100 times slower than cysteine, and 2-hydroxymethyl-2-(2'-mercaptoethylamino)-1,3-propanediol three times faster.

CHAPTER IV

APPENDIX

Since Chapter III was written in a manner suitable for publication in a journal, it was subject to brevity requirements. The details which have there been omitted are presented in this Chapter, which is subdivided into two parts. The first part gives more information concerning calculation of the rate constants by least-square analysis using a Fortran program for an IBM 1410 computer. The second part discusses other experimental data omitted from Chapter III.

Calculation of k by Least-Square Method

The specific rate constant was calculated from the equation of the straight line

$$\underline{y} = C + D\,\underline{x} \qquad (1)$$

Equation (2), Chapter III, can be rewritten in terms of natural logarithms as:

$$\ln[\underline{A}_t/(\underline{A}_t - \underline{A}_\infty)] = \ln[\underline{A}_o/(\underline{A}_o - \underline{A}_\infty)] + \underline{A}_\infty \underline{t}k/\underline{\varepsilon} \qquad (2a)$$

This expression corresponds to eq. (1):

$$y = \ln[\underline{A}_t/(\underline{A}_t - \underline{A}_\infty)];\ C = \ln[\underline{A}_o/(\underline{A}_o - \underline{A}_\infty)];$$

$$D = \underline{A}_\infty \cdot \underline{k}/\varepsilon;\ \text{and}\ \underline{x} = \underline{t}.$$

Fig. 2 is a photographed copy of the computer output. The original data are obtained from experiment. The following symbols are used:

X represents time $\underline{t}$ in sec

CYSH PH 4.95 27/4 A(INF) = .200

ORIGINAL DATA

POINT	X	NO. OF Y VALUES	Y (MEAN)	STD. DEV.
1	.00000E-99	1	.14640E 01	.00000E-99
2	.11000E 03	1	.23310E 01	.00000E-99
3	.12000E 03	1	.24290E 01	.00000E-99
4	.13000E 03	1	.25400E 01	.00000E-99
5	.14000E 03	1	.26650E 01	.00000E-99
6	.15000E 03	1	.28190E 01	.00000E-99
7	.16500E 03	1	.30000E 01	.00000E-99
8	.18000E 03	1	.32210E 01	.00000E-99

COEFFICIENTS OF FITTED EQUATION

TERM NO.	COEFFICIENT
1	.37321515E 00
2	.43681229E-02

PLOTTED DATA

POINT	X	LN (Y)	W	F	DEV.
1	.00000E-99	.38117E 00	.10000E 01	.37321E 00	-.79572E-02
2	.11000E 03	.84629E 00	.10000E 01	.85370E 00	.74113E-02
3	.12000E 03	.88747E 00	.10000E 01	.89738E 00	.99102E-02
4	.13000E 03	.93216E 00	.10000E 01	.94107E 00	.89070E-02
5	.14000E 03	.98020E 00	.10000E 01	.98475E 00	.45483E-02
6	.15000E 03	.10363E 01	.10000E 01	.10284E 01	-.79486E-02
7	.16500E 03	.10986E 01	.10000E 01	.10939E 01	-.46568E-02
8	.18000E 03	.11696E 01	.10000E 01	.11594E 01	-.10214E-01

STANDARD DEVIATION OF ORDINATE POINTS .84994820E-02

Fig. 2 Representative copy of computer output for least-square calculation of rate constant

Y stands for the ratio $\underline{A}_t/(\underline{A}_t - \underline{A}_\infty)$

Terms no. 1 and 2 of fitted equation represent the coefficients C and D, respectively, in eq. (1).

The plotted data also contain the terms:

W, the weight of the experimental points, that was set equal to unity in every case;

F, which represents the distance on the ordinate axis from any particular experimental point to the straight line. The standard deviation σ of ordinate points is given by

$$\sigma^2 = \sum_{i=1}^{n} (\underline{F}_i - \underline{y}_i)^2 \cdot \underline{w}^2/(\underline{n} - 1)$$

where $\underline{n}$ is the number of experimental points.

Additional Details Concerning the Reaction of Mercaptans with NEM

Table IV gives again the data presented in Table I, but includes the calculated values of the terms 2.303 $\underline{\epsilon}/\underline{At}$ and $\log \underline{A}_t(\underline{A}_t - \underline{A}_\infty)/\underline{A}_o(\underline{A}_t - \underline{A}_\infty)$; also it reports one additional experiment.

Fig. 3 presents the experimental data obtained in representative experiments done with cysteine and 2-aminoethanethiol. From the graph it can be seen that the half-times for reaction of NEM with cysteine and 2-aminoethanethiol are approximately 100 sec and 70 sec, respectively.

Table V reports the results of the individual experiments, from which the averages reported in Table II were calculated; the average deviation is calculated and given in the last column. A representative calculation using equation (3) is given below; it shows the adjustment of the rate obtained at pH 4.95 to pH 4.92, given in note (b) to Table II:

For pH 4.95

$$\underline{k}_{4.95} = \underline{k}_o + (1.41 \times 10^{-4}/1.12 \times 10^{-5})$$

Similarly

$$\underline{k}_{4.92} = \underline{k}_{o} + (1.41 \times 10^{-4}/1.20 \times 10^{-5})$$

Subtracting, one gets

$$\underline{k}_{4.95} - \underline{k}_{4.92} = 12.6 - 11.8 = 0.8$$

$$\underline{k}_{4.92} = \underline{k}_{4.95} - 0.8 = 14.2 - 0.8 = 13.4 \text{ liter mole}^{-1} \text{ sec}^{-1}$$

Table VI reports the results of the individual experiments from which the averages reported in Table III were obtained.

TABLE IV

KINETICS OF REACTION OF CYSTEINE WITH NEM, pH 4.78

Time, sec	A_t	$\frac{2.3 \times \varepsilon}{A_\infty \times t}$	$\log \frac{A_t(A_o - A_\infty)}{A_o(A_t - A_\infty)}$	k, liter mole^{-1} sec^{-1}
		1.0×10^{-3} M NEM and 7.0×10^{-4} M cySH		
0	.636	—	—	—
90	.450	64.5	.130	8.40
105	.432	55.5	.152	8.40
120	.418	48.4	.175	8.45
135	.402	43.0	.195	8.40
150	.390	38.6	.220	8.50
180	.368	32.2	.265	8.50
∞	.245	—	—	—
				Avg, 8.44[a]
		1.0×10^{-3} M NEM and 3.5×10^{-4} M cySH		
0	.636	—	—	—
75	.542	48.0	.148	7.20
90	.530	40.0	.175	7.00
105	.512	34.2	.222	7.55
120	.500	30.0	.260	7.80
135	.490	26.6	.294	7.80
150	.484	24.0	.315	7.55
180	.465	19.9	.405	8.05
∞	.396	—	—	—
				Avg. 7.56

TABLE IV (Continued)

Time, sec	A_t	$\frac{2.3 \times \epsilon}{A_\infty \times t}$	$\log \frac{A_t(A_o - A_\infty)}{A_o(A_t - A_\infty)}$	k, liter mole^{-1} sec^{-1}
		5.0×10^{-4} M NEM and 3.5×10^{-4} M cySH		
0	.323	—	—	—
75	.270	185	.042	7.78
105	.252	132	.061	8.08
120	.245	115	.070	8.10
135	.238	102	.080	8.20
165	.225	84	.098	8.20
180	.220	76.5	.110	8.40
∞	.103	—	—	—
				Avg. 8.13

[a] k from least-square fitting 8.43

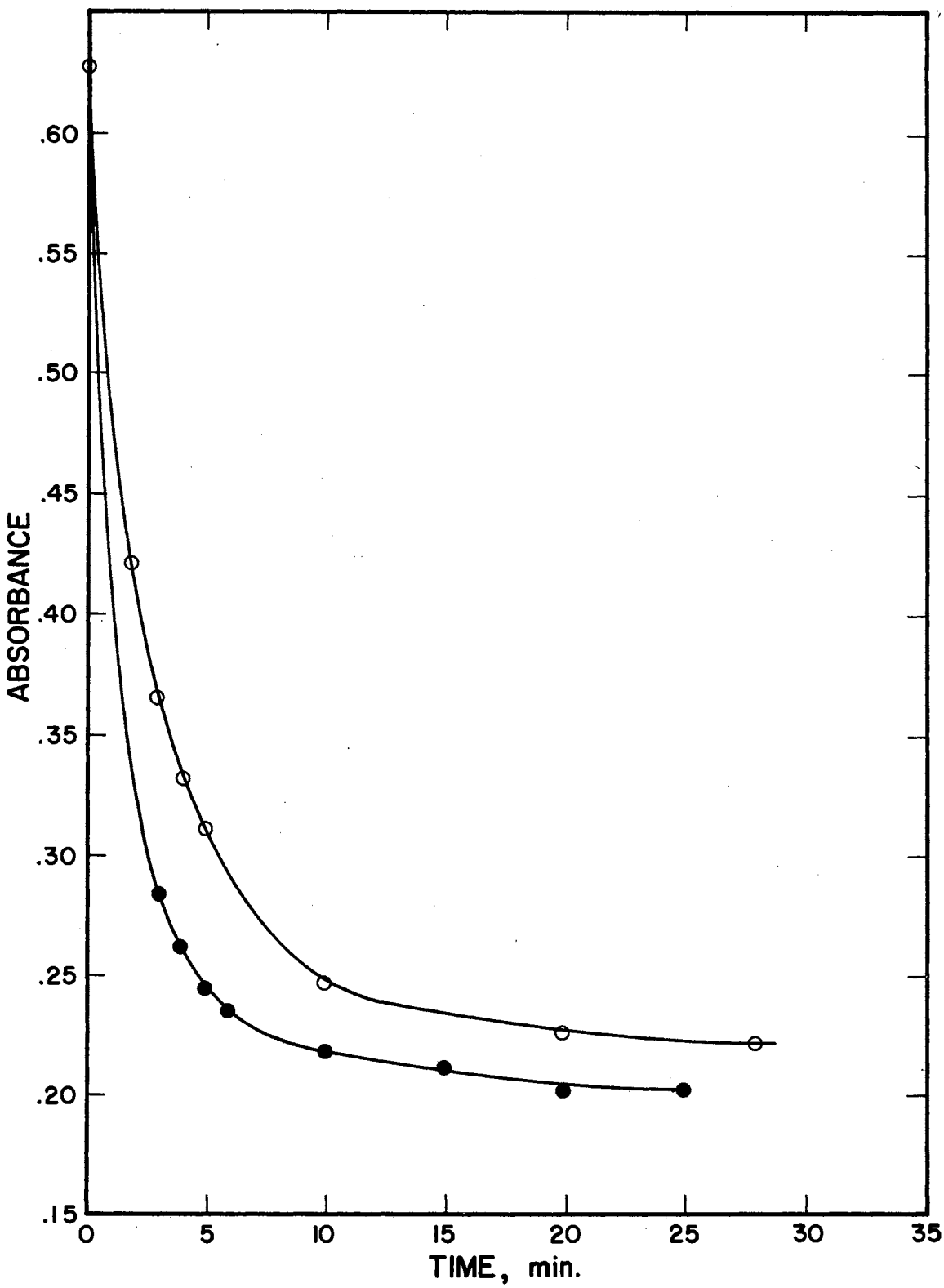


Fig. 3 Rate of reaction of NEM at pH 4.78 with cysteine (empty circles) and aminoethanethiol (full circles)

TABLE V

RATE CONSTANTS FOR REACTION OF NEM WITH CYSTEINE AT VARYING pH

pH	A_o	A_∞	k_{exp}	deviation, d
4.95	.595	.180	15.0	.4
4.95	.585	.170	15.1	.4
4.95	.613	.200	13.7	.2
4.95	.632	.210	15.5	.6
4.95	.595	.360	13.4	.4
4.95	.620	.200	12.1	.1
4.95	.618	.185	14.7	.4
4.95	.632	.200	13.1	.1
			Avg. 14.1 ± .3	
4.92	.632	.235	13.1	.3
4.92	.641	.242	13.2	.4
4.92	.640	.230	13.3	.3
4.92	.640	.430	13.4	.2
4.92	.640	.425	12.3	.2
			Avg. 13.1 ± .9	
4.78	.636	.245	8.43	.06
4.78	.636	.235	8.65	.15
4.78	.629	.222	(7.55)	
			Avg. 8.54 ± .11	
4.38	.632	.235	3.62	.14
4.38	.640	.230	3.84	.06
			Avg. 3.73 ± .11	

TABLE V (Continued)

pH	A_o	A_∞	k_{exp}	d
3.94	.632	.235	1.41	.13
3.94	.632	.220	1.78	.20
			Avg. 1.59 ± .18	
3.00	.632	.235	.323	.044
3.00	.645	.450	.369	.010
			Avg. .346 ± .023	

TABLE VI

RATE CONSTANTS FOR REACTION OF NEM WITH VARIOUS MERCAPTANS

pH	A_o	A_∞	k_{exp}	d
		$HSCH_2CH_2NC(CH_2OH)_3$		
4.78	.632	.221	24.85	.30
4.78	.632	.217	23.55	.25
			Avg. 24.20 ± .35	
		$HS\text{-}C(CH_3)_2\text{-}CH_2NH_2$		
6.98	.620	.176	13.10	.30
6.98	.620	.205	13.20	.45
			Avg. 13.15 ± .05	
		$HS\text{-}CH_2CH_2\text{-}NH_2$		
4.78	.629	.202	14.75	.25
4.78	.629	.207	14.60	.20
			Avg. 14.67 ± .07	
		Glutathione		
4.95	.627	.215	11.40	.30
4.95	.632	.205	9.80	.20
			Avg. 10.60 ± .80	
3.94	.632	.220	1.05	.35
3.94	.636	.228	1.29	.30
			Avg. 1.17 ± .12	
		$HS\text{-}CH(CH_3)CH_2NH_2$		
4.78	.636	.222	5.30	.12
4.78	.620	.244	5.41	.12
4.78	.629	.215	5.07	.22
			Avg. 5.26 ± .19	

BIBLIOGRAPHY

1. Supported by Grant AM 06 and Career Development Award 5-K3-GM (to Dr. Gorin) from the National Institute of Health. We are indebted to Drs. D. Jacobus and T. E. Sweeney, Walter Reed Army Institute of Research for samples of the mercaptans used in this research.

2. Friedmann, E., Marrian, D. H., and Simon-Reuss, I., Brit. J. Pharmacol. 4, 105 (1949).

3. Gregory, J. D., J. Am. Chem. Soc. 77, 3922 (1955).

4. Roberts, E., and Rouser, G., Anal. Chem. 30, 1291 (1958).

5. Alexander, N. M., Anal. Chem. 30, 1292 (1958).

6. Tsao, T. C., and Bailey, K., Biochim. Biophys. Acta 11, 102 (1953).

7. Barron, E. S. G., and Singer, T. P., Science 97, 356 (1943).

8. Swenson, A. D., and Boyer, P. D., J. Am. Chem. Soc. 79, 2174 (1957).

9. Cecil, R., "The Proteins", Neurath, H. (ed), 2nd Ed. vol. 1., Academic Press, New York, 1963 p. 380.

10. Barron, E. S. G., Advan. in Enzymol. 11, 201 (1951).

11. Boyer, P. D., "The Enzymes", Boyer, Lardy, and Myrbäck (eds.), 2nd ed., Vol. 1, Academic Press Inc. New York, 1959 p. 511.

12. Flett, L. H., and Gardner, W. H., "Maleic Anhydride Derivatives; Reactions of the Double Bond", Wiley, New York, 1952 p. 216.

13. Ashworth, F., and Burkhardt, G. N., J. Chem. Soc. 1791 (1928).

14. Ipatieff, V. N., and Friedman, B. S., J. Am. Chem. Soc. 61, 71 (1939).

15. Jones, S. O., and Reid, E. E., J. Am. Chem. Soc. 60, 2452 (1938).

16. Kharasch, M. S., Read, A. T., and Mayo, F. R., Chemistry and Industry, 752 (1938).

17. Carothers, W. H., J. Am. Chem. Soc. 55, 2008 (1933).

18. Robertson, P. W., et al., J. Chem. Soc. 335 (1937).

19. Pryor, W. A., "Mechanism of Sulfur Reactions", McGraw-Hill, Inc., New York 1962 p. 71.

20. Cram, D. J., and Hammond, G. S., "Organic Chemistry", 2nd ed., McGraw-Hill, New York, 1964 p. 232.

21. Morgan, E. J., and Freidmann, E., Biochem. J. 32, 733, 832 (1938).

22. Smyth, D. G., Nagamatsu, A., and Fruton, J. S., J. Am. Chem. Soc. 82, 4600 (1960).

23. Riggs, A. F., and Wells, M., Federation Proc. 19, 78 (1960).

24. Benesch, R., and Benesch, R. E., J. Biol. Chem. 236, 405 (1961).

25. Leslie, J., Williams, D. L., and Gorin, G., Anal. Biochem. 3, 257 (1962).

26. Habeeb, A. F. S. A., Can. J. Biochem. Physiol. 38, 269 (1960).

27. Benesch, R., et al., Science 123, 981 (1956).

28. Price, C. A., and Campbell, C. W., Biochem. J. 65, 512 (1957).

29. Rhinesmith, H. R., Schroeder, W. A., and Pauling, L., J. Am. Chem. Soc. 79, 4682 (1957).

30. Shell, J. M., and Weissberg, A., J. Am. Chem. Soc. 61, 450 (1939).

31. Truce, W. E., et al., J. Am. Chem. Soc. 78, 2743 (1956).

32. Dmuchovsky, B., Vineyard, B. D., and Zienty, F. B., J. Am. Chem. Soc. 86, 2874 (1964).

33. Lee, C. C., and Samuels, E. R., Can. J. Chem. 42, 168 (1964).

34. Leslie, J., Anal. Biochem. 10, 162 (1965).

35. Frost, A. A., and Pearson, R. G., "Kinetics and Mechanism", Wiley, Inc., New York, 1953.

36. Borsook, H., Ellis, E. L., and Huffman, H. M., J. Biol. Chem. 117, 281 (1937).

37. Ryklan, R. L., and Schmidt, C. L. A., Arch. Biochem. 5, 89 (1944).

38. Gorin, G., J. Am. Chem. Soc. 78, 767 (1956).

39. Grafius, M. A., and Neilands, J. B., J. Am. Chem. Soc. 77, 3389 (1955).

40. Laidler, K. J., "Chemical Kinetics", 1st ed., McGraw-Hill Inc; New York, 1950.

41. Li, N. C., Gavron, O., and Bascuas, G., J. Am. Chem. Soc. 76, 225 (1954).

VITA

Petar A. Martic

Candidate for the Degree of

Master of Science

Thesis: KINETICS OF REACTION OF N-ETHYLMALEIMIDE WITH CYSTEINE AND SOME CONGENERS

Major Field: Physical Chemistry

Biographical:

Personal Data: Born in Split, Yugoslavia, on July 6, 1938, the son of the late Ante Martic and Mrs. Tonci Martic.

Education: Graduated from High School, Split, Yugoslavia, in 1957. Received the Bachelor of Science degree from the University of Belgrade, Yugoslavia in June, 1962.

Professional Experience: Graduate Research Assistant, Oklahoma State University, January to August, 1964, and Summer, 1965; Graduate Teaching Assistant, Oklahoma State University, Fall, 1964 and Spring, 1965.