REACTION OF 5,5'-DITHIOBIS(2-NITROBENZOIC ACID) WITH MERCAPTANS AND PROTEIN MERCAPTO GROUPS

By

SHAKTI KUMAR AIREE

Bachelor of Science
(Honours School)
Panjab University, Solan
Panjab, India
1956

Master of Science
(Honours School)
Panjab University, Chandigarh
Panjab, India
1958

Submitted to the Faculty of the Graduate College
of the Oklahoma State University
in partial fulfillment of the requirements
for the degree of
DOCTOR OF PHILOSOPHY
May, 1967

REACTION OF 5,5'-DITHIOBIS(2-NITROBENZOIC ACID)

WITH MERCAPTANS ANS PROTEIN MERCAPTO GROUPS

Thesis Approved:

George Gorin
Thesis Adviser

E J Eisenbraun

J. Paul Hevlin

Clarence M. Cunningham

J Brickl

N N Durham
Dean of the Graduate College

ACKNOWLEDGMENTS

I wish to express my gratitude to Professor George Gorin for his invaluable guidance, counsel, and patience during the course of the research work and the preparation of this thesis. My gratitude is also extended to Dr. M. Quintiliani for invaluable help during the work on yeast alcohol dehydrogenase. Thanks are due Messrs. Gavin Doughty and Walter Godwin for assistance in calculations and for helpful discussions.

Financial assistance from the National Institutes of Health is gratefully acknowledged.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS AND SYMBOLS

$A_{(\ )}$	absorbance at ( )
BMD	3,6-bis(acetatomercurimethyl)dioxane
EDTA	disodium ethylenedinitrilotetraacetate
G-HCl	guanidine hydrochloride
GSSG	glutathione (oxidized)
NAD^+	nicotinamide-adenine dinucleotide
nbSSbn	5,5'-dithiobis(2-nitrobenzoic acid)
NEM	N-ethylmaleimide
PCMB	p-chloromercuribenzoate
$P(SH)_n$	mercaptan or mercapto containing protein
SDS	sodium dodecyl sulfate
YADH	yeast alcohol dehydrogenase
μ	ionic strength

CHAPTER I

INTRODUCTION

This thesis deals with the reactions of 5,5'-dithiobis(2-nitrobenzoic acid) (nbSSbn), which was first synthesized by Ellman (1) and used by him for the determination of mercapto compounds in biological samples. Since then the reagent has found considerable use for the same purpose and also for the determination of mercapto groups in proteins. Its use as a mercapto-group reagent is based on the fact that each -SH group which reacts with this reagent liberates one nbS^- anion which can be determined spectrophotometrically. The stoichiometry of the reaction might be represented by one of the following three equations:

$$\text{P-SH} + \text{nbSSbn} \rightleftharpoons \text{P-SS-bn} + \text{nbSH} \qquad (1)$$

$$2\text{P-SH} + \text{nbSSbn} \rightleftharpoons \text{P-SS-P} + 2\text{nbSH} \qquad (2)$$

$$\text{P}\begin{matrix}\text{-SH}\\ \text{-SH}\end{matrix} + \text{nbSSbn} \rightleftharpoons \text{P}\begin{matrix}\text{-S}\\ \text{-S}\end{matrix} + 2\text{nbSH} \qquad (3)$$

where P represents either a simple radical or a protein. The reaction is a special case of mercaptan-disulfide interchange, which plays an important role in protein chemistry, e.g., in enzyme catalysis (2), radiation protection (3), protein aggregation (4), cell division (5), etc. The following aspects of mercaptan-disulfide interchange, involving nbSSbn, are examined in this thesis: (a) the stoichiometry of the reactions, i.e., determination of whether they accord with the

aforementioned possibilities, and its significance; (b) effect of the reaction on the catalytic activity of the enzyme yeast alcohol dehydrogenase (YADH); and (c) utilization of the reaction in a study of the effect of X-irradiation.

Before carrying out these objectives, certain other studies were done with nbSSbn and with YADH. The stability of nbSSbn solutions was checked at different pH values and the pK_a of nbSH was determined. In regard to YADH, various assay methods were critically tested and a reliable assay method developed. The effect of other mercapto group reagents was compared to that of nbSSbn.

The main findings and conclusions are summarized in a form suitable for publication in journals and are presented in the Appendices. Chapter III presents the methods and results of the studies done with nbSSbn, except its reaction with YADH. This and other studies on the enzyme are described in Chapter IV.

Chapter II gives a review of the pertinent literature.

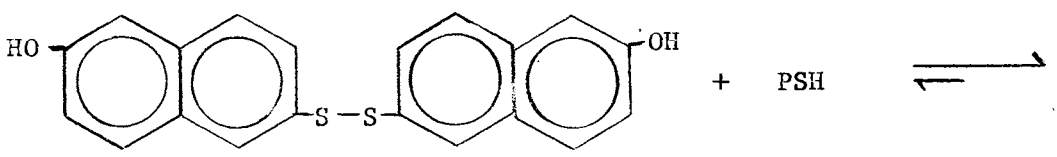
CHAPTER II

REVIEW OF PERTINENT LITERATURE

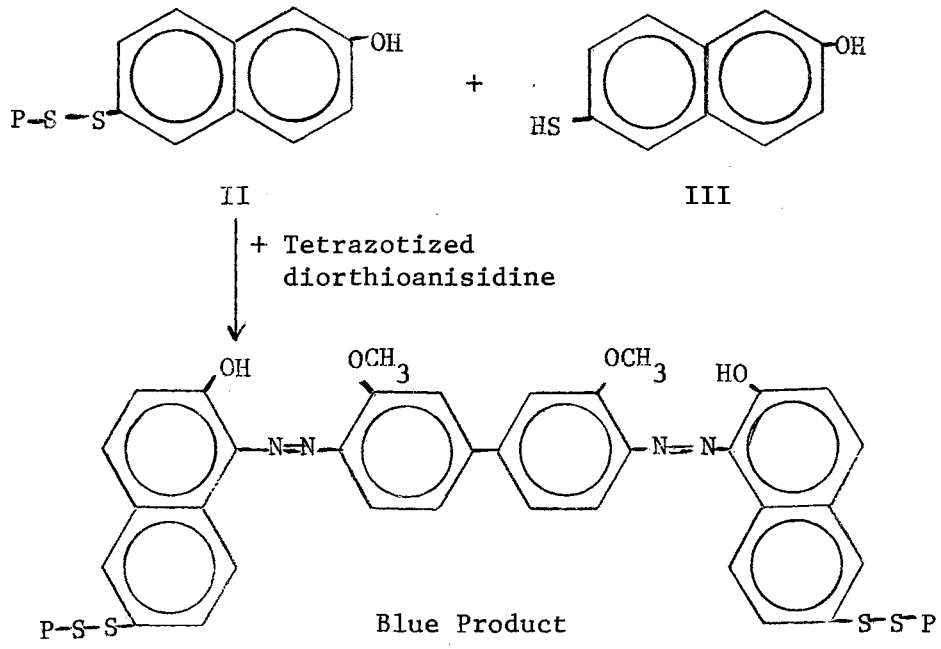
The vastness of the literature dealing with the use of nbSSbn and with the enzyme, yeast alcohol dehydrogenase can be appreciated from the facts that in 1965 alone, there were 70 papers making reference (6) to Ellman's paper on nbSSbn (1) and that four reviews (7-10) have appeared on the enzyme since 1959, each emphasizing, of course, a different aspect of the subject. In order to limit the review to manageable proportions only the most pertinent aspects of the extant literature are reviewed; it is felt that they provide an adequate background to the thesis.

Interaction of Aromatic Disulfides with Proteins

The first study of this interaction seems to have been made by Barnett and Seligman (11), who used 2,2'-dihydroxy-6,6'-dinaphthyl disulfide (I) in order to demonstrate protein-bound sulfhydryl groups histochemically. The following equations illustrate the sequence of reactions involved:



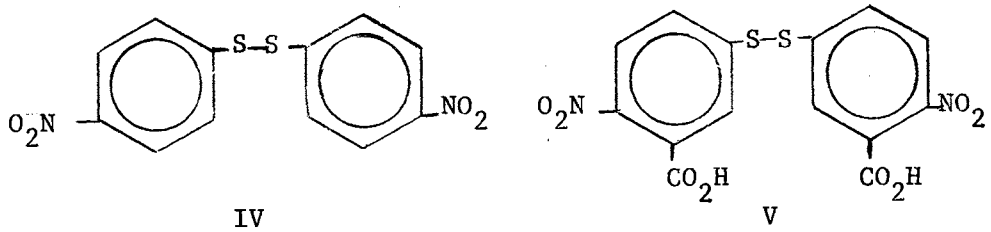
I



Blue Product

Since I and III are soluble in water as well as in ether and alcohol, and II is not, the procedure was to react I with tissue bound sulfhydryl groups, wash away excess I and resultant III and develop color or stain by reacting II with tetrazotized diorthoanisidine.

In 1958, Ellman (12) introduced another reagent, bis(p-nitrophenyl) disulfide (IV); it was demonstrated that the new reagent reacts quantitatively with mercaptans to release p-nitrobenzenethiol. The anion

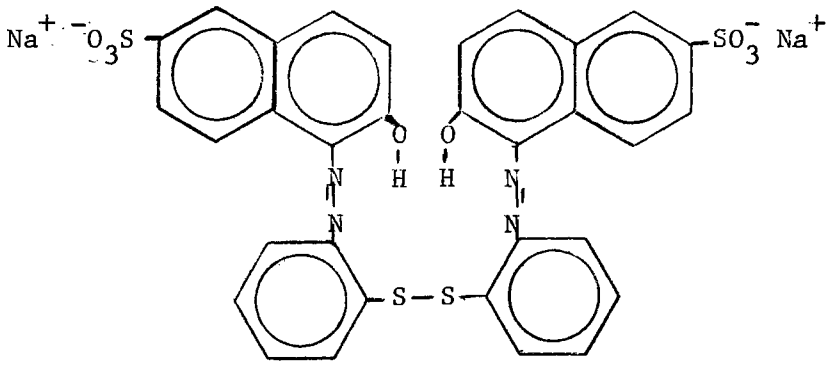


IV V

obtained from p-nitrobenzenethiol is colored--it has an extinction of 13,600 cm^2 $mole^{-1}$ at 412 mμ--and therefore the extent of reaction could be demonstrated without adding any other chemical. This reagent was

further improved by Ellman in 1959 (1), who synthesized a water soluble disulfide, nbSSbn (V); IV had to be used in acetone solution.

Klotz et al., studied the interaction of 2,2'-(2-hydroxy-6-sulfonaphthyl-1-azo)diphenyl disulfide (VI) with serum albumin, ovalbumin and β-lactoglobulin (13). The course of the reaction was followed



VI (apSSap)

by measuring the spectral changes that occur when the free disulfide VI reacts with the protein mercapto groups. It was found that bovine serum albumin reacted with apSSap in 2:1 ratio and that the molecular weight of the protein remained unchanged, as determined by ultracentrifugal and equilibrium dialysis experiments. This stoichiometry was explained by the following equations:

$$\begin{array}{l} \text{-SH} \\ \text{P-S} \\ \text{-S} \end{array} + \text{apSSap} \longrightarrow \begin{array}{l} \text{-S-S-ap} \\ \text{P-S} \\ \text{-S} \end{array} + \text{HS-ap}$$

$$\begin{array}{l} \text{-SH} \\ \text{P-S} \\ \text{-S} \end{array} + \text{HS-ap} \longrightarrow \begin{array}{l} \text{-S-S-ap} \\ \text{P-SH} \\ \text{-SH} \end{array}$$

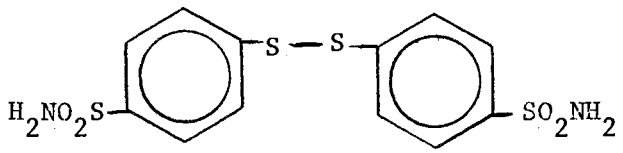
It was assumed that thiol groups produced in the above reactions were "masked" and hence that no further reaction would take place; a Grotthus-type mechanism was implicated for their production. For the

case of ovalbumin, 2 moles of VI were cleaved for each protein molecule. This protein contains four mercapto groups and the mechanism in this case presumably was the same as for small molecular weight mercaptans

$$2\ \text{P-SH} + \text{apSSap} \longrightarrow \text{P-S-S-P} + 2\ \text{apSH}$$

A protein dimer was assumed as the product because some precipitation occurred. In the presence of the sodium dodecyl sulfate no precipitation was reported; since no sedimentation analyses were done the basis for postulating an intermolecular reaction is uncertain. In the case of β-lactoglobulin, the reaction proceeded only to the mixed disulfide stage.

Brighenti *et al.* studied the reaction of sulfanilamide disulfide (VII) and its derivatives with some enzymes and the effect on the enzymatic activity (14,15). The course of reaction was followed by



VII

measuring spectral changes. They found, for example, that VII and its derivatives strongly inhibited dehydrogenases; that the inhibition was pH dependent, non-competitive and of mixed type with respect to co-enzyme and substrate respectively. The inhibition could not be reversed by cysteine and it was ascribed to mixed disulfide formation.

Uses of nbSSbn

As was mentioned in the last section, Ellman (1) used this reagent for determining mercapto compounds in biological fluids. This reagent

has some advantages over other mercapto group reagents and it can be adapted to making some other studies of protein structure and reactions. One advantage of this reagent is that the color change produced after reaction is in a part of the spectrum where the other reacting species do not absorb. Also, the reaction with mercapto groups should be quite specific.

Table I gives a list of well characterized pure proteins (including enzymes) that have been studied with nbSSbn; other pertinent data that were given in the references are included. It can be seen that a denaturant was usually required in order to get a reasonably fast reaction. Comparison with the results of other reagents indicates that nbSSbn was more specific. The last seven references show how nbSSbn has been used to study the relationships between the enzymatic activity and -SH content. It may be noted, for example, that L-arginine phosphokinase was completely inhibited by only one equivalent of nbSSbn, while in yeast hexakinase 4-6 groups could be titrated without loss of any activity. It may also be noted that the inhibition by nbSSbn did not always parallel the inhibition by other reagents.

Some of the other uses of nbSSbn include the estimation of coenzyme A (48,49) released during reactions, e.g.

$$\text{Acetyl-Co-A} + \text{Isopropyl } \beta\text{-D-thiogalactoside} \rightarrow \text{Co-A-SH} + \text{acetylated isopropyl } \beta\text{-D-thiogalactoside}$$

$$\text{nbSSbn} + \text{CoA-SH} \longrightarrow \text{nbSSCoA} + \text{nbSH}$$

The reagent has been used in an improved method for determining glutathione in blood (50). An ingenious adaptation of the method has been utilized for the assay of nicotinamide-adenine dinucleotide phosphate

TABLE I

SURVEY OF PROTEINS REACTED WITH nbSSbn

Protein (Reference)	Pertinent Findings
Acetoacetate Decarboxylase (16)	2.2 -SH groups reacted for each of 8 subunits of 33,000 M.W. Amino acid analysis indicated 3 -SH groups; one reacted in the native state, the second one only after denaturation, the third one was not accounted for.
Adenosine Triphosphate Creatine Phosphotransferase (17)	Eight half-cystines were found by amino acid analysis, 6-7 reacted with nbSSbn.
Arginino Succinase (18)	1.07 -SH groups reacted for the fully active enzyme, 1.37 for the reversibly inactivated enzyme and 1.52 for the irreversibly inactivated enzyme.
ATPase (Mitochondrial) (19)	Most of the -SH groups in the native enzyme were unavailable to nbSSbn, but reacted rapidly in the presence of urea or SDS.
ATPase (Rat Brain) (70)	'A significant portion' of the -SH groups was unmasked in the presence of Triton-X-100. The inhibitory effects of PCMB and NEM on ATPase activity were different in the detergent-treated and untreated preparations.
ATP-Lombricine Phosphotransferase (21)	Six -SH groups reacted with nbSSbn (pH 6.8), NEM and PCMB (pH 7.0) in the presence of urea. Only one -SH group was found essential for activity.
ATP-Taurocyamine Phosphotransferase (21)	8 to 9 -SH groups reacted with nbSSbn, NEM and PCMB; only four were required for activity.
Carbamyl Phosphate Synthetase (22)	39 -SH groups were found for 315,000 M.W. as compared to the earlier value of 41-44. Kinetics were first order with respect to nbSSbn. Base produced an orange color and absorbance at 412 mμ increased with time even at pH 7.0. Some -SH groups reacted preferentially with PCMB and others with nbSSbn.

TABLE I (Continued)

Protein (Reference)	Pertinent Findings
Chymotrypsin (23)	nbSSbn was used to measure -SH groups while studying disulfide interchange catalyzed by a microsomal enzyme.
Citrate Condensing Enzyme (24)	In the presence of urea 4 -SH groups reacted for 80,000 M.W., no reaction without urea. The reaction was used to measure unfolding of the protein; the rate of unfolding was found to be first order, dependent on urea concentration and temperature.
D-Amino Acid Oxidase (25)	Reaction with nbSSbn, PCMB, Ag^{110} and amperometric titration all gave the same result: 11.8 ± 0.5 for 100,000 M.W.
Fatty Acid Synthetase (Pigeon Liver) (26)	50 -SH groups were found per mole.
γ-Globulin (Human) (27)	0.48 -SH groups and 27 -SS- groups were found. nbSSbn was judged to be more sensitive than NEM and PCMB.
Glutamate Dehydrogenase (28)	No reaction took place with the native enzyme. In the presence of 0.0008M SDS or 4M guanidine hydrochloride, 114 -SH groups reacted for 10^6 M.W. Earlier, 120 -SH groups were found by amperometric titration.
α-Glycerophosphate Dehydrogenase (29)	11.9 -SH groups were found with nbSSbn, 11.8-16.6 groups with PCMB, for 78,600 M.W.
Hemoglobin (Human) (30)	Pseudo-first order curves were obtained in the presence of 5-fold molar excess of nbSSbn.
Insulin (23)	nbSSbn was used to measure -SH groups while studying disulfide interchange catalyzed by a microsomal enzyme.
Isoleucyl Ribonucleic Acid Synthetase (31)	Out of 15 half-cystines for 112,000 M.W., 8-11 reacted with NEM or nbSSbn in 6M guanidine hydrochloride.
Isophenoxazine Synthetase (32)	2 -SH groups reacted for 24,000 M.W. but only in the presence of the substrate, o-aminophenol.

TABLE I (Continued)

Protein (Reference)	Pertinent Findings
β-Lactoglobulin (33)	nbSSbn reacted with 1.1 -SH groups in the native state, 1.9 in the denatured protein. NEM did not react with the native protein, but gave a value of 1.88 with SDS-denatured protein. PCMB reacted with 1.8 -SH groups and iodine with 2.0 to 2.1.
Lysine-Transfer Ribonucleic Acid (34)	2 -SH groups reacted in the native state, one more on denaturation; 0.0001 EDTA was used, as metal ions interfered with nbSSbn reaction.
Ovalbumin (33)	3.8 -SH groups reacted with nbSSbn in the presence of SDS, no reaction for the native protein. NEM reacted with 4.0 -SH groups in the denatured protein, but only with 0.4-0.6 in the native protein; the corresponding values for the PCMB reaction were 2.7-2.8 and 3.8-4.1. Iodine reacted with 3.0 $\pm$ 5% -SH groups in three minutes
Potato Virus X (35)	NEM did not react, PCMB was not satisfactory, nbSSbn precipitated the protein. Value obtained by latter reagent gave 1 -SH $\pm$ 25%.
Pyruvate Carboxylase (36)	55 -SH groups and no disulfides were found for 655,000 $\pm$ 20,000 M.W.
Pyruvate Transaminase (37)	nbSSbn did not react in the native state, but in the presence of 0.5% SDS, 5.5 -SH groups found; 4 equivalents of PCMB caused turbidity at pH 6.9 in 8M urea, but 6 -SH groups were found at pH 8.0.
Ribonuclease (23,28)	Progress of oxidation of the reduced protein was followed by reaction with nbSSbn and compared with the extent of reactivation (38). nbSSbn was used to measure the -SH content while studying disulfide interchange catalyzed by a microsomal enzyme (23).
Serum Albumin (Bovine) (27,33)	0.3-0.75 -SH groups were found for different preparations; denaturation caused no difference (33). 0.62 -SH groups and 17 disulfide groups were found (27).

TABLE I (Continued)

Protein (Reference)	Pertinent Findings
Serum β-Lipoprotein (Human) (39)	'Excellent' agreement was found between the results obtained by nbSSbn and by iodoacetate after reduction of the -SS-bonds.
Urease (40)	10.5-11.0 -SH groups reacted with 50 molar excess of nbSSbn.
α-Amylase (from Soya Beans) (41)	nbSSbn reacted with one -SH and the activity was not lost even when an excess of nbSSbn was used. PCMB reacted with 5 -SH groups causing 99% inactivation.
α-Amylase (Hog Pancreas) (42)	1.7 -SH groups reacted only if both EDTA and SDS were present, none reacted if either were absent. NEM and nbSSbn caused 80% inhibition.
Carboxydismutase (43)	nbSSbn reacted over a period of 30 min and gave an equivalent weight of 131,000 at pH 8.0. PCMB reacted by first order and iodoacetamide by second order kinetics; both inhibited the enzyme.
Citrate Condensing Enzyme (44)	nbSSbn did not react with the native enzyme at pH 8.1, but in the presence of urea 3.8 -SH groups reacted. PCMB gave a value of 1.9 for the native and 4.4 for the denatured enzyme. Value obtained by amino acid analysis was 6.0. NEM and iodoacetamide did not inhibit, PCMB caused inhibition.
α-Isopropylmalate Synthetase (45)	nbSSbn strongly inhibited the enzyme, NEM had no effect on activity.
L-arginine Phosphokinase (46)	Reaction with nbSSbn was fast at pH 8.5, but at pH 7.0 three groups reacted slowly and the other three fast. When reacted with 1-6 equivalents of nbSSbn, it was found that one equivalent of nbSSbn completely inhibited the enzyme. Three equivalents of NEM reacted faster and caused complete inhibition. PCMB, NEM and nbSSbn all gave a value of 6 -SH groups per mole.

TABLE I (Continued)

Protein (Reference)	Pertinent Findings
Yeast Hexokinase (47)	Both nbSSbn and PCMB gave the same results, 7.8 -SH groups for 96,000 M.W. Activity was decreased as a function of the reaction with nbSSbn; however, 4-6 groups could be titrated without loss of activity.

in blood (51) by the following series of reactions:

$$GSSG + NADPH_2 \xrightarrow[\text{Reductase}]{\text{Glutathione}} 2GSH + NADP$$

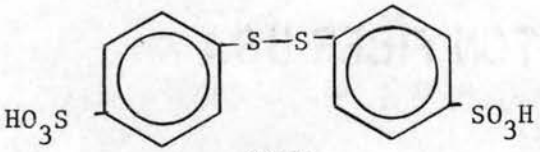
$$2\,GSH + nbSSbn \longrightarrow GSSG + 2nbSH$$

Another application of nbSSbn has been made in the study of the enzyme acetylcholinesterase by substituting acetylthiocholine for the substrate, thus:

$$H_2O + (CH_3)_3N^+CH_2CH_2S\overset{\overset{O}{\|}}{C}CH_3 \xrightleftharpoons{\text{Acetylcholinesterase}} (CH_3)_3N^+CH_2CH_2S^- + CH_3CO_2^- + 2H^+$$

$$(CH_3)_3N^+CH_2CH_2S^- + nbSSbn \rightleftharpoons (CH_3)_3N^+CH_2CH_2SSbn + nbS^-$$

One of the problems that will be considered in the thesis is the possible reversal of the mercaptan disulfide interchange reactions. A general discussion of the equilibria involved for a very similar disulfide, 4,4'-dithiobis(benzenesulfonic acid) (VIII) has been given by Smith et al., (53), and by Gorin et al., (54). Another problem which



VIII

will be considered is the hydrolytic cleavage of nbSSbn. A review of the various chemical reactions that cleave disulfide bonds has been given by Parker and Kharasch (55). Swan (56) has also given a brief review of the subject. It emerges from these reviews that the aromatic disulfides easily undergo hydrolytic decomposition but that the amount and even the nature of the products is not known. The following initial steps have usually been postulated:

$$ArSSAr + H_2O \longrightarrow ArSOH + ARSH$$

or

$$ArSSAr + OH^- \longrightarrow ArSOH + ArS^-$$

but this may be followed by further reaction.

Stereochemical conformation plays an important role in determining the reaction of nbSSbn with proteins; thus study of the latter can throw useful light on the relative position of mercapto groups. nbSSbn may be found useful for the oxidation of fully reduced proteins, e.g., for combining the component chains of reduced insulin or for regenerating the activity of reduced lysozyme or ribonuclease.

Yeast Alcohol Dehydrogenase

The systematic name of this enzyme, as given by the International Union of Biochemistry, is alcohol-NAD^+ oxidoreductase, EC 1.1.1.1. (57). It catalyzes the following general reaction:

$$R\text{-}CH_2OH + NAD^+ \rightleftharpoons NADH + R\text{-}CHO + H^+$$

where $R\text{-}CH_2OH$ may be a primary or a secondary alcohol and NAD^+ stands for a pyridine nucleotide. For extensive reviews on this enzyme, one may consult Sund and Theorell (7), Hoch and Vallee (8), Wallenfels et al., (9), and McKinley-McKee (10). In this section only a summary of the pertinent parts of the aforementioned reviews is given as well as references to the subsequent literature.

The equilibrium constant for ethyl alcohol and NAD^+, nicotinamide-adenine dinucleotide, at 20°, between pH 7 and 10, $\mu = 0.1$, is

$$K_{eq} = \frac{[NADH][CH_3CHO][H^+]}{[NAD^+][CH_3CH_2OH]} = (8.01 \pm 0.14) \times 10^{-12} M$$

The temperature variation at $\mu = 0.1$ is (58)

$$\log K = \frac{1554}{T} - 5.80$$

$$\text{or} \quad K = 1.6 \times 10^{-6} \, e^{7130/RT}$$

$$\Delta H^{o} = 7130 \pm 200 \text{ cal mole}^{-1}$$

$$\Delta S^{o} = -26.5 \text{ cal mole}^{-1} \text{ degree}^{-1}$$

$$\Delta F^{o} = 1489 \text{ kcals mole}^{-1} \text{ at } 20^{o}$$

The equilibrium obviously is in favor of the left side, i.e., the reduction of acetaldehyde. Since for the assays of the enzymatic activity it is desirable to have NAD^{+} reduced to NADH, which has a characteristic high absorption at 340 mμ, an alkaline pH (8.8) and a high concentration of ethanol are used. The various conditions employed by different investigators for assaying activity are summarized in Table B-1 in Appendix B.

YADH is a "metallo enzyme" as well as a "sulfhydryl enzyme", i.e., the enzymatic activity depends both on the zinc content and on the mercapto group content. Each molecule (M.W. 150,000) contains 4-5 zinc atoms (59,60), the removal of which causes dissociation of the enzyme into four sub-units (61) which are inactive. The enzyme contains many mercapto groups, but the actual number is in question.

There have been conflicting reports regarding the inactivation of this enzyme by reaction with p-chloromercuribenzoate, a mercapto group reagent. According to Barron and Levine (62) all the -SH groups were essential for activity; Keleti (63) reported that all the activity was destroyed on reaction with only two -SH groups. Hoch and Vallee (8) studied activity and sulfhydryl content as a function of various parameters, but could not decide whether all or all but seven -SH groups were essential for activity. Wallenfels and Sund (64)

found that activity was linearly related to the number of -SH groups. McKinley-McKee (65) reported that activity did not vary linearly with -SH content but did become zero when all the -SH groups had reacted with p-chloromercuribenzoate. Whitehead and Rabin (66) found that iodoacetamide had a much higher selectivity than PCMB; in fact, they hypothesized that it reacted only with the thiol group in the catalytic site. No work has so far been done with nbSSbn.

In a most significant paper on this enzyme, Harris (67) has reported the amino acid sequence around the reactive cysteine. He has concluded that both in liver and in yeast enzyme, the fundamental structural unit consists of a protein chain of molecular weight 36,000. This structural unit binds NAD/NADH and zinc and contains a reactive -SH group. The sequence around the -SH group is different in the two enzymes, but the changes do not radically alter the sequence.

Enzyme Source	Sequence
Liver	. . . Thr.Gly.Ile.Cys.Arg.Ser.Asp. Asp. His. Val. . .
Yeast	. . . Ser.Gly.Val.Cys.His.Thr.Asp. Leu. His. Ala. . .

Discrepant results have been reported regarding the inactivation of this enzyme by X-irradiation. Barron and Johnson (68) reported a very high ionic yield whereas Lange, Pihl and Eldjarn (69) repeated the experiments under similar conditions and obtained a very low yield. Romani and Tappel (70) studied the loss of both the activity and the sulfhydryl groups on X-irradiation under anaerobic conditions. The ionic yield obtained by them was comparable to that obtained by Lange et al. They also found that complete enzymatic inactivation was achieved when only a third of the available -SH groups had been destroyed, i.e., a decrease from about 18 to 12. In a later publication,

Shimazu and Tappel (71) reported an ionic yield of 0.15 molecules/100 e.v. A very high value (3.0 molecules/100 e.v.) was reported by Hutchinson and Preston (72) as late as 1963. Table 2 gives a summary of the ionic yields obtained by various investigators.

TABLE II

INACTIVATION YIELDS OF YADH ON X-IRRADIATION

Reference	G-Value (Molecules/100 e.v.)
Barron and Johnson (68)	1.30
Romani and Tappel (70)	0.06
Lange et al. (69)	0.06
Hutchinson and Preston (72)	3.0
Shimazu and Tappel (71)	0.15

CHAPTER III

REACTIONS OF 5,5'-DITHIOBIS(2-NITROBENZOIC ACID)

In this chapter will be described the methods and findings in regard to the stability of the nbSSbn solutions at different pH values, reduction with sodium borohydride, pK_a of the thiol (nbSH), rates of reactions with cysteine, β-mercaptoethanol and glutathione at pH 5.0 and the stoichiometry of the reactions with ovalbumin, β-lactoglobulin and cysteine.

Stability of nbSSbn Solutions

A 10^{-3}M nbSSbn solution was prepared by dissolving 39.6 mg of nbSSbn in 100 ml of 0.1 M phosphate buffer, pH 6.8. This solution was then diluted 20-fold with additional buffer and varying amounts of 1 M NaOH was added to realize the desired final pH. The absorbances at 410 mμ found at various intervals for solutions of different pH are presented in Table III; the extent of decomposition can be deduced from the magnitude of the absorbance.

To check whether this decomposition is catalyzed by trace metal ions that might be present in the reagents, experiments were conducted with and without added EDTA. In the presence of EDTA, the rate is considerably reduced as is obvious from the Table.

TRIS buffer was used at pH 8 to 10, since phosphate does not buffer well in this range. The results of these experiments were the same as

TABLE III

ABSORBANCE AT 410 mμ OF 5 x 10^{-5}M nbSSbn SOLUTIONS

AT DIFFERENT pH VALUES

pH (buffer)	A_{410} at 2 hrs.	1 day	2 days
6.86 (0.1M phosphate)	.016	.018	.030
7.65 (0.1M phosphate)	.017	.027	.035
7.9 (0.1M phosphate)	.015	.035	.055
10.0 (0.1M phosphate)	.285	.860	.770
11.0 (0.1M phosphate)	.940	.960	.87
11.2 (0.1M phosphate)	1.0	.960	.87
7.0 (0.1M phosphate, no EDTA)	.022	.028	.035
7.0 (0.1M phosphate, 10^{-3}M EDTA)	.020	.018	.023
8.0 (0.1M phosphate, no EDTA)	.025	.038	.052
8.0 (0.1M phosphate, 10^{-3}M EDTA)	.018	.018	.025
7.40 (TRIS)	.022	.030	----
7.95 (TRIS)	.023	.033	----
8.45 (TRIS)	.025	.050	----
8.90 (TRIS)	.033	.092	----
9.10 (TRIS)	.045	.125	----

in phosphate buffer, i.e., the rate of decomposition or of hydrolytic splitting of the disulfide bond increases with pH.

Reduction of nbSSbn with Sodium Borohydride

The reductions were carried out in boric acid buffer, for the reason that the products of reaction, viz. NaOH and boric acid, are the same as the constituents of the buffer. Besides, it will be seen that this buffer provides the right pH range. Buffer was prepared (73) by taking 3.10 g (0.05 moles) of boric acid (Fischer Reagent Grade), 3.73 g (0.05 moles) of KCl and 21.3 ml of 1 M NaOH and making the volume 1 liter with air-free distilled and de-ionized water.

For making a rate study, 0.0375 g of sodium borohydride (Matheson, Coleman and Bell, 98%) was put in a 100-ml volumetric flask and 85-90 ml of boric acid buffer added. Then 5 or 10 ml of approximately 2.5×10^{-5} M nbSSbn solution in boric acid buffer was added and the volume quickly made 100 ml. Aliquots of the solution were withdrawn at intervals and the absorbance measured at 410 mμ. The reaction was complete in less than 10-15 min and the half-life was 1.1 min. The data for three experiments are plotted in Fig. 1.

In order to establish the optimum pH for the reduction, the same procedure was repeated in buffers of pH 7.70, 8.10, 8.65, 9.20 and 9.30. Incomplete reduction was realized at the two lowest pH values. At the lower pH values sodium borohydride was decomposed very quickly.

In a pair of experiments done to prepare cysteine from cystine by reduction with sodium borohydride by above procedures, only about 60% yield was realized. Even at 50° for 1 hr, the yield increased only to 75%. There seems to be some difficulty in the reaction with cystine,

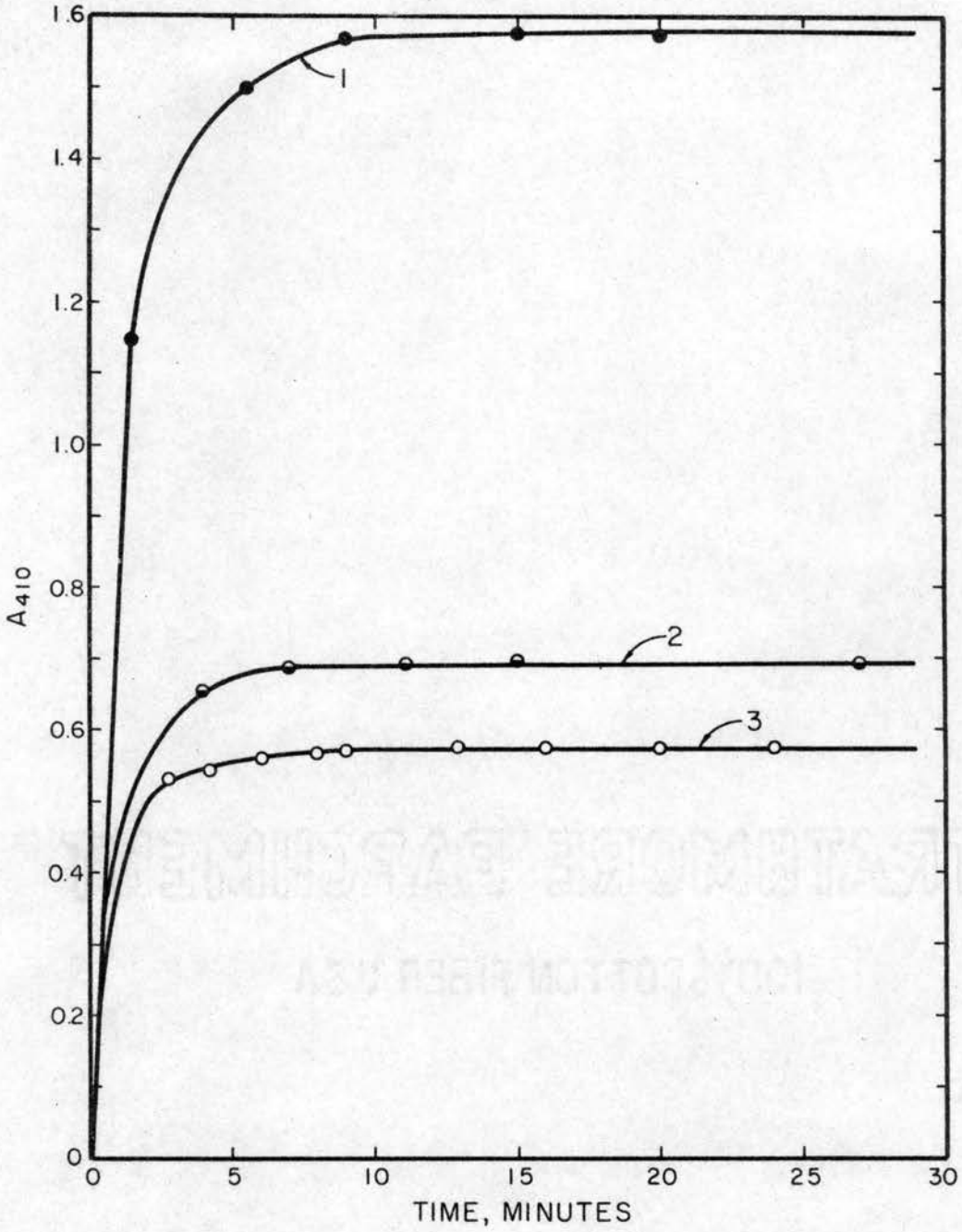


Fig. 1. Reduction of nbSSbn with sodium borohydride at pH 9.0. (1) 5.80 x 10^{-5}M nbSSbn (2) 2.58 x 10^{-5}M nbSSbn (3) 2.13 x 10^{-5}M nbssbn

which was not investigated further.

Determination of pK_a for nbSH

The procedure consisted essentially of producing nbS^- by the reduction of nbSSbn with sodium borohydride and then titrating it in acetate buffer of known ionic strength (=μ)

Determinations of the pK_a at μ = 1.1 were done in the following manner: 45.25 mg (1.14×10^{-4} moles) of nbSSbn in 100 ml boric acid buffer was treated with 0.037 g (10^{-2} moles) of sodium borohydride for 1 hr. Five milliliters of the resulting solution was diluted to 250 ml with acetate buffer (0.1 M sodium acetate, 1 M NaCl, 10^{-3} M EDTA, 10 ml of 1 M HCl in one liter) and then placed in a 500-ml Erlenmyer flask. Oxygen-free nitrogen was bubbled slowly through the solution. Varying amounts of 1 M HCl were added; after each addition an aliquot was withdrawn, its pH and its absorbance at 410 mμ were measured.

The same procedure was employed at μ = 0.12, except that the acetate buffer used this time was 0.02 M in sodium acetate, 0.1 M in NaCl and 10^{-3} M in EDTA. The titrations were done both by starting at pH 4.1 and going to pH 5.05 by adding 0.1 M NaOH and by starting from pH 5.15 and going to 3.48 by adding 0.1 M HCl.

The pK_a was calculated from the following expression (74):

$$pH = pK_a + \log \frac{[nbSH]}{[nbS^-]} = pK_a + \log \frac{A'-A}{A}$$

where A is the absorbance at given pH and A' that at pH 7.0 or above. The value of pK_a at μ = 0.12 was 4.41 and that at μ = 1.2 was 4.13; an "infinite dilution" value of 4.65 may be estimated from the extended Debye-Huckel equation (74). The results for the titration at μ = 0.12

TABLE IV

TITRATION OF nbSH AT μ = 0.12

$pH_{(observed)}$	$\log \frac{A'-A}{A}$	$pH_{(calcd)}$[a]	pH
5.15	.65	5.06	-.09
5.01	.55	4.96	-.05
4.83	.39	4.80	-.04
4.70	.25	4.66	-.04
4.50	.10	4.51	+.01
4.29	-.14	4.27	-.02
3.70	-.65	3.76	+.06
4.10	-.37	4.04	-.06
4.17	-.20	4.21	+.04
4.23	-.12	4.29	+.06
4.30	-.07	4.34	+.04
4.38	-.02	4.39	+.01
4.45	+.04	4.45	.00
4.58	.15	4.56	-.03
4.65	.25	4.66	+.01
4.82	.31	4.72	-.10
5.05	.52	4.93	-.12

[a]Calculated from pK_a = 4.41

are tabulated in Table IV, together with the values calculated on the basis of the pK_a determined.

Reaction of nbSSbn with Cysteine, β-Mercaptoethanol and Glutathione

These reactions were studied in acetate buffer of pH 5.0. This was prepared by placing 2.722 g (0.02 moles) of sodium acetate, 5.845 g (0.1 moles) of NaCl, 0.373 g of EDTA in a 1-l. volumetric flask, displacing the air with oxygen-free nitrogen, then adding 5.50 ml of 1 M HCl and the required amount of air-free de-ionized distilled water.

In the experiment, 10^{-3} M nbSSbn solution was prepared in boric acid buffer, 5 or 10 ml of this solution was diluted to 100 ml with acetate buffer. In a 100-ml flask was placed 25 or 50 ml of this solution, acetate buffer was added nearly to the mark, the calculated amount of 10^{-3} - 10^{-4} M PSH solution in acetate buffer was added to give the desired reagent ratio and the volume quickly made up to 100 ml. After shaking well, an aliquot was placed in a stoppered cuvette and its absorbance measured at 410 mμ versus a blank containing all the reagents except PSH.

Representative data are plotted in Fig. 2. Concentration of the mercaptan, C_{PSH}, and that of nbSSbn, C_{nbSSnb}, at any time were calculated from knowledge of the initial concentrations C^o_{PSH} and C^o_{nbSSbn} and the concentration of total nbSH (unionized and ionized thiol), $C_{(nbSH)_T}$, on the basis of the following stoichiometric equation:

$$nbSSbn + 2PSH = 2nbSH + PSSP$$

$C_{(nbSH)_T}$ ($= C_{nbS^-} + C_{nbSH}$) was obtained from A_{410} and from knowledge of the ratio of C_{nbS^-} and C_{nbSH}. Since, at $\mu = 0.12$, pK_a is 4.41

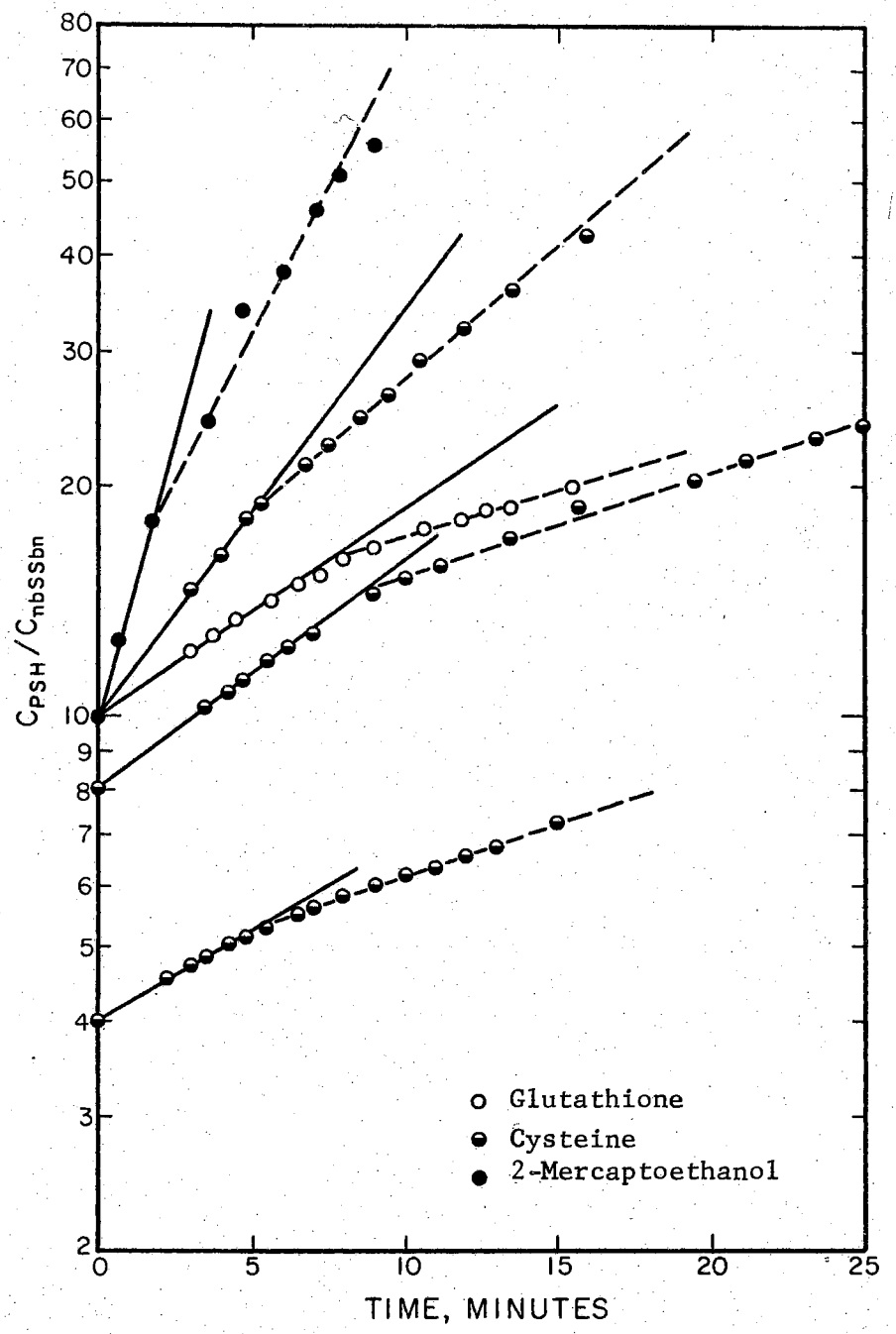


Fig. 2. Rates of reaction of mercaptans.

$$K_a = \frac{C_{H^+} \times C_{nbS^-}}{C_{nbSH}} = 4.0 \times 10^{-5}$$

At pH 5.0, or, $C_{H^+} = 10^{-5}$ M, $\frac{C_{nbS^-}}{C_{nbSH}} = 4.0$

$$\text{or } C_{(nbSH)_T} = C_{nbS^-} + C_{nbSH} = \frac{5}{4} C_{nbS^-}$$

$$\text{where } C_{nbS^-} = \frac{A_{410}}{1.36 \times 10^4}$$

$$\text{so that } C_{(nbSH)_T} = \frac{A_{410}}{1.36 \times 10^4} \times \frac{5}{4}$$

A sample set of results is presented in Table V for the case of reaction where initially 10-molar excess of cysteine was used. The second order rate constants were calculated from the initial slopes using the following equation (75):

$$\text{initial slope} = \frac{\Delta \ln (C_{PSH}/C_{nbSSbn})}{\Delta t} = \frac{(C^o_{PSH} - 2C^o_{nbbSSbn})}{2} k_{PSH}$$

where k_{PSH} is the rate constant for the disappearance of the mercaptan (PSH). With cysteine, the reaction rate was studied at different initial ratios of cysteine to nbSSbn and a value of $(3.6 \pm .8) \times 10^3$ liter mole^{-1} min^{-1} was obtained. When PSH was in 10-fold molar excess, the rate constant for glutathione (1.9×10^3 l.mole^{-1} min^{-1}) was smaller than that for cysteine or for β-mercaptoethanol (6.3×10^3 l.mole^{-1} min^{-1}); however, the order of magnitude is the same in all cases. It may be pointed out that above treatment of the data is not rigorous because a precise analysis of the data would require consideration of at least two consecutive, competitive and reversible reactions; the

TABLE V

REACTION OF nbSSbn WITH 10-FOLD EXCESS OF CYSTEINE

$C^{o}_{nbSSbn} = 2.43 \times 10^{-5}M \qquad C^{o}_{PSH} = 2.43 \times 10^{-4}M$

Time Min	A_{410}	$D_{(nbSH)_T} \times 10^5$	$C_{PSH} \times 10^5$	$C_{nbSSbn} \times 10^5$	$\frac{C_{PSH}}{C_{nbSSbn}}$
3.0	.198	1.82	22.48	1.52	14.8
4.0	.232	2.13	22.17	1.37	16.2
4.83	.265	2.44	21.86	1.21	18.1
5.25	.280	2.57	21.73	1.15	19.0
6.75	.310	2.85	21.45	1.01	21.3
7.5	.325	2.99	21.31	0.94	22.8
8.5	.342	3.14	21.16	0.86	24.6
9.5	.355	3.26	21.04	0.80	26.3
10.5	.373	3.43	20.87	0.72	29.2
12.0	.390	3.58	20.72	0.64	32.4
13.5	.405	3.72	20.58	0.57	36.1
16.0	.425	3.91	20.39	0.48	43.0
58.0	.493				

treatment here assumes that the first step is rate determining, i.e., the slower step:

$$\text{nbSSbn} + \text{PSH} \underset{\text{slow}}{\rightleftharpoons} \text{nbSSP} + \text{nbSH}$$

$$\text{nbSH} \underset{\text{fast}}{\rightleftharpoons} \text{nbS}^- + \text{H}^+$$

$$\text{nbSSP} + \text{PSH} \underset{\text{fast}}{\rightleftharpoons} \text{P-SS-P} + \text{nbSH}$$

$$\text{nbSH} \underset{\text{fast}}{\rightleftharpoons} \text{nbS}^- + \text{H}^+$$

Table VI presents, besides the rate data, the extents of reaction obtainable at pH 5.0. The extent of reaction is the maximum amount of total thiol $C_{(nbSH)_T}$ at equilibrium divided by the amount expected on the basis of the concentration of the limiting constituent. It is seen that the reaction does not go to completion even when a large excess of mercaptan is used at this pH.

Stoichiometry of the Reaction of nbSSbn with Cysteine, β-Lactoglobulin and Ovalbumin

The procedure consisted essentially of taking a certain amount of the nbSSbn solution in each of a series of volumetric flasks, adding different amounts of the protein or the cysteine solution and then solvent to make the desired volume; thus different ratios of the reagents were obtained.

Table VII shows the data and the $nbS^-/P(SH)_n$ ratio finally obtained. The concentration of nbS^- was calculated from the absorbance at 410 mμ read against a blank which contained all the reagents except the

TABLE VI

EXTENTS AND THE RATES OF REACTION OF nbSSbn WITH MERCAPTANS

Mercaptan PSH	$C^{o}_{nbSSbn} \times 10^{5}$	Molar Excess of PSH	% Reaction[a] ϕ	Initial 2nd order rate[b] ($l\text{-mole}^{-1}\text{min}^{-1}$)
Cysteine	2.43	10	92	3.6×10^{3}
Glutathione	2.43	10	92	1.9×10^{3}
β-Mercaptoethanol	2.36	10	92	6.3×10^{3}
Cysteine	2.43	8	86	3.8×10^{3}
Cysteine	3.88	4	82	3.8×10^{3}

[a] $\phi = C^{equil.}_{(nbSH)_T} / 2C^{o}_{nbSSbn}$

[b] $k_{PSH} = \left(\frac{2}{C^{o}_{PSH} - 2C^{o}_{nbSSbn}}\right) \Delta\left(\ln \frac{C_{PSH}}{C_{nbSSbn}}\right) / \Delta t$

TABLE VII

STOICHIOMETRY OF REACTION OF nbSSbn WITH CYSTEINE,

OVALBUMIN AND β-LACTOGLOBULIN

nbSSbn		$P(SH)_n$			Initial nbSSbn/ $P(SH)_n$		Final nbS^-/ $P(SH)_n$
Concn. x 10^4M	Volume taken ml	Concn. x 10^4	Volume taken ml	Final Volume	Ratio	A_{410}	Ratio
		(a) $P(SH)_n$ = Cysteine					
2.28	5	9.12	20	25	.63	1.03	1.0
2.28	5	9.12	15	25	.84	.755	1.0
2.28	5	9.12	10	25	1.25	.522	1.0
2.28	5	1.18	2.9	25	.33	1.20	.645
2.28	5	1.19	15.5	100	.063	.313	.13
2.28	5	9.12	7.5	25	1.67	.374	1.0
2.28	5	1.18	11.7	100	.083	.303	.163
2.28	5	1.18	5.8	100	.33	.600	.643
		(b) $P(SH)_n$ = β-Lactoglobulin					
3.07	1	1.42	0.4	10	.586	.118	1.67
3.07	1	1.42	1.2	10	1.95	.330	1.62
3.07	1	1.42	2.0	10	1.17	.408	1.15
3.07	1	1.42	1.5	10	1.56	.374	1.40
3.07	1	1.42	3.5	10	0.67	.410	0.66
3.07	1	1.42	9.0	10	0.26	.434	0.27
		(c) $P(SH)_n$ = Ovalbumin					
3.60	2	.776	1	25	9.28	.154	3.65
3.60	2	.776	2	25	4.64	.320	3.78
3.60	2	.776	3	25	3.09	.467	3.69
3.60	2	.776	4	25	2.32	.620	3.67
3.60	2	.776	6	25	1.55	.730	2.88
3.60	2	.776	8	25	1.16	.758	2.24
3.60	2	.776	12	25	0.77	.758	1.50

protein or cysteine, $P(SH)_n$. A plot of the data appears in Fig. A-5 of Appendix A, where it is seen that the initial slopes for cysteine and for ovalbumin are the same.

To distinguish between an intramolecular reaction and an intermolecular reaction, sedimentation analyses were made for ovalbumin before and after reaction with nbSSbn in the presence of 1% SDS. For comparison, ultracentrifugal analyses were done also for the native protein (no SDS) and for the product of reaction with another -SH binding reagent, 3,6-bis(acetatomercurimethyl)dioxane, BMD.

Sedimentation data were obtained with the Beckman Analytical Ultracentrifuge Model E using a speed setting of 59780 rpm and the temperature 20°. A typical determination was the following. One milliliter of 6.0×10^{-4} M ovalbumin solution was placed in each of four 5-ml volumetric flasks. To three of these 1 ml of 5% SDS solution was added. To one of the latter flasks 1 ml of 2.5×10^{-3} M nbSSbn solution was added and to another 1 ml of 2.5×10^{-3} M BMD solution. The volume was then made 5 ml in each of the four flasks with phosphate buffer. Approximately 0.35 ml of above solutions were taken in a pair of cells which were then placed in the rotor. Fig. 3 is a photograph of the plates taken during this analysis. The first picture in each case was taken after 50 min from start of spinning; subsequent pictures at 30 min intervals thereafter.

The sedimentation constant, S_T was calculated in the following manner:

Distance of reference hole from center of rotation r = 7.300 cm

$$X_1 = r - b_1$$

$$X_2 = r - b_2$$

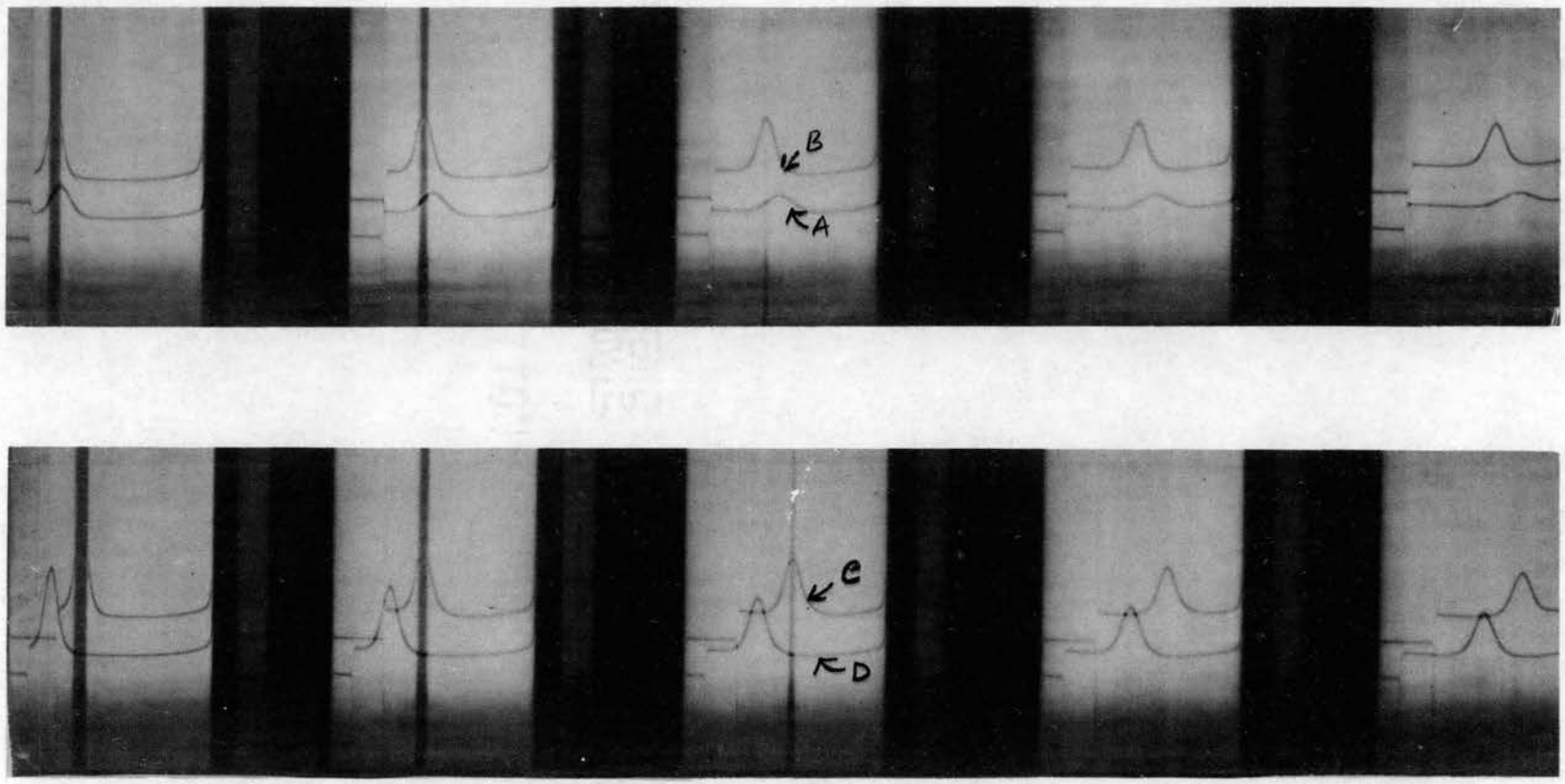


Fig. 3. Sedimentation analysis of ovalbumin.
(A) Native ovalbumin (B) Ovalbumin + 1% SDS
(C) Ovalbumin + 1% SDS + nbSSbn
(D) Ovalbumin + 1% SDS + BMD

TABLE VIII

SEDIMENTATION CONSTANTS OF OVALBUMIN

[Ovalbumin] = 1.2×10^{-4}M

Denaturant	Added Reagent	Distance, X	t min	S_{20}
-	-	6.2018	50	
		6.3337	81	2.89
		6.4656	110	3.09
		6.6107	140	3.03
		6.7556	170	3.07
1% SDS	-	6.1605	50	
		6.2762	81	2.55
		6.3795	110	2.39
		6.4901	140	2.44
		6.6172	170	2.75
1% SDS	5×10^{-4}M nbSSbn	6.2652	50	
		6.3755	80	2.48
		6.4916	110	2.56
		6.6006	140	2.36
		6.6723	170	1.52(?)
1% SDS	5×10^{-4}M BMD	6.0677	50	
		6.1636	80	2.24
		6.2639	110	2.29
		6.3639	140	2.24
		6.4760	170	2.48

where b is obtained by dividing the distance measured on the slide, B, by camera enlargement ratio M (=2.1082 in this experiment)

$$\Delta\chi = \chi_2 - \chi_1$$

$$\chi = \frac{\chi_1 + \chi_2}{2}$$

$$S_T = \frac{1}{\omega^2 \chi} \cdot \frac{\Delta\chi}{\Delta t}$$

$$\omega = \frac{2\pi(\text{rpm})}{60} \text{ radians per sec}$$

Table VIII presents the data obtained. It is seen that the sedimentation constant does not change when ovalbumin reacts with nbSSbn in the presence of 1% SDS. The same is true in the case of 3,6-bis-(acetatomercurimethyl)dioxane.

Determination of Mercapto Groups with nbSSbn in the Presence Guanidine Hydrochloride

Take 5.73 g of guanidine hydrochloride in a 20 ml beaker and add to this 5 ml of 10^{-3} M nbSSbn solution in 0.1 M phosphate buffer, pH 7.0. Readjust the pH to 7.0 with 1 M NaOH, transfer the solution to a 10-ml volumetric flask and make the volume 10 ml with phosphate buffer. Weigh the protein in a 5-ml volumetric flask and make up the volume with nbSSbn-guanidine hydrochloride solution. Measure the absorbance at 410 mμ of the latter solution versus the former. The number of mercapto groups equals

$$\frac{A_{410}}{1.36 \times 10^4 \times \text{Protein Concn.}}$$

Determination of Mercapto Groups with NEM in the Presence Guanidine Hydrochloride

(1) Make 10 ml of 6 M guanidine hydrochloride (G-HCl) by taking 5.73 g in a 20-ml beaker, adding 5 ml of 0.1 M phosphate buffer, pH 7.0, adjusting the pH back to 7.0 after dissolution of G-HCl, and finally making the volume 10 ml in a volumetric flask with phosphate buffer.

(2) Take 2.87 g of G-HCl, add 2 ml of 5×10^{-3} M NEM solution in phosphate buffer, pH 7.0, adjust the pH back to 7.0 and make volume 5 ml.

(3) Take 2.87 g of G-HCl, add the calculated amount of protein (so that final mercapto group concentration would be about 10^{-4} M), add 2 ml of phosphate buffer, adjust the pH to 7.0 and make the volume 5 ml.

(4) Mix 2 ml of solution (1) with 2 ml of solution (2) and transfer an aliquot to a curvette. This gives the NEM solution.

(5) Mix 2 ml of (1) with 2 ml of (3). This gives the protein solution.

(6) Mix 2 ml of (2) with 2 ml of (3) to obtain the reaction mixture, containing protein as well as NEM.

(7) Measure the absorbance at 300 mμ of solution (6) versus solution (5). Subtract this absorbance from the absorbance obtained for solution (4) versus solution (1). The number of -SH groups reacted is obtained from

$$\frac{\Delta A_{300}}{620 \times \text{Protein Concn.}}$$

Measurements may also be made at 315 mμ, at which wavelength a molar absorbancy of 535 should be used.

Table IX presents the representative results for comparison purposes.

TABLE IX

DETERMINATION OF -SH GROUPS WITH nbSSbn AND WITH NEM

Protein	Sample Origin	Denaturant	Reagent	# SH Group
Ovalbumin	Calbiochem #36293	1% SDS	nbSSbn	3.9
Ovalbumin	D. W. #B	6M-G-HCl	nbSSbn	3.7
Ovalbumin	D. W. #B	6M G-HCl	NEM	3.7
β-Lactoglobulin	Koch Light #11203	1% SDS	nbSSbn	1.6
β-Lactoglobulin	Koch Light	6M G-HCl	nbSSbn	1.5
β-Lactoglobulin	Kogh Light	6M G-HCl	NEM	1.7

CHAPTER IV

STUDIES ON YEAST ALCOHOL DEHYDROGENASE

The first section of this chapter describes the preparation of the enzyme and deals with the result of some preliminary experiments done to identify the best source material and other conditions which effect the yield. The next section gives some of the procedures for assaying the enzyme. Three sections that follow deal with the reaction of the enzyme with various mercapto group reagents, the inactivation by X-irradiation, and the relationships between activity and titrable -SH groups.

Preparation

As the name implies, the enzyme is prepared from yeast. Three kinds of material were used: Fleishman's Active Dry Yeast (Standard Brands, Inc., N.Y.) (Y-1); the same material, ball-milled for 16 hours at 4^{o}C and strained (Y-2); and Fleischman's Yeast Cakes (Standard Brands, Inc., N.Y.), crumbled, dried for 3 days, ball-milled at 4^{o} for 16 hours, and strained (Y-3).

The first step in the precedure as used by Racker (76) or Wallenfels *et al.* (64) calls for extraction with 0.067 M phosphate. Table X reports the results obtained, i.e., the activity appearing in the first extract. It is seen that using larger volume of buffer than prescribed by those authors (3 ml per gram of yeast), 5×10^{-4}M EDTA and

TABLE X

YADH-ACTIVITY OBTAINED AFTER FIRST EXTRACTION FROM YEAST

Number	Yeast Sample	ml buffer / g yeast	EDTA in .067M phosphate buffer	Activity (U_{WB}/g of Yeast)
727	Y-1	3	--	37.9[a]
730_A	Y-1	3	--	28.2[a]
730_B	Y-1	5.6	$5x10^{-4}M$	78.8
731	Y-2	6	$5x10^{-4}M$	577
802	Y-2	6	$5x10^{-4}M$	698
812	Y-3	3	$5x10^{-4}M$	58.0
813	Y-3	6	$5x10^{-4}M$	55.6
814_A	Y-3	6	$5x10^{-4}M$	84.0
814_B	Y-2	5	$5x10^{-4}M$	478
VIII-2	Y-2	5	$5x10^{-4}M$	580
VIII-3	Y-2	6	$5x10^{-4}M$	556[b]
827	Y-2	8	--	27.4[b]
VIII-3	Y-2	8	--	458[c]

[a] No stirring or only occasional stirring was used in expt. nos. 727 and 730.

[b] Incubation was 2 hours at 37° and 3 hours at room temperature for VII-3,827 and VIII-3. Incubation was 3½ hours at 35° and 2 hours at room temperature in experiments 727 through VIII-2.

[c] Deionized distilled water was redistilled in all glass apparatus.

stirring during extraction helps to recover activity. Y-2 gave better yield than Y-1 or Y-3. These results were useful in carrying out the isolations. Two typical procedures are described in the following sections.

Preparation of Sample VIII-2:

This procedure is similar to that of Wallenfels et al. (64).

Extraction.--One hundred grams of yeast powder Y-2 and 500 ml of 0.067 M phosphate -5 x 10^{-4}M EDTA buffer, pH 8.0, were mixed thoroughly and incubated first at 35°C for 3½ hours and then at room temperature for 2 hours, with constant stirring. The slurry was transferred to 250-ml centrifuge bottles and centrifuged at 9000 rpm for 30 min in a Servall Refrigerated Centrifuge. The residue was rejected.

Acetone Precipitation.--The supernatant, 370 ml, was cooled to approximately -2° and 185 ml of cold acetone (Baker Analyzed Reagent, kept in a cold room, temperature -18°) was added slowly, making sure that the temperature stayed below 2°C. The mixture was left at -18° C in a cold room for 2 hours. It was then centrifuged in 250-ml bottles at 9000 rpm in a Servall Refrigerated Centrifuge. The residue were rejected.

To the supernatant, 200 ml of -18°C acetone was added, taking care that the temperature stayed low; the flask to which the additions were made was kept in an ice-salt bath. The solution was stirred well after each addition and at the end centrifuged at 9000 rpm for 30 min in four 250 ml bottles. The supernatant was rejected.

The precipitate was suspended in 30 ml of 0.067 M phosphate-5 x 10^{-4}M EDTA buffer. The bottles were allowed to stand in an ice

bath for two hours. Then 25 ml of double distilled water was added, and the whole suspension centrifuged in 10 ml tubes (small rotor) at 15000 rpm for 30 min. The precipitate was rejected.

Heating Step.--The supernatant was heated to 53-55° for 15 min and then quickly placed in an ice bath. It was then centrifuged at 15000 rpm for 30 min. The residue was rejected; the volume of supernatant was 53 ml.

$(NH_4)_2SO_4$ Precipitation.--To 50 ml of this supernatant, 18.2 g of $(NH_4)_2SO_4$ was added slowly and the mixture was left overnight. Then it was centrifuged at 15000 rpm for 30 minutes. The supernatant was rejected.

First Crystallization.--The precipitate was dissolved in 11 ml of 0.025% EDTA (25 mg of EDTA in 100 ml, pH adjusted to 8.0 with NaOH). To this solution an equal volume (11 ml) of a saturated $(NH_4)_2SO_4$ solution (pH adjusted to 8.0 with NH_4OH) was added in small portions (0.5 ml) over a period of 2½ hours. The suspension was then centrifuged at 15000 rpm for 30 min.

Second Crystallization.--The precipitate was taken up in 10 ml of 0.025% EDTA. Six milliliters of saturated $(NH_4)_2SO_4$ solution was added in 0.5 ml portions over a 2 hour period. The suspension was allowed to stay overnight in refrigerator, $\sim 4^{\circ}C$. The suspension was then centrifuged for 30 min at 15000 rpm.

Third Crystallization.--The precipitate from the last step was taken up in 10 ml of 0.025% EDTA solution and 10 ml of saturated $(NH_4)_2SO_4$ solution added. The crystals that formed at this stage were stored in refrigerator, $\sim 4^{\circ}C$, for future use.

The enzyme activity obtained at each step is reported in Table XI.

TABLE XI

YADH-ACTIVITY AT EACH STEP OF ISOLATION OF VIII-2

Step	U_{IUB}/g of yeast		U_{IUB}/mg of protein	
	Wallenfels et al.	This Lab.	Wallenfels et al.	This Lab.
1st Extract	469	580	8.76	-
Acetone Precipitation	442	318	11.9	-
Heating Precipitation	428	367	48.6	-
$(NH_4)_2SO_4$ Precipitation	383	(199)	95.6	31.0
1st Crystallization	310	272	258.	95.0
2nd Crystallization	280	232	264	186
3rd Crystallization	258	242	266	197

The activity units correspond to the assay described in Appendix B; sometimes the Wallenfels assay procedure was used for measuring activity, and the units reported in the Table were obtained using the conversion factors reported in the Appendix. The specific activity was calculated on the basis of protein concentration determined from $E_{280}^{1\%} = 12.62$.

Preparation of Sample VIII-3:

This procedure is similar to that of Racker (76).

Extraction from Yeast.--One hundred grams of yeast powder, Y-2, was extracted with 600 ml of 0.067 M phosphate-5 x 10^{-4}M EDTA buffer, pH 8.0, at 37°C for 2 hours and at room temperature for an additional 3 hours with constant stirring. The yeast residue was then removed by centrifugation at 9000 rpm for 50 min.

Heat Precipitation.--455 ml of liquid from the last step was heated to 55° for 15 minutes and then cooled quickly in an ice bath. After cooling it was centrifuged at 9000 rpm for 50 min. The residue was rejected and the supernatant allowed to stay overnight in refrigerator.

Acetone Precipitation.--To 328 ml of the supernatant liquid, 164 ml of -18° acetone (Baker Reagent) was added slowly and with stirring; the Erlenmeyer flask containing the solution was kept in an ice-salt mixture. The precipitate was rejected after centrifuging at 9000 rpm for 40 min. To the supernatant, 175 ml of cold acetone was added in the same manner as above. The supernatant solution was discarded after centrifuging at 9000 rpm for 40 min. The precipitate was suspended in 25 ml of double distilled water and the suspension transferred to dialysis tubing which had been soaked and washed in phosphate buffer for several weeks and washed with distilled water just before use. Dialysis

was carried out by suspending the tubing in 8 liters of 0.001M potassium phosphate-5 x 10^{-4}M EDTA buffer pH 8.01 in cold room, 5°C. Buffer was constantly stirred and the tubing shaken up occasionally. Buffer was changed once and the total time allowed for dialysis was 3 hours. The suspension from the dialysis tube was centrifuged at 10000 rpm for 30 min. The residue was discarded. The volume of supernatant was 46 ml.

Crystallization.--To the supernatant from last step 16.55 g of $(NH_4)_2SO_4$ was added and the mixture kept at 0°C for ½ hour. It was then centrifuged at 15000 rpm for 30 min and the supernatant was rejected.

Recrystallization.--The precipitate was dissolved in 10 ml of double distilled water. To this 2 g of $(NH_4)_2SO_4$ was added and the suspension centrifuged at 15000 rpm for 15 min. The precipitate was rejected. The supernatant was seeded with crystals from preparation VIII-2, i.e., about .050 ml of YADH VIII-2 suspension were added to the supernatant, and allowed to stay in refrigerator.

Second Recrystallization.--Suspension from the last step was centrifuged at 5000 rpm for 30 min. The precipitate was dissolved in 5 ml of water and saturation with respect to $(NH_4)_2SO_4$ was brought to 35%.

Table XII gives the activity obtained after each step. For comparison, the values reported by Racker are also included. Specific activity reported for the first three steps was calculated after determining the protein concentration by the Biuret Method (77) since in the earlier stages of the preparation lots of colloidal material was present that prevented measurement of the ultraviolet absorption.

TABLE XII

YADH-ACTIVITY AT EACH STAGE OF ISOLATION OF VIII-3

Step	U_{IUB}/g of yeast		U_{IUB}/mg of protein	
	Racker	This Lab	Racker	This Lab
1st Extract	851	556	11.5	5.15
Heating Precipitation	959	459	--	8.0
Acetone Precipitation	866	424	171	16.9
1st Crystalline Preparation	588	130	374	213
Recrystallized Preparation	402	84.3	492	242

Biuret Procedure for Determination of Protein Content:

The Biuret reagent was prepared (77) by dissolving 9 g of Rochelle salt, potassium sodium tartrate tetrahydrate (Baker Reagent) in about 400 ml of 0.2 M NaOH. To this was then added 3 g of $CuSO_4.5H2O$ (Baker Reagent), after this had dissolved, 5 g of KI (Fisher Reagent) was then added and the volume made up to 1 liter from 0.2 M NaOH.

The following procedure was used for estimating the protein content:

(1) Precipitate the protein by adding 0.15 ml of 3 M trichloroacetic acid solution to 0.85 ml of the "crude" solution of protein. Centrifuge at 1000 rpm for 20 min.

(2) Dissolve the precipitate in 5 ml of water. Add 5 ml of Biuret reagent and let stand for at least ½ hour. For the blank, add 5 ml of Biuret reagent to 5 ml of water.

(3) To eliminate any fatty or colloidal organic material, add 2 ml of ether to a 5 ml portion of reaction mixture. Shake well. Then centrifuge on a clinical centrifuge to completely separate the ether layer, which is then pipetted off.

(4) Measure absorbance at 546 mμ for both the blank and the reaction mixture. ΔA_{546} multiplied by 34 gives the protein concentration in mg/ml.

Activity Assays

Since Appendix B presents the major findings in a form suitable for publication in a journal, only those details and considerations are

described that are not explicit there.

Preliminary studies were made in order to determine the optimum conditions for the assay. As is true for some other enzymes, YADH may be inactivated on dilution. Earlier investigators have used, and commercial suppliers recommended, the use of serum albumin or gelatin in diluting the enzymes for assay. We have found that 10^{-3} or 5×10^{-4}M EDTA was as effective as, or even more effective, than serum albumin. Whereas lower and somewhat erratic activity values were obtained if no protector was used, the values obtained in the presence of 5×10^{-4}M EDTA were linearly related to concentration and reproducible.

Effect of NAD^+:

The concentration of NAD^+ affects the activity measurement in at least two ways. One, if the NAD^+ concentration is too low, the rate measured is constant for only a shorter time and slows down considerably due to the reverse reaction; this may be seen from representative curves in Figure 4, where four different assay procedures results are plotted, each having widely different (NAD^+) concentration as may be seen from Appendix B, Table B-1. Two, at lower concentrations of NAD^+, small changes in its concentration can cause larger changes in the rate; this effect is illustrated in Fig. IV-2, where specific activity of two enzyme samples is plotted as a function of NAD^+ concentration.

Experimental Details for Various Assays:

Reagents used during the study were: NAD^+ from Sigma Chemical Company (Lot #15B7310); ethanol, USP grade from U. S. Industrial Chemicals Company; semicarbazide hydrochloride, Matheson Coleman & Bell;

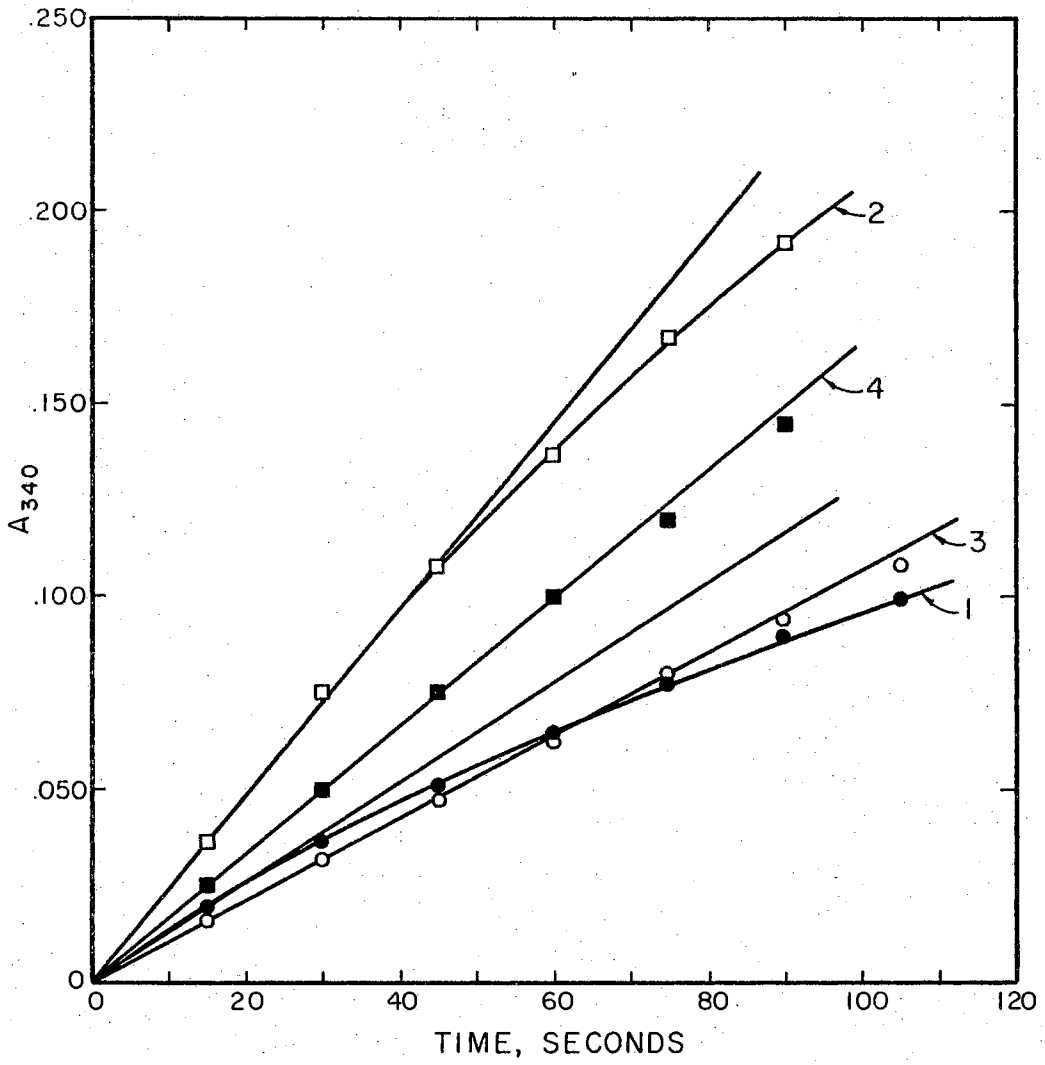


Fig. 4. Representative curves from different assays. (1) Racker, (2) Wallenfels et al., (3) This work, (4) Hoch and Vallee. Enzyme Sample, VIII-3. Concn. 2.67 x 10^{-8}M for (1) and (3) and 3.3. x 10^{-9}M for (2) and (4).

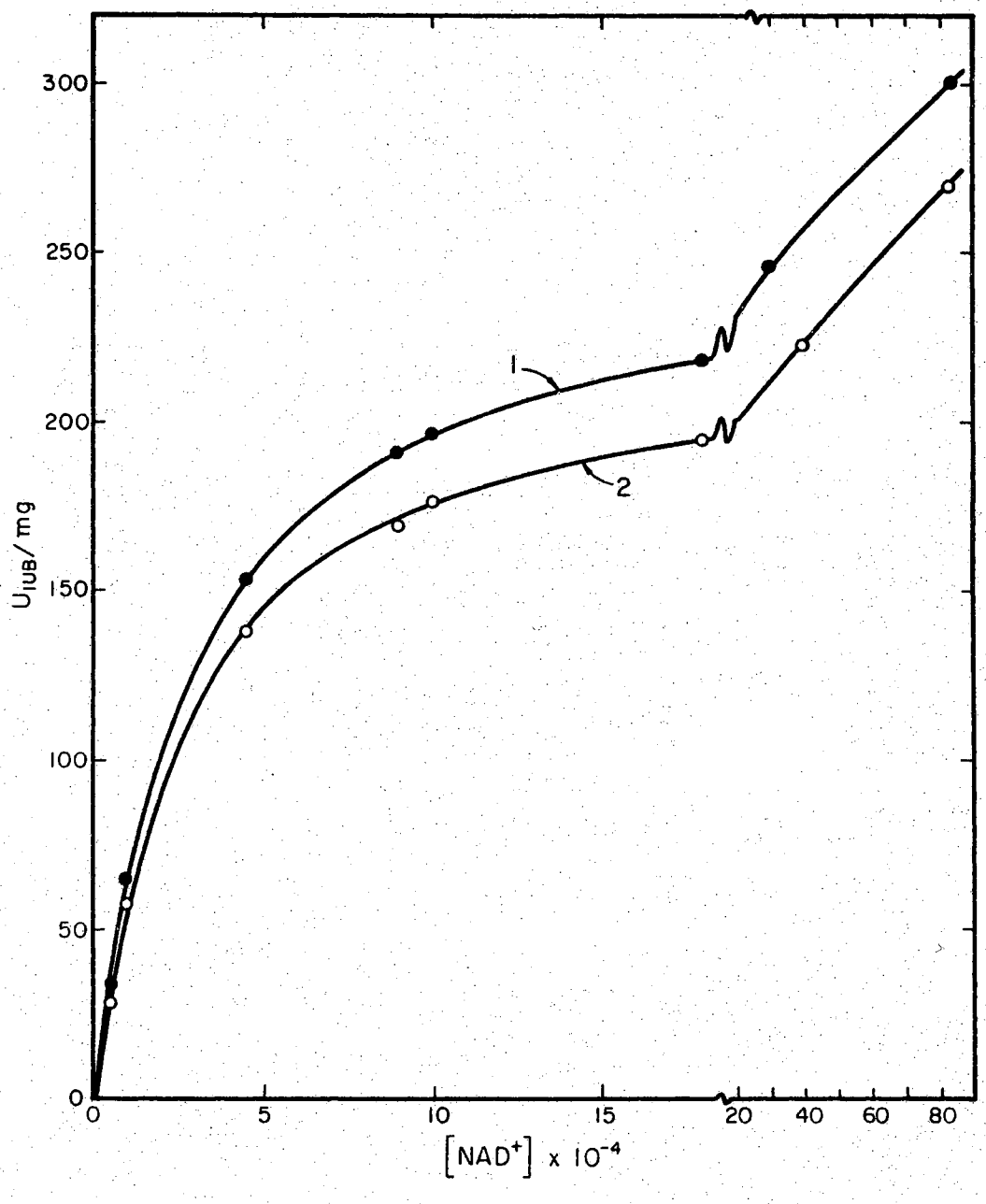


Fig. 5. Dependence of activity on NAD^+ concentration. Other concentrations in assay mixture: 0.57 M ethanol, 0.05 M Na-pyrophosphate, 5 x 10^{-4}M EDTA. pH 8.80. (1) Sample VIII-3, (2) Sample W-1.

sodium pyrophosphate decahydrate, Baker Reagent; glycine and EDTA disodium salt, Eastman Kodak Reagent; boving albumin, Pentex (Lot #11).

For the assays, 2.9 ml of stock assay solution was taken in the cuvette and 0.1 ml of enzyme solution added. Stock solutions were prepared as follows: the Racker's assay was mixed from 5 ml of 0.06 M pyrophosphate buffer, pH 8.5, 22 ml of double distilled water, 1 ml of 1.5×10^{-3} M NAD^+ and 1 ml of 3 M ethanol. Similarly, the stock solution for Wallenfels and Sund's assay was mixed from 27 ml of 0.075 M pyrophosphate-0.075 M semicarbazide-hydrochloride-0.022 M Glycine buffer, pH 8.6, 1 ml of 3 M ethanol and 1 ml of 1.35×10^{-3} M NAD^+. Other stock solutions were mixed in a similar manner taking appropriate concentrations and volumes. NAD^+ concentration was usually checked by measuring the absorption at 260 mμ ($\varepsilon = 1.8 \times 10^4$).

The dilution medium for Racker's assay was 0.1% Bovine Albumin solution in 0.01 M phosphate.

For the data in Fig. 5, the assay mixture was mixed in the cuvette by taking 1.6 ml of ethanol-pyrophosphate (2 ml absolute ethanol added to 30 ml of 0.1 M pyrophosphate, pH 8.8), X ml of NAD^+ solution and (1.3 - X) ml of water, so that final volume would be 3.0 ml after 0.1 ml of enzyme solution was added.

Reaction of YADH with Various Mercapto Reagents

In this section the experimental procedures used to study the reactions of p-chloromercuribenzoate, nbSSbn, N-ethylmaleimide, ferricyanide and iodine with YADH are described. The major findings are summarized in a form suitable for publication in a journal and

Presented in Appendix C.

Materials:

The following reagents were obtained from the sources indicated and used without further purification; nbSSbn, Aldrich Chemical Company; N-ethylmaleimide, Calbiochem; sodium dodecyl sulfate, Fisher Scientific Company; p-chloromercuribenzoic acid, Sigma Chemical Company. Other chemicals were of analytical reagent grade, except guanidine hydrochloride. The latter was of "Aero" (technical) grade from the American Cyanamid Co., Bound Brook, N. J., and was purified as follows: Make a slurry of a kilogram of crude material with 200 ml of water with stirring at 45°C, adjust the pH to 4.5, filter and cool the filtrate overnight at -5°C to obtain crystals (evaporate on a rotary evaporator if necessary to get enough crystals). Filter and then recrystallize from absolute methanol repeatedly, treating each time with 'Norite A', until the absorbance at 240 mμ is no more than 0.10 and that at 300 mμ was negligible. Usually three recrystallizations were required.

Reaction with p-chloromercuribenzoate, PCMB:

Stoichiometry was usually determined from successive addition of a protein solution (~1 mg/ml or 6.7×10^{-6}M) in 0.1 ml portions to 3 ml of PCMB solution (~1.5×10^{-5}M); after each addition the absorbance at 255 mμ was measured. After correcting A_{255} for dilution, it is plotted versus the milliliters of protein solution added and the equivalence point is determined. The PCMB concentration is calculated from $E_{232} = 1.69 \times 10^{4}$.

For example, in Fig. 6, the equivalence point corresponds to

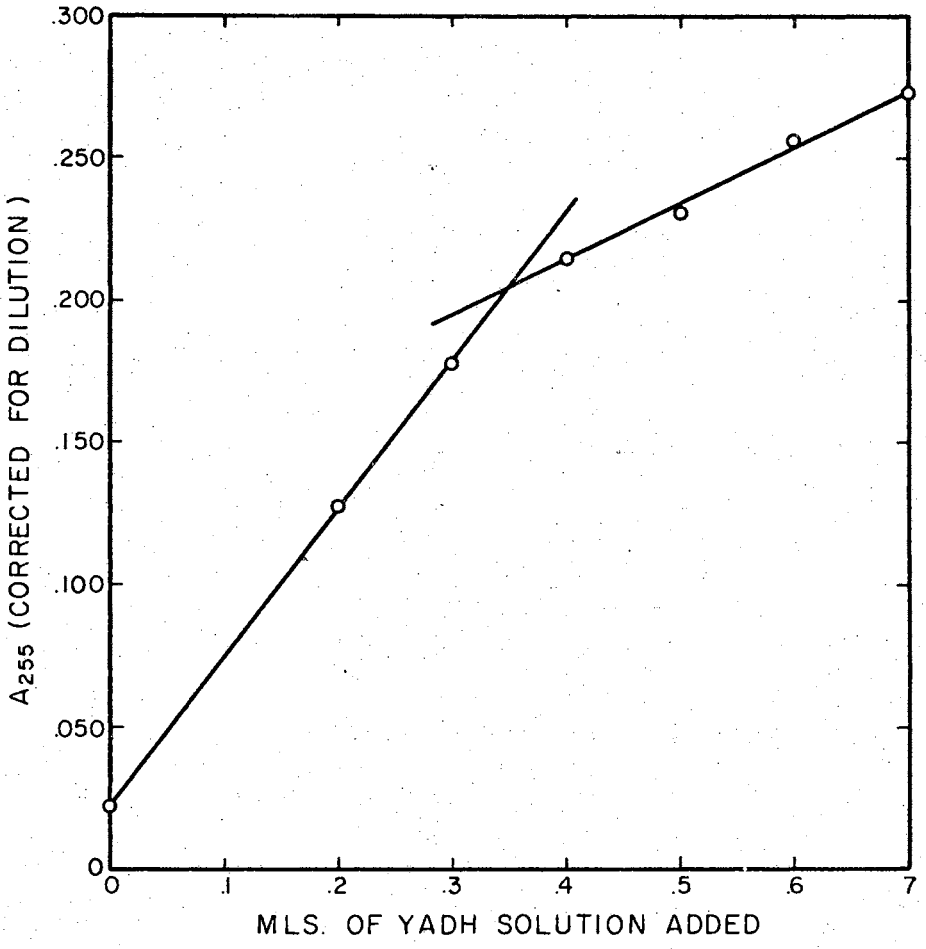


Fig. 6. A typical titration with PCMB.
[YADH] = 7.39×10^{-6}M
[PCMB] = 1.41×10^{-5}M

0.047 ml of YADH added. Concentrations at the equivalence point are

$$[\text{PCMB}] = \frac{3.0}{3.347} \times 1.41\times10^{-5} = 1.26\times10^{-5}\text{M}$$

$$[\text{YADH}] = \frac{.347}{3.347} \times 7.39\times10^{-6} = 7.65\times10^{-6}\text{M}$$

$$\text{SH groups per mole of YADH} = \frac{1.26\times10^{-5}}{7.65\times10^{-6}}$$

$$= 16.5$$

Reaction of N-Ethylmaleimide (NEM) with YADH:

When the native enzyme solution and NEM solution are brought together, the usual decrease in absorbance at 300 mμ cannot be measured because of the precipitation which results usually within about ten minutes. This precipitation was first observed in a solution containing 2.67×10^{-5}M YADH and 1.09×10^{-3}M NEM. After removing the precipitate by centrifugation, the supernatant was found to have lost at least 80% of its protein as determined from absorbance at 280 mμ. Decreasing the protein concentration to 6.65×10^{-6}M and the NEM concentration to 1.09×10^{-4} still gave a visible precipitate.

The precipitate was not visible when the reaction was carried out in the presence of 1% SDS. The usual procedure was to mix 1 ml of 3% SDS, 1 ml of 3.0×10^{-3}M NEM and 1 ml of 3.0×10^{-5}M YADH and measure the absorbance of this solution at 300 mμ *versus* a blank containing 1 ml of buffer instead of YADH solution. The absorbance of NEM alone was measured by mixing 1 ml of NEM solution, 1 ml of buffer and 1 ml of 3% SDS and determining A_{300} versus a blank containing 1 ml of 3% SDS and 2 ml of buffer. YADH sample (W-2) which was found to contain

16-17 mercapto groups with (nbSSbn) and PCMB, but the result with NEM in the presence of 1% SDS was only 10-12 SH groups.

To carry out the reaction in the presence of guanidine hydrochloride, first, 7.2 M guanidine solution was prepared at pH 7.0 (the pH is lowered on dissolution, but was adjusted back to 7.0 by adding NaOH). Then 2.5 ml of this solution was placed in each of two cuvettes. To one 0.5 ml of 6×10^{-3}M NEM was added and to the other 0.5 ml of buffer. The absorbance measured at 300 mμ; then 0.5 ml of YADH solution was added to each cuvette and A_{300} measured. The initial A_{300} for NEM was corrected for dilution. The results varied between 16-18 for SH content for YADH sample W-1.

Number of mercapto groups was calculated from

$$\frac{\#\text{-SH groups}}{\text{mole of YADH}} = \frac{\Delta A_{300}}{620} \times \frac{1}{[\text{YADH}]}$$

Reaction Between YADH and (nbSSbn):

Appropriate amounts of the protein, (nbSSbn) and denaturant solutions were mixed in the cuvette and A_{410} measured versus a reference solution containing the same reagents except protein. Unless indicated otherwise, all the solutions were made in 0.1 M phosphate-10^{-3}M EDTA buffer, pH 7.0.

For example, when the reaction was carried out in 1% SDS, the reagents used were 1 ml of 3% SDS, 1 ml of approximately 6.67×10^{-6}M ($\frac{1 \text{ mg}}{\text{ml}}$) YADH and 1 ml of 2.5×10^{-3}M nbSSbn (1 mg/1 ml). The blank contained the same reagents except that 1 ml of plain buffer was substituted for 1 ml of YADH solution. When the reaction was to be carried in 6 M guanidine hydrochloride solution, 2.5 ml of 1×10^{-3}M nbSSbn solution

in 7.2 M guanidine hydrochloride was taken in each cuvette. To one 0.50 ml of enzyme was added and to other buffer only.

Number of mercapto groups was calculated from

$$\frac{A_{410}}{1.36 \times 10^{4} \times [YADH]}$$

Reaction between Ferricyanide and YADH:

The same procedure was followed as for the reaction with nbSSbn, except that the ferricyanide solution replaced nbSSbn, the buffer contained no EDTA. The decrease in absorbance at 410 mμ was determined. The number of -SH groups was calculated from

$$\Delta A_{410}/990X[YADH]$$

Reaction between Iodine and YADH:

One usually determines the stoichiometry for the iodine reaction by adding iodine in potassium iodide solution to the protein and noticing at each step the increase in absorbance at 355 mμ. When iodine remains unreacted there are large increases in absorption and the point of intersection gives the stoichiometric ratio.

During an experiment in which 3 ml of 1.9×10^{-6} M YADH was taken in a cuvette and 2.1×10^{-4} M iodine in 0.2 M KI added in 0.1 ml portions, A_{355} started to rise after 0.5 ml had been added, and continued to do so until an unusually high value was reached; visual inspection revealed that a gel had precipitated.

An experiment was carried out in the presence of 1% SDS. Again, a precipitate appeared a little before the calculated equivalent

amount of iodine had been added.

Effect of X-Irradiation on YADH

Two effects of x-irradiation were examined, inactivation and the destruction of mercapto groups. Theoretical treatments of the general subject of x-irradiation of enzymes with specific applications are given by Bacq and Alexander (78), Augenstine (79), Hutchinson and Ross (80), Sanner and Pihl (80), and Gorin and Quintiliani (82). Since in the case of YADH the inactivation dose relationship was found to be exponential, the following equation may be used to interpret the data:

$$\ln \frac{E}{E_o} = k_i \, D/n \, (k_e E_o + k_d) \qquad \text{IV-1}$$

It is postulated that the species X produced in aqueous solution by X-irradiation reacts with active enzyme $\underline{E}_a$ to produce inactive enzyme E_i with rate k_i; k_e is the rate with which X reacts with the inactive enzyme or with active enzyme and k_d is the rate at which X disappears due to reaction with other species in solution and represents the "scavenging ability of the solvent." According to this equation $\underline{D}_{37}$, the dose at which $\ln \frac{E}{E_o} = -1$, is a linear function of E_o. The slope gives the inactivation yield and the intercept, k_d, 'the scavenging ability of the solvent.'

Experimental:

Enzyme solutions were prepared in 0.05 M phosphate buffer, pH 7.0 containing no EDTA. The irradiation source used was a Westinghouse industrial X-ray unit. One-half milliliter aliquots of the enzyme solution were put in 10 x 75 mm Pyrex culture tubes, and then were

placed in a holder and surrounded by ice-water mixture. The holder was then positioned in the path of X-ray beam. The dose rate was determined for the positioned tubes under same circumstances by the method of Weiss et al. (83). When lower concentrations of the enzyme were irradiated, the filament current was reduced from 10 ma to 5 ma and a 2-mm thick aluminum filter was put in the path of the beam. The average dose without filter was 1550 rads min^{-1} at 110 kV and 10 ma, with filter 293 rads min^{-1} at 110 kV and 5 ma. An ionic yield of 13.9 ferric ions per 100 e.v. was used as the basis of calculation.

The assays for the activity of irradiated and control samples were done at the same time, usually within 6 hours after irradiation. The dilutions were made with 0.01 M phosphate-5 x 10^{-4}M EDTA buffer, pH 7.5.

The mercapto groups were determined in the presence of 1% SDS, 10^{-3} M EDTA-0.1 M phosphate buffer of pH 7.0 and 8.3 x 10^{-4}M nbSSbn; usually 0.25 ml of the enzyme solution was added to 3 ml of nbSSbn solution.

Results:

Fig. 7 shows a typical set of data. D_{37} was the dose when only 37% activity or mercapto groups would be left. The inactivation yield or G-value was obtained by dividing the concentration by D_{37}; both D_{37} and G-values were obtained by least square fitting of the data with an appropriate computer program. G(-SH) was similarly obtained from D_{37} and the concentration of mercapto groups. Table XIII gives the G-values for two samples of YADH studied, W-1 and VIII-4. Plotting the D_{37} values according to the equation IV-1 gives negligible

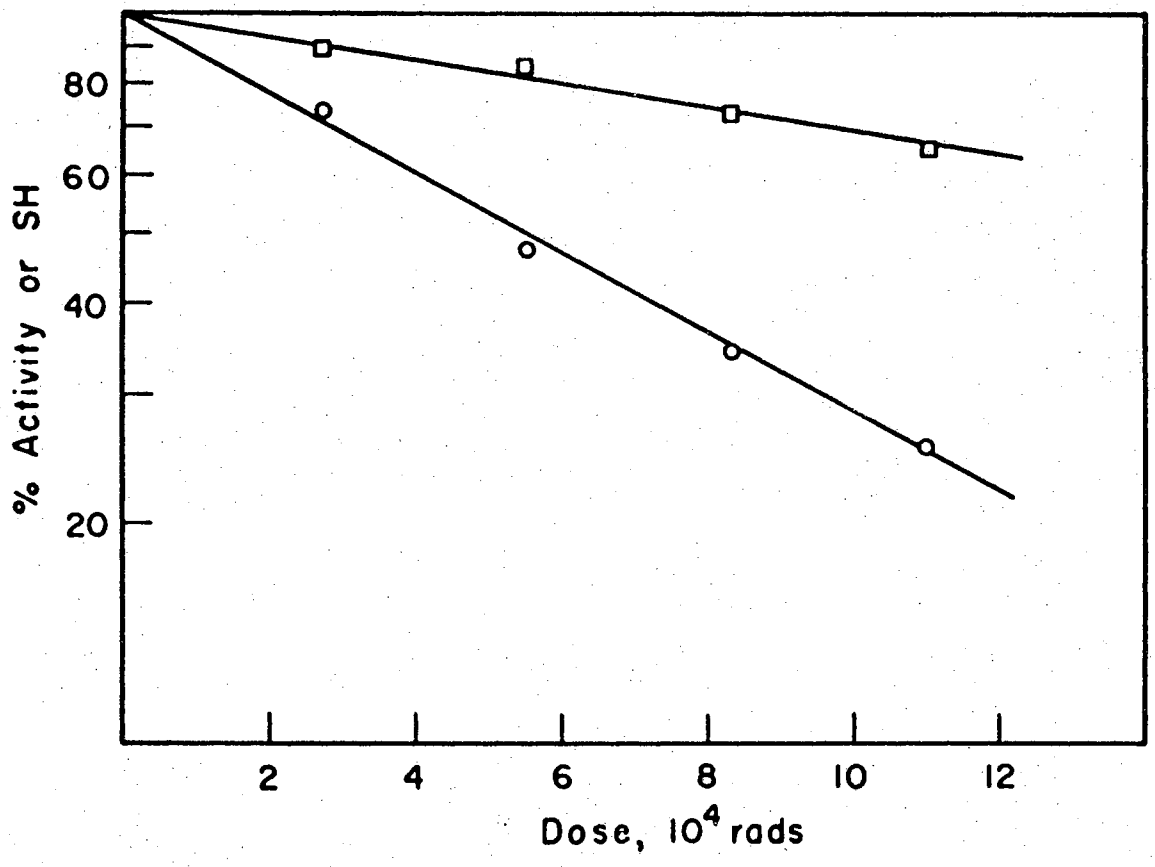


Fig. 7. X-irradiation of YADH. Sample W-1, Conc. 1.66 mg/ml (1) % -SH groups left, (2) %-activity left.

TABLE XIII

G-VALUES FOR YADH

Sample	Conc. mg/ml	Conc. Molecules/ml x 10^{-14}	D_{37} k rads	D_{37} 100 e.v.	$G_{calc.}$ (Molecules/100 e.v.)	Calculated Values from Equation IV-1
	.039	1.566	1.72	1.08×10^{15}	.145	
	.07	2.801	2.18	2.15×10^{15}	.13	G = .137
VIII-4	.10	4.017	6.13	3.83×10^{15}	.11	k_d = -1.2
	.215	8.613	9.93	6.21×10^{15}	.14	
	.855	34.33	39.80	24.9×10^{15}	.14	
	.045	1.825	2.465	1.54×10^{15}	.12	
	.069	2.771	2.704	1.69×10^{15}	.16	G = 11
W-1	.084	3.391	5.894	3.68×10^{15}	.09	k_d = 0.7
	.197	7.890	13.697	8.56×10^{15}	.09	
	1.66	66.86	79.816	49.9×10^{15}	.13	

intercepts, see for example Fig. 8 . This indicates that the medium has almost no scavenging effect for the inactivation process. For three different experiments, it was found that $D_{37}(-SH)/D_{37}(Inact.)$ was 4.3, 4.2 and 4.0, respectively. Since the number of -SH groups is 16-18, it might have been expected that this ratio would be 16-18. The actual result indicates that destruction of all -SH groups is not required for inactivation or, conversely that not all the -SH groups are essential for activity.

Relationships between Activity and Titrable Mercapto Groups

In the last section it was shown that destruction of all mercapto groups does not occur prior to, or after, irradiation and inactivation. In Appendix C, the results presented show that activity is not lost when 3-4 -SH groups react with nbSSbn; it seems that whereas nbSSbn shows preference for non-essential -SH groups, PCMB is non-selective (64,65) and iodoacetamide prefers essential -SH groups (66,67). The conclusion that may be drawn is that 3-4 mercapto groups which react with nbSSbn faster or preferentially are not essential for activity and that these are probably a part of the set of -SH groups which are not destroyed on irradiation with X-rays.

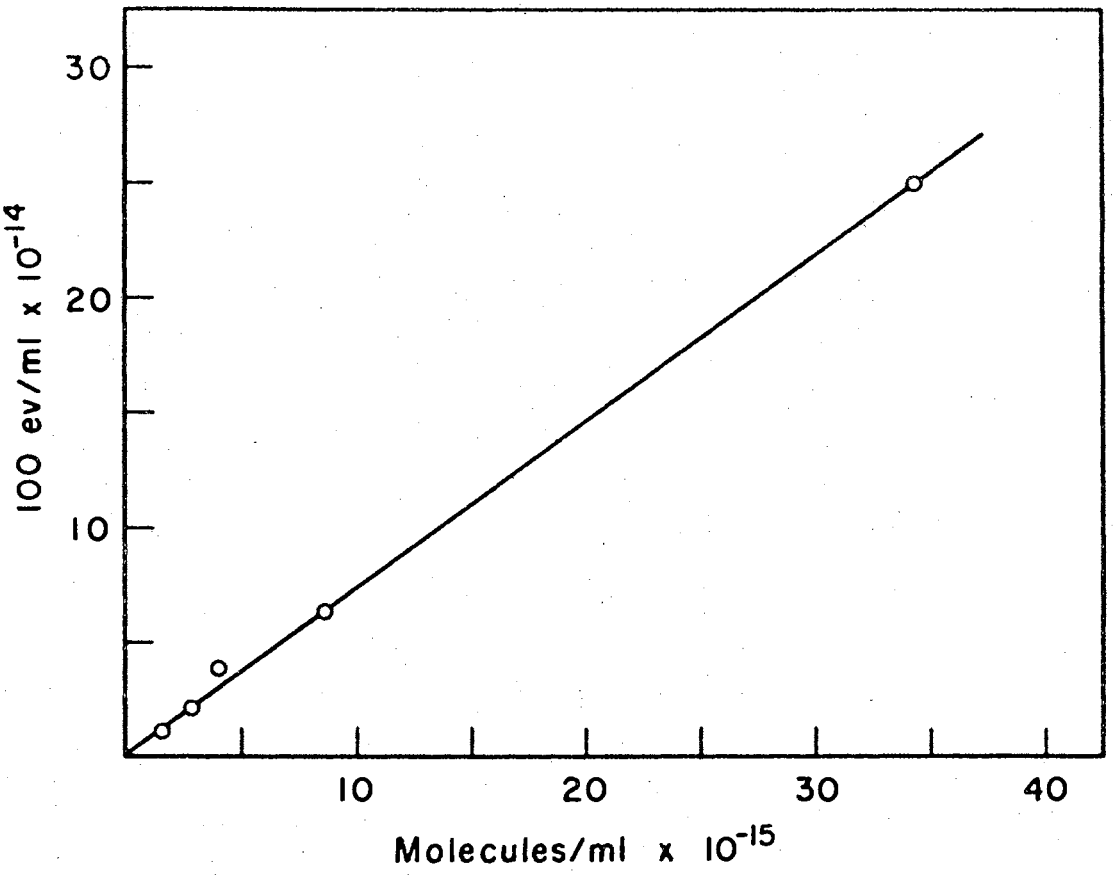


Fig. 8. Plot of D_{37} against concentration (cf. equation IV-1). Sample VIII-4.

BIBLIOGRAPHY

1. Ellman, G. L., Archiv. Biochem. Biophys. 82, 70 (1959).

2. Boyer, P. D., "The Enzymes", (Boyer et al., Eds.), Vol. 1, Academic Press, N. Y., 1959, p. 511.

3. Gorin, G., Progr. Biochem. Pharmacol. 1, 142 (1965).

4. Jensen, E. V., Science 130, 1319 (1959).

5. Mazia, D., "Sulfur in Proteins", (Benesch et al., Eds.), Academic Press, N. Y., 1959, p. 357.

6. "Science Citation Index", Institute for Scientific Information, 325 Chestnut Street, Philadelphia, Pa.

7. Sund, H., and Theorell, H., "The Enzymes", (Boyer et al., Eds.), Vol. 7, Academic Press, N. Y., 1963, p. 25.

8. Hoch, F. L., and Vallee, B. L., "Sulfur in Proteins", (Benesch et al., Eds.), Academic Press, N. Y., 1959, p. 245.

9. Wallenfels, K., Sund, H., Zarnitz, M. L., Malhotra, O. P. and Fischer, J., ibid., p. 215.

10. McKinley-McKee, J. S., Progr. Biophys. Mol. Biol. 14, 223 (1964).

11. Barnett, J. R., and Seligman, A. M., Science 116, 323 (1952).

12. Ellman, G. L., Archiv. Biochem. Biophys. 74, 443 (1958).

13. Klotz, I. M., Ayers, J., Ho, J. Y. C., Horowitz, M. G. and Heiny, R. E., J. Am. Chem. Soc. 80, 2132 (1958).

14. Brighenti, L., Boll. Chim. Farm. 103, 257 (1964); Chem. Abstr. 61, 8575f.

15. Brighenti, L., Boll. soc. ital. biol. sper. 35, 560 (1959); Chem. Abstr. 55, 15586e.

16. Lederer, F., Coutis, S. M., Laursen, R. A., and Westheim, F. H., Biochemistry 5, 823 (1966).

17. Thomson, A. R., Eveleigh, J. W., and Miles, B. J., Nature 203, 267 (1964).

18. Havir, E. A., Hadassah, T., Ratner, S., and Warner, R. C., J. Biol. Chem. 240, 3079 (1965).

19. Penefsky, H. S., Fed. Proc. 23, 533 (1964).

20. Somogyi, J., and Gaal, J. G., Experientia 21, 573 (1965).

21. Kassab, R., Pradel, L. A., and Thoi, N. V., Biochem. Biophys. Acta, 99, 397 (1965).

22. Novoa, W. B., Tigier, H. A., and Grisolia, S., ibid., 113, 84 (1966).

23. Givol, D., Lorenzo, F. D., Goldberger, R. F., and Anfinsen, C. B., Proc. Natl. Acad. Sci., U. S. 53, 676 (1965).

24. Srere, P. A., Archiv. Biochem. Biophys. 110, 200 (1965).

25. Hellerman, L., Coffey, D. S., and Neims, A. H., J. Biol. Chem. 240, 290 (1965).

26. Hsu, R. Y., Wasson, G., and Porter, J. W., ibid., 240, 3736 (1965).

27. Habeeb, A. F. S., Biochem. Biophys. Acta 115, 440 (1966).

28. Bitensky, M. W., Yielding, K. L., and Tomkins, G. M., J. Biol. Chem. 240, 1077 (1965).

29. Vaneys, J., Judd, J., Ford, J., and Womack, W. B., Biochemistry 3, 1755 (1964).

30. Guidotti, G., J. Biol. Chem. 240, 3924 (1965).

31. Baldwin, A. N., and Berg, P., ibid., 241, 831 (1966).

32. Nair, P. M., and Vining, L. C., Biochem. Biophys. Acta 96, 318 (1965).

33. Diez, M. J. F., Osuga, D. T., and Feeney, R. E., Archiv. Biochem. Biophys. 107, 449 (1964).

34. Stern, R., Deluca, M., Mehler, A. H., and Mcelroy, W. D., Biochemistry 5, 126 (1966).

35. Reichmann, M. E., and Hatt, D. L., Biochem. Biophys. Acta 49, 153 (1961).

36. Scrutton, M. C., and Utter, M. F., J. Biol. Chem. 240, 1 (1965).

37. Fujioka, M., and Snell, E. E., ibid., 240, 3050 (1965).

38. Givol, D., Goldberger, R. F., and Anfinsen, C. B., ibid., 239, 3114 (1964).

39. Margolis, S., and Langdon, R. G., ibid., 241, 477 (1966).

40. Fishbeim, W. N., Carbone, P. P., and Hochstein, H. D., ibid., 240, 2407 (1965).

41. Gerther, A., and Birk, Y., Biochem. J., 95, 621 (1965).

42. Schramm, M., and Bdolah, A., Biochemistry 3, 1231 (1964).

43. Rabin, B. R., and Trown, P. W., Nature 202, 1290 (1964).

44. Srere, P. A., Biochem. Biophys. Res. Commun. 18, 87 (1965).

45. Webster, R. E., and Gross, S. R., Biochemistry 4, 2309 (1965).

46. Pradel, L. A., Kassab, R., Terrosian, E. D. and Thoai, M. N. V., Compt. Rend. 260, 3212 (1965).

47. Kenkare, U. W., and Colowick, S. P., J. Biol. Chem. 240, 4570 (1965).

48. Alpers, D. H., Appel, S. H., and Tomkins, G. M., ibid., 240, 10 (1965).

49. Srere, P. A., Biochem. Biophys. Acta 106, 445 (1965).

50. Beutler, E., Duron, O., and Kelly, B. M., J. Lab. & Clin. Med. 61, 882 (1963).

51. Thompson, R. H., Clin. Chem. Acta 12, 198 (1965); Chem. Abstr. 63, 8667c (1965).

52. Ellman, G. L., Courtney, K. D., Andres, V., and Featherstone, R. M., Biochem. Pharmacol. 7, 88 (1961).

53. Smith, H. A., Doughty, G., and Gorin, G., J. Org. Chem. 29, 1484 (1964).

54. Gorin, G., Gideon, R., and Doughty, G., J. Chem. Soc. to be published.

55. Parker, A. J., and Kharasch, N., Chem. Rev. 59, 583 (1959).

56. Swan, J. M., "Sulfur in Proteins", (Benesch et al., Eds.), Academic Press, N. Y., 1959, p. 3.

57. International Union of Biochemistry, "Report of the Commission on Enzymes", Pergamon Press, Oxford, 1961.

58. Backlin, K. I., Acta Chem. Scand. 12, 1279 (1958).

59. Vallee, B. L., and Hoch, F. L., Proc. Natl. Acad. Sci. U. S. 41, 327 (1955).

60. Wallenfels, K., Sund, H., Faessler, A., and Burchard, W., Biochem. Z. 329, 31 (1957).

61. Kagi, H. R., and Vallee, B. L., J. Biol. Chem. 235, 3188 (1960).

62. Barron, E. G. S., and Levine, Archiv. Biochem. Biophys. 41, 175 (1952).

63. Keleti, T., Acta Physiol. Acad. Sci. Hung. 13, 309 (1958).

64. Wallenfels, K., and Sund, H., Biochem. Z. 329, 17 (1957).

65. McKinley-McKee, J. S., Biochem. J. 87, 42P (1964).

66. Whitehead, E. P., and Rabin, B. R., ibid., 90, 532, 539 (1964).

67. Harris, I., Nature, 203, 30 (1964).

68. Barron, E. G. S., and Johnson, P., Archiv. Biochem. 48, 149 (1954).

69. Lange, R., Pihl, A., and Eldjarn, L., Intl. J. Rad. Biol. 1, 73 (1959).

70. Romani, R. J., and Tappel, A. L., Archiv. Biochem. Biophys. 79, 323 (1959).

71. Shimazu, F., and Tappel, A. L., Radiation Res. 23, 210 (1964).

72. Hutchinson, F., and Preston, A., "Actions Chimiques et Biologique des Radiations", Sixieme Serie, Masson et C., 1963.

73. Britton, H. T. S., "Hydrogen Ions", 4th ed., Vol. 1, Van Nostrand, Princeton, N. J., 1956, p. 352.

74. Laitinen, H. A., "Chemical Analysis", McGraw-Hill Book Co., N. Y., 1960, pp. 21, 81.

75. Benson, S. W., "The Foundations of Chemical Kinetics", McGraw-Hill Book Co., N. Y., 1960, p. 15.

76. Racker, E., "Methods in Enzymology", (Colowick & Kaplan, Eds.,), Vol. 1, Academic Press, N. Y., 1955, p. 500.

77. Beisenherz, G., Boltze, H. J., Bucher, T. H., Usw, R. C., Z. Naturforsch. 8b, 555 (1953).

78. Bacq, Z. M., and Alexander, P., "Fundamentals of Radiobiology", Pergamon Press, The MacMillan Co., N. Y., 1961.

79. Augenstine, L. G., Radiation Res. 10, 89 (1959).

80. Hutchinson, F., and Ross, D. A., Radiation Res. 10, 477 (1959).

81. Sanner, T., and Pihl, A., Radiation Res. 19, 12 (1963).

82. Gorin, G., and Quintiliani, M., Radiation Res. to be published.

83. Weiss, J., Allen, A. O., and Schwarz, H. A., Proc. Intern. Conf. Peaceful Uses Atomic Energy, Geneva 14, 179 (1956).

APPENDIX A

Determination of Mercapto Groups with 5,5'-Dithiobis(2-nitrobenzoic Acid)

GEORGE GORIN AND SHAKTI K. AIREE

From the Chemistry Department, Oklahoma State University, Stillwater, Oklahoma

Received_____

The disulfide 5,5'-dithiobis(2-nitrobenzoic acid) (nbSSbn)[1] was first synthesized by Ellman and used by him for the determination of mercapto groups in biological fluids (1). Diez et al. (2) were among the first to utilize this reagent to determine the mercapto-group content of purified proteins. (nbSSbn) has certain advantages over other mercapto group reagents, and recently it has become very popular; in the Science Citation Index (3) there are some 28 entries for Ellman's paper in 1964, 70 for 1965 (for a brief review of the pertinent literature, see reference 4).

This paper reports determinations of the stoichiometry of the reaction with cysteine, β-lactoglobulin and ovalbumin. It will be seen that, in the conditions chosen, three different equations represent the respective reactions (B is the basic component of the buffer medium):

---

[1] Abbreviations and symbols: (nbSSbn), 5,5'-dithiobis(2-nitrobenzoic acid) or its salt; $P(SH)_n$, cysteine or mercapto-containing proteins; SDS, sodium dodecyl sulfate; EDTA, disodium ethylenedinitrilotetraacetate; μ, ionic strength.

$$2PSH + nbSSbn + 2B \longrightarrow PSSP + 2nbS^- + 2BH^+ \quad (1)$$

$$PSH + nbSSbn + B \longrightarrow PSSbn + nbS^- + BH^+ \quad (2)$$

$$P\begin{matrix}-SH \\ -SH\end{matrix} + nbSSbn + 2B \longrightarrow P\begin{matrix}-S \\ -S\end{matrix} + 2nbS^- + 2BH^+ \quad (3)$$

The significance of these findings is considered in the DISCUSSION.

In order to realize fully the potentialities of this reagent, it is important to recognize that the pH of the medium affects the reaction in several distinct ways. Raising the pH increases the rate of reaction, which may be very low for proteins, especially in the native state. However, at too high pH hydrolytic splitting of the disulfide bond may occur and give misleading results. As the pH is lowered, on the other hand, the product (nbS^-) eventually becomes protonated, with a concomitant decrease in absorbancy; furthermore, the reaction may not attain as high a degree of completion, since it is the ionization of (nbSH) which provides a major portion of the driving force for the reaction. The present paper gives some consideration to all these matters.

MATERIALS AND METHODS

(nbSSbn) was obtained from Aldrich Chemical Co., recrystallized twice from glacial acetic acid, and dried in a vacuum dessicator for one week. Ovalbumin (B grade, batch 36293) and L-cysteine hydrochloride hydrate (A grade, 32467) were products of Calbiochem. Bovine β-lactoglobulin was from Koch-Light Laboratories (batch 11203), glutathione from Nutritional Biochemical (lot 8755), β-mercaptoethanol from Evans Chemetics (batch 657102) and sodium borohydride from Matheson, Coleman and Bell. SDS[1] was of U.S.P. grade from Fisher Scientific and other chemicals were of ACS-reagent grade. All the buffers were made in

distilled deionized water, which had been deaerated by boiling and then cooling under nitrogen.

Reduction of (nbSSbn) with Sodium Borohydride

Sodium borohydride, 36 mg, was added to 25 ml of 10^{-3} M (nbSSbn) solution in pH 9.0 borate buffer (0.01 M boric acid, 0.1 M NaCl, 10^{-3} M EDTA, adjusted to pH 9.0 with 0.1 M NaOH) in a 100-ml volumetric flask. Effervescence and an intense yellow color developed almost immediately. The mixture was allowed to react for 0.5 hr (the reaction was complete in 15-20 min). Enough 0.1 M HCl was added to make the yellow color just disappear, then the same volume of 0.1 M NaOH and enough borate buffer to make 100 ml. The spectrum was determined after diluting this solution twenty times.

Determination of pK_a

Sodium borohydride, 36 mg, was added to 100 ml of 10^{-3} M (nbSSbn) and the mixture allowed to react for about 1 hr. A 5-ml portion of this solution was diluted to 250 ml with (a) a solution containing 0.02 M sodium acetate, 0.1 M NaCl and 10^{-3} M EDTA (μ = 0.12) or (b) a solution containing 0.1 M sodium acetate, 1 M NaCl and 10^{-3} M EDTA (μ = 1.1). Small amounts of 0.1 M HCl were added, an aliquot was removed after each addition, and its pH and absorbance at 410 mμ were measured; the blank contained all reagents except sodium borohydride. A stream of nitrogen was passed over the solution. The pK_a was calculated from the following expression:

$$pK_a = pH - \log([nbSH]/[nbS^-]) = pH + \log([A' - A]/A)$$

where A is the absorbance at given pH and A' that at pH 7 or above

(i.e. the absorbance of completely ionized thiol, nbS^-).

Reaction between Mercaptans and (nbSSbn) at pH 5

The buffer contained 0.02 M sodium acetate, 0.1 M NaCl, 10^{-3} M EDTA and sufficient 0.1 M HCl to give pH 5.0. A 25 or 50-ml portion of 5×10^{-5} M (nbSSbn) solution was placed in a 100-ml volumetric flask, mixed with the amount of 10^{-3}-10^{-4} M mercaptan solution to give the desired ratio, and buffer was added to make 100 ml. Aliquots were withdrawn at intervals and the absorbance measured at 410 mμ versus a blank containing all the reagents except the mercaptan until a constant value was attained.

Determination of Stoichiometry

Cysteine solutions were prepared in buffer of pH 6.8 (0.02 M phosphate, 0.1 M NaCl, 10^{-3} M EDTA). The ovalbumin and β-lactoglobulin solutions were prepared in buffer of pH 8.0 (0.1 M phosphate, 10^{-3} M EDTA, 1% SDS). The usual procedure was to place 1 ml of approximately 5×10^{-4} M (nbSSbn) solution in a series of 25-ml volumetric flasks, to add different volumes of the mercaptan or protein solution (approximately 10^{-4} M with respect to mercapto groups) and then enough buffer to make 25 ml. The absorbance was measured at 410 mμ against a blank containing all the reagents except the mercaptan or the protein until a constant value was attained.

Determination of the sedimentation coefficient for ovalbumin were made with a Beckmann Analytical Ultracentrifuge, Model E, using a speed setting of 59,780 rpm and a temperature of 20°. The concentration was 6 mg/ml ovalbumin (1.2×10^{-4} M) with and without 5×10^{-4} M

(nbSSbn).

RESULTS

Fig. 1 shows the spectrum obtained by dissolving (nbSSbn) in phosphate buffer of pH 7.0 (curve A). When (nbSSbn) was treated with borohydride or with a large excess of cysteine, the spectra of the products were identical (curve B); clearly this is the spectrum of nbS^-, the position of the maximum (410 mμ) and molar absorbancy coefficient (13,600 l./mole-cm) are essentially those reported by Ellman (1).

A difficulty was caused by the fact that (nbSSbn) dissolved rather slowly in buffer solutions. By the time solution was complete a yellow tinge could be clearly seen by eye, which intensified somewhat with the passing of time; this of course indicates that a spectral change was taking place. The change was very slow, however; curve A in Fig. 1 was determined as soon as possible after complete solution of the sample, and 24 hr later the maximum was only a few per cent lower and the absorbance at 410 mμ was about 0.035 (for 5×10^{-5} M solution). At pH 8-9 the change was somewhat faster, e.g., in tris(hydroxymethyl)aminomethane buffer of pH 9, the absorbance at 410 mμ was 0.045 1 hr after mixing the buffer and the solid (nbSSbn) and 0.125 after 24 hr.

At pH 11, the spectrum shown in Fig. 1, curve C, developed in 1 hr, and it can be seen that this is the same as the spectrum of (nbS^-); in this case, the absorbance at 410 mμ did not intensify subsequently, indeed it decreased slowly with time.

The pK_a was calculated as explained in the METHODS section. Fig. 2 represents the data obtained for $\mu = 0.12$; the pK_a was found to be 4.41; at $\mu = 1.1$, the pK_a was 4.13. On the basis of these data it may

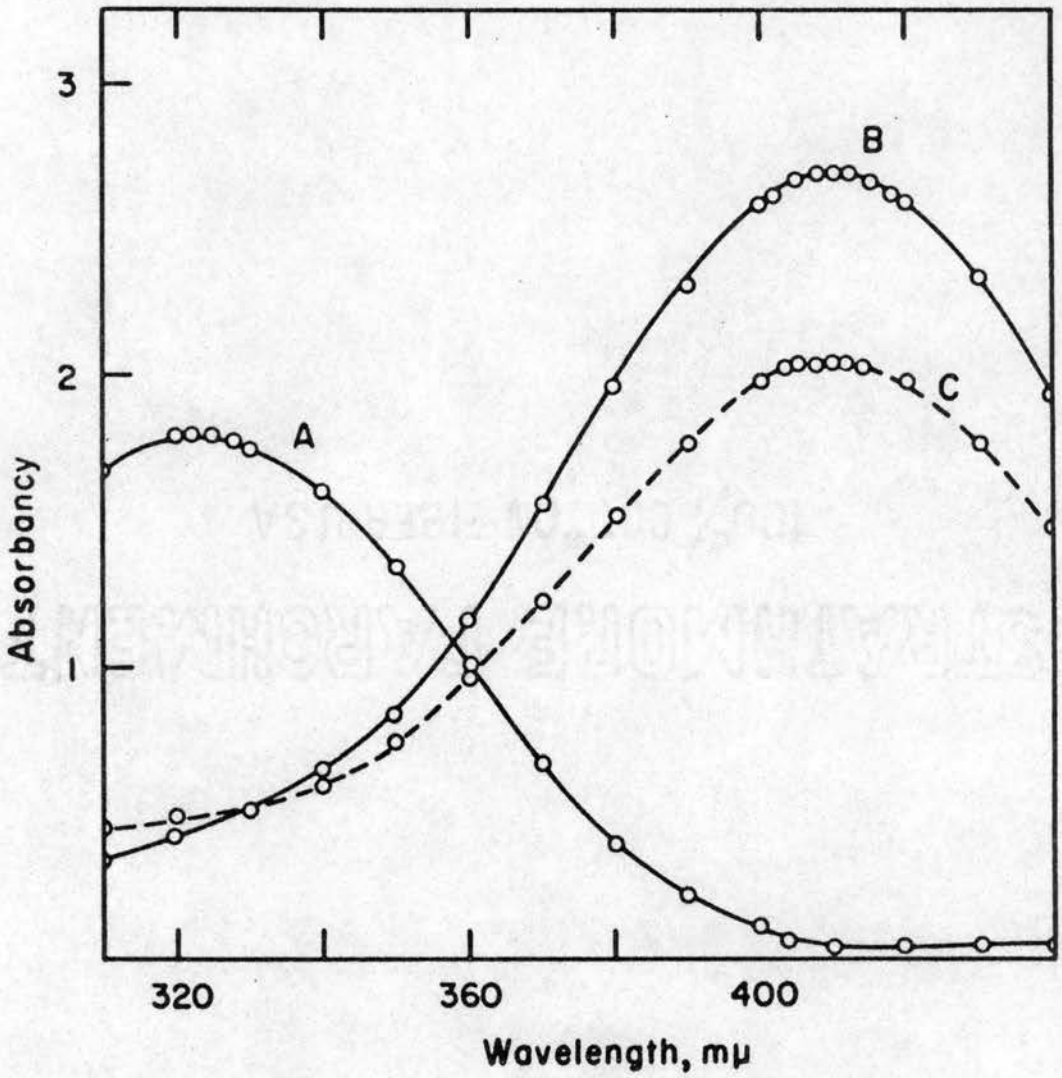


Fig. A1. Absorbancy in (1./mole (nbSSbn)-cm) x 10^{-4}: (A) (nbSSbn) in phosphate buffer, pH 7.0; (B) (nbSSbn) after reduction with borohydride or cysteine; (C) (nbSSbn) in phosphate buffer of pH 11.

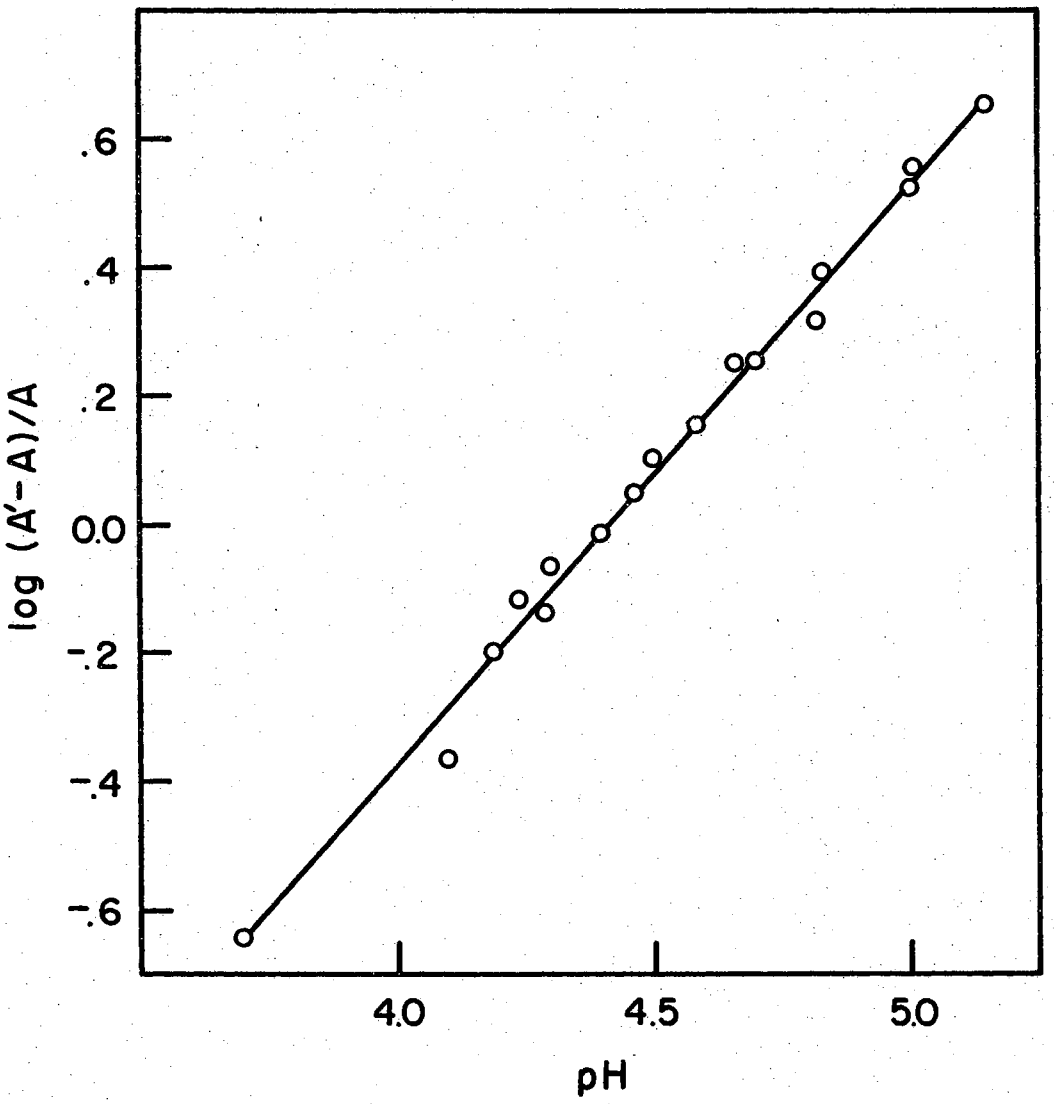


Fig. A2. Determination of pK_a.

be estimated (5) that the "infinite dilution" value would be about 4.6. At μ = 0.1, (nbSH) would be more than 99.5% ionized at pH 6.7, but only 80% ionized at pH 5.0

The reaction of (nbSSbn) with cysteine at pH was investigated both with respect to the degree of completion and the rate. Fig. 3, curve A, shows the effect of increasing the cysteine concentration upon a given initial quantity of (nbSSbn). The rate of the reaction was not related in a simple way to the reactant concentration (see DISCUSSION for some consideration of this point); the half-life period with a ten-fold molar excess of cysteine was 4.5 min. The rate of reaction with glutathione was also measured in the same conditions; the half-life time was 9 min. At pH 6.8, the half-life time for cysteine was less than 1 min at all the reactant ratios investigated, and the absorbances attained are shown in Fig. 3, line B; the two segments of this line intersect at the ratio 2.

Native ovalbumin or β-lactoglobulin gave no appreciable reaction with (nbSSbn) at pH 8.0. In the presence of 1% SDS, which denatures ovalbumin rapidly (6,7), this protein reacted rather slowly at pH 6.8, more quickly at pH 8 (Fig. 4, curves A and B). The reaction of β-lactoglobulin with 1% SDS at pH 8 is shown by the curve C.

Fig. 5 shows the plots of the number of (nbS^-) ions obtained per mole of the mercapto compound against the ratio of (nbSSbn) to the mercapto compound. The initial segment for cysteine and ovalbumin coincide, and the slope of this line is 2: the two segments intersect at 3.7 (nbS^- ions liberated/mole, M.W. 45,000). The mercapto group content of this sample determined by the NEM method gave 3.7_7. The sedimentation constant in the presence of 1% SDS of the protein was

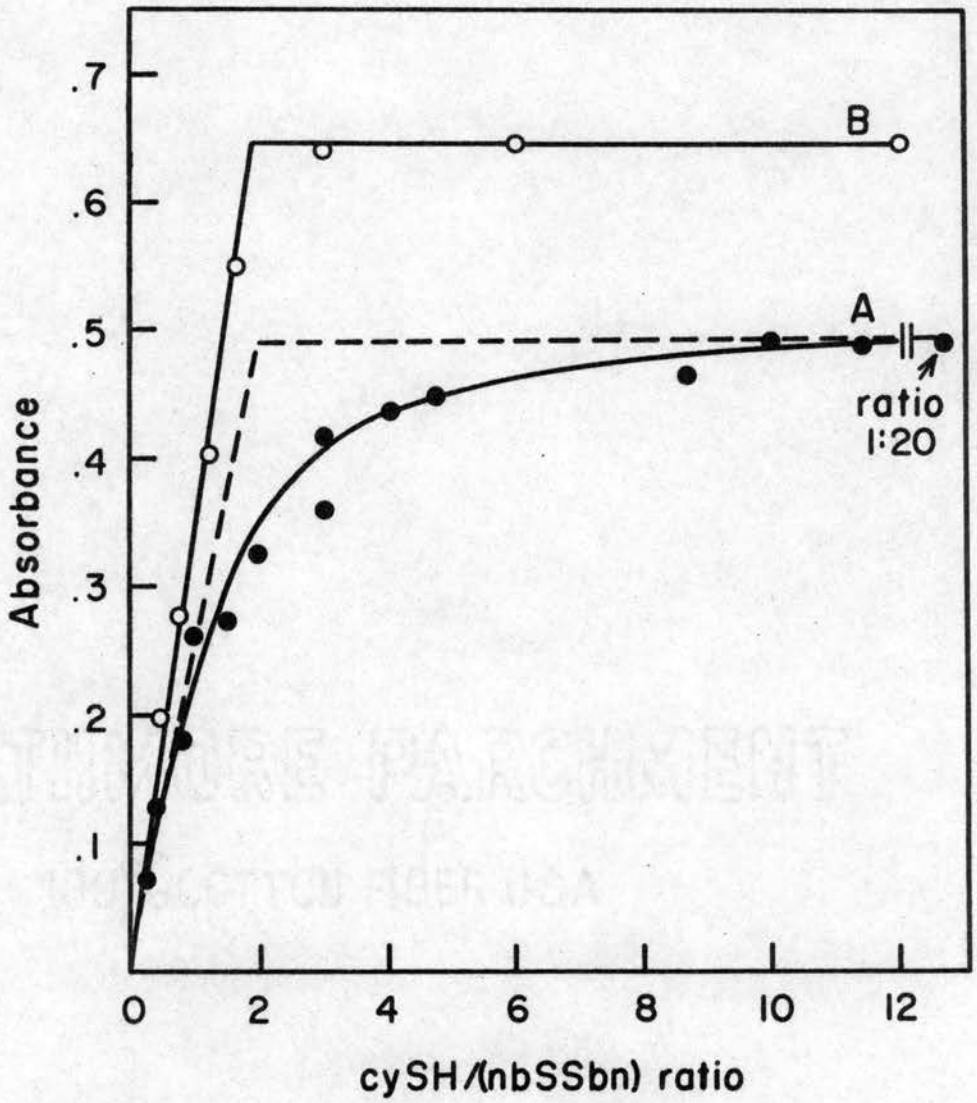


Fig. A3. Stoichiometry of the reaction of (nbSSbn) with cysteine: absorbance vs. cysteine/(nbSSbn) ratio. (nbSSbn) concn; 2.4×10^{-5} M. (A) pH 5.0; (B) pH 6.8.

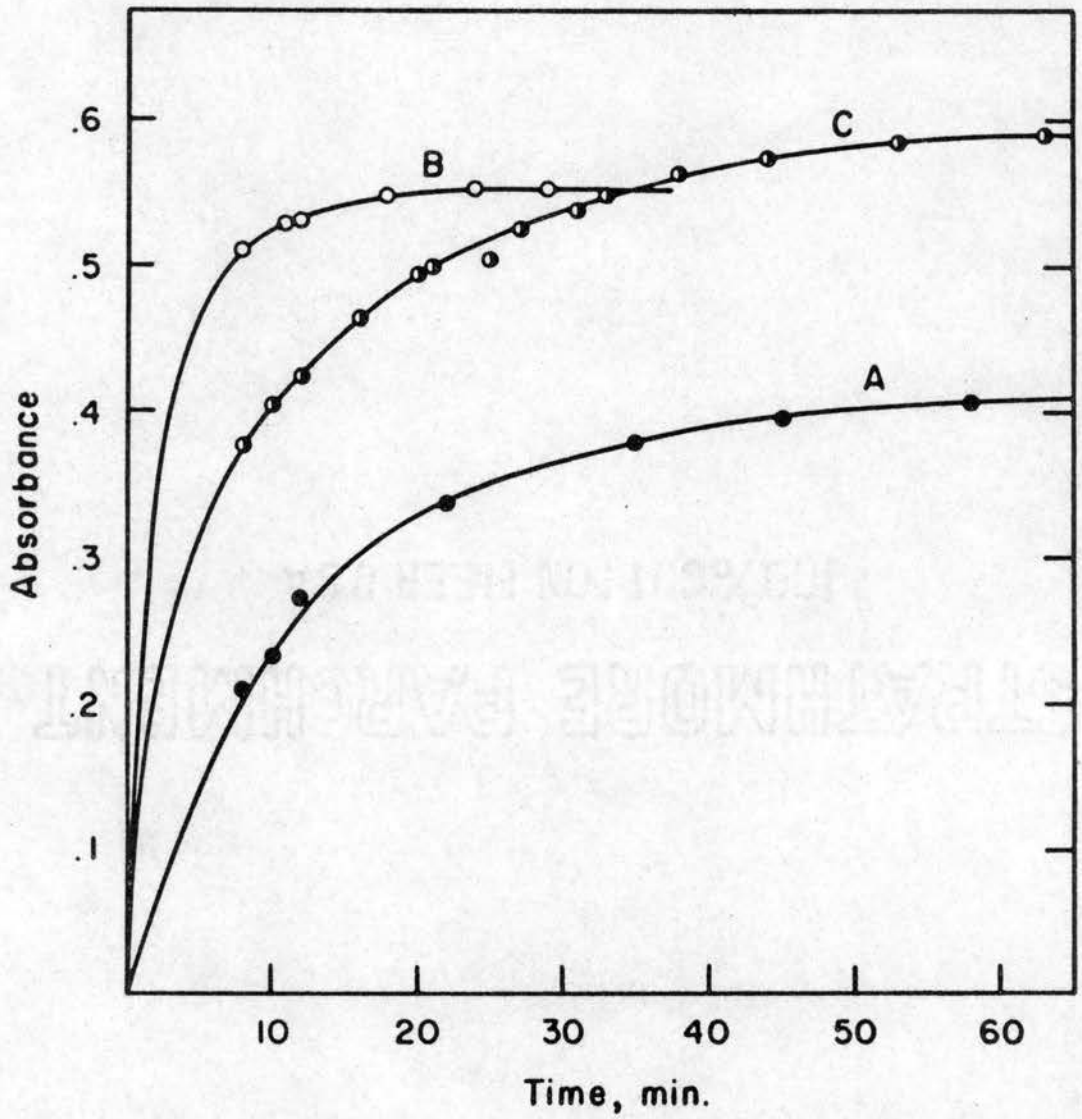


Fig. A4. Rates of reaction of excess (nbSSbn) with proteins: (A) 7 x 10^{-6} M ovalbumin, pH 6.8; (B) 1.04 x 10^{-5} M ovalbumin, pH 8.0; (C) 2.67 x 10^{-5} M β-lactoglobulin, pH 8.0.

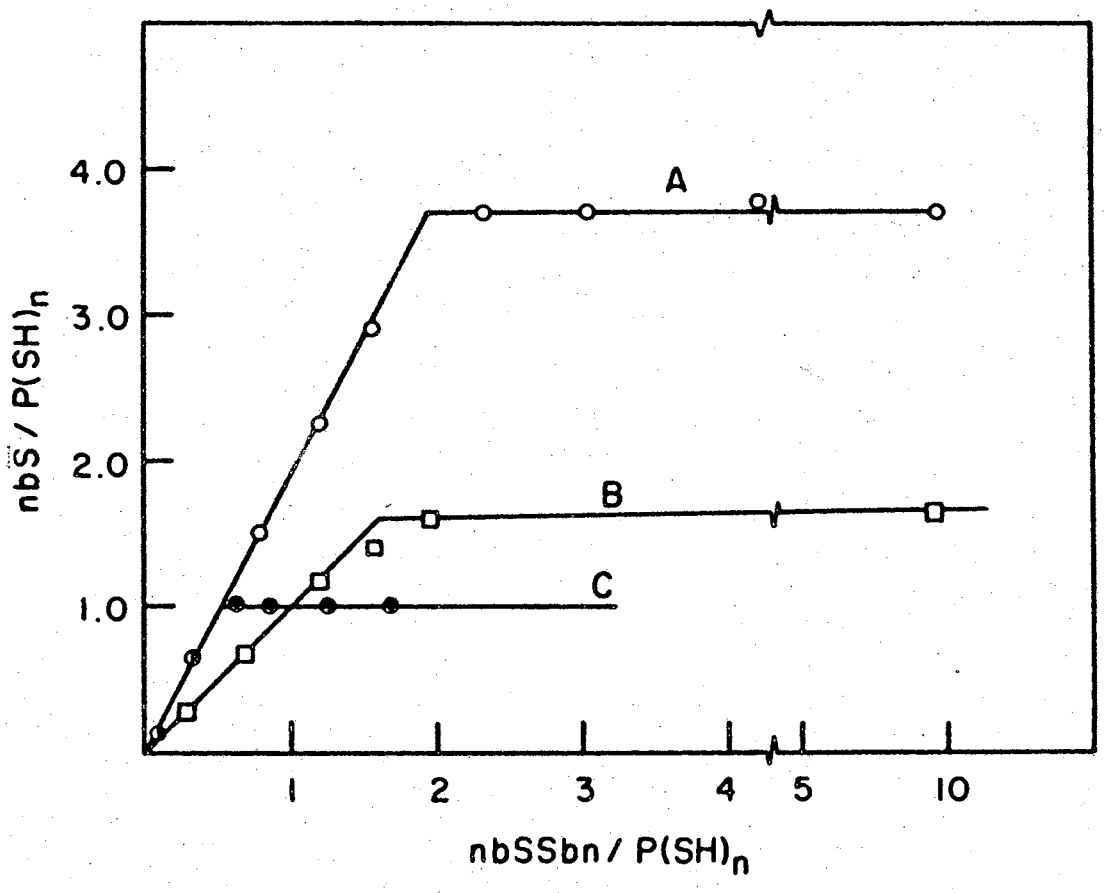


Fig. A5. Stoichiometry of the reaction of (nbSSbn) with (A) ovalbumin, pH 8.0, 1% SDS; (B) β-lactoglobulin, pH 8.0, 1% SDS; (C) Cysteine, pH 6.8.

determined before and after treatment with (nbSSbn); the same result (2.5 ± 0.05) was obtained.

For the reaction with β-lactoglobulin, Fig. 5, line C, the initial slope was 1, and the point of intersection 1.6 (mercapto groups/mole, M.W. 36,000). The mercapto group content of this protein sample by the NEM method was 1.6_7.

DISCUSSION

The results of the present work confirm the utility of (nbSSbn) as a mercapto-group reagent. Some caution must be exercized, however, in its utilization for the study of proteins. Probably its greatest limitation is the slowness of the reaction with protein mercapto-groups. Some other reagents, e.g. p-hydroxymercuribenzoate, react with ovalbumin and β-lactoglobulin in the native state (2), while (nbSSbn) does not; furthermore, many mercapto-group reagents react rapidly with SDS-denatured ovalbumin (6,7), while the rate with (nbSSbn) is relatively slow. In this respect, (nbSSbn) resembles NEM (7); but the former has the advantage of being applicable at lower concentrations.

When the reaction is slow, attention must be given to avoiding the reoxidation of the (nbS^-) produced by air, else low results will be obtained. The importance of adding EDTA to the reaction mixtures might be noted at this point; EDTA inhibits the oxidation, presumably by complexing with traces of metal ions that catalyze the reaction. If EDTA was not added, the absorbance due to (nbS^-) reached a maximum and then decreased, and never attained the level observed in the presence of EDTA. In the application of the reagent to biological fluids, it should be kept in mind that a long time might be needed for complete

reaction, and that oxidation might cause difficulty; since such mixtures might contain considerable amounts of metal ions, it could not be taken for granted that the concentration of EDTA ions in the present work would be sufficient in other applications.

Raising the pH increases the rate of reaction; this could be anticipated, since such effects have been observed in other mercaptan-disulfide systems (8). Unfortunately, raising the pH causes a side-reaction, which also liberates (nbS^-). The other products of the reaction are not known form the present evidence, but the following are likely possibilities (9)

$$nbSSbn + 2OH^- \longrightarrow nbSO^- + nbS^- + H_2O$$

$$2nbSSbn + 4OH^- \longrightarrow nbSO_2^- + 3nbS^- + 2H_2O$$

In the range pH 7-8, the extent of reaction is negligible for freshly prepared solutions, but the employment of higher pH values or of aged solutions should be avoided.

For some applications, it may be desirable to employ a lower pH; as was reported in the preceding section, conveniently measurable rates were realized with cysteine and glutathione at pH 5. At this pH, however, (nbSH) is only partially ionized and hence exhibits a lower effective absorbance at 410 mμ; this must be taken into account in estimating the extent of reaction.

In this connection, another problem also arises. Mercaptan-disulfide interchange reactions generally involve two reversible steps:

$$XSH + YSSY \rightleftharpoons YSH + XSSY$$

$$XSH + XSSY \rightleftharpoons YSH + XSSX$$

and the respective equilibrium constants are of the order of 1. For this reason, such reactions do not proceed to completion with stoichiometric proportions of the reagents (8,10). The case is different with (nbSSbn) at pH 5-7 because the product mercaptan is extensively ionized, whereas the reactant is not; then, interaction with the buffer base tends to drive the reaction toward completion (10). This is well illustrated by the data in Fig. 3; at pH 5.0, it is seen that the amount of (nbS^-) liberated keeps increasing past the stoichiometric ratio and that near-completion is attained only with 10-fold excess of cysteine. At pH 6.8, on the other hand, reaction is sensibly complete at the stoichiometric ratio. The fact that the reaction involves two steps, and hence four specific-rate constants, complicates the interpretation of rate data. No quantitative kinetics studies have been made, but qualitatively the data accord with what might be expected a priori, that the rate of each elementary reaction would be of second-order, i.e. proportional to the concentration of each reagent. This is important, because it follows that the time needed for "complete" reaction will increase as the absolute concentration decreases.

Fig. 5 presents the data that relate to the stoichiometry of the reaction with cysteine at pH 6.8 in a different way: the ordinate represents number of (nbS^-) ions liberated per mole of cysteine, as calculated from the absorbance, and the abscissa the (nbSSbn)/cysteine ratio. The slope of the initial segment is 2, which indicates that 2 (nbS^-) ions are produced per (nbSSbn) molecule in the presence of excess cysteine, i.e., the stoichiometry is represented by equation (1) in the INTRODUCTION.

The same reasoning can now be applied to the study of proteins. With ovalbumin, the slope of the initial line is also 2, showing that 2 moles of nbS^- are produced per mole of nbSSbn. In this case, an uncertainly still existed with regard to whether protein disulfide bonds were formed intermolecularly or intramolecularly. For this reason, measurements of sedimentation coefficients were made before and after the treatment with (nbSSbn); since no change was found, it is possible to conclude that the bonds were formed intramolecularly, and that the reaction is represented by equation (3), at least for the most part (formation of some intermolecular bonds might not be detected by the ultracentrifugal analysis).

In the case of β-lactoglobulin, it has been wel established that the fundamental structural unit is a polypeptide chain of M.W. 18,000, which contains a single cysteinyl residue. Except in some extreme conditions, two of these units occur in solution strongly bound to one another, and consequently a molecular weight of 36,000 has been obtained by a number of physical methods (11,12); this value has also been taken as the basis for the stoichiometric calculation. The sample of β-lactoglobulin used in this work has less than the theoretical content of mercapto groups; the value given by the NEM procedure was 1.6_7 and the same result was obtained with (nbSSbn). This case differs from cysteine and ovalbumin in an important way, in that the slope of the initial segment is 1 instead of 2; this indicates that only one (nbS^-) was liberated per mole of (nbSSbn) even in the presence of excess protein. This indicates that the reaction may be represented by equation (2); i.e., that the reaction did not proceed beyond formation of the mixed disulfide. It is likely that there would be considerable electrostatic and steric

repulsion between the polypeptide chains, which prevents their becoming joined by a disulfide fond.

It may be well explicitly to point out that the determination of stoichiometry involved measurements of the reaction between (nbSSbn) and excess protein, which is the opposite from the proportion which must be used to determine the number of mercapto groups. As long as a sufficient excess of (nbSSbn) is used, the latter may be determined without knowing the former. However, the additional information provided by the stoichiometry should be of interest in many cases.

SUMMARY

(1) The stoichiometry of the reaction of (nbSSbn) with cysteine at pH 6.8 and with ovalbumin and 1% SDS at pH 8.0 is 2 mercapto groups per (nbSSbn).

(2) With β-lactoglobulin and 1% SDS at pH 8.0 the stoichiometry is 1 mercapto group per (nbSSbn); apparently, a mixed disulfide is formed.

(3) (nbSSbn) can be easily reduced to (nbSH) with sodium borohydride. The pK_a of (nbSH) is 4.4 at $\mu = 0.1$

(4) (nbSSbn) is rapidly hydrolyzed at pH 11 to give (nbS^-); at lower pH the reaction is slower but it may be appreciable above pH 8 or in aged solutions.

ACKNOWLEDGEMENT

This investigation was aided by a Career Development Award (to G.G.) from the National Institutes of Health, Department of Health, Education and Welfare (HEW) and by Grant 00305 from the Division of

Radiological Health, HEW.

REFERENCES

1. ELLMAN, G. L., Archiv. Biochem. Biophys. 82, 70 (1959)

2. DIEZ, M. J. F., OSUGA, D. T., AND FEENEY, R. E., Archiv. Biochem. Biophys. 107, 449 (1964).

3. "Science Citation Index", Institute for Scientific Information, Philadelphia, Pa.

4. AIREE, S. K., Ph. D. Thesis, Oklahoma State University (1967).

5. LAITINEN, H. A., "Chemical Analysis, An Advanced Text and Reference", McGraw-Hill Book Co., N.Y., 1960, p. 20, 81.

6. KATYAL, J. M., LESLIE, J., BUTLER, M. F., AND GORIN, G., Archiv. Biochem. Biophys. 94, 54 (1961).

7. LESLIE, J., WILLIAMS, D. L., AND GORIN, G., Anal. Biochem. 3, 257 (1962).

8. SMITH, H. A., DOUGHTY, G., AND GORIN, G., J. Org. Chem. 29, 1484 (1964).

9. PARKER, A. J., AND KHARASCH, N., Chem. Rev. 59, 583 (1959).

10. GORIN, G., AND DOUGHTY, G., J. Chem. Soc., to be published.

11. GORDON, W. G., BASCH, J. J., AND KALAN, E. B., J. Biol. Chem. 236, 2908 (1961).

12. TOWNSEND, R., WEINBERGER, L., AND TIMASHEFF, S. N., J. Am. Chem. Soc. 82, 3175 (1960).

APPENDIX B

ASSAY OF YEAST ALCOHOL DEHYDROGENASE

A variety of conditions have been used in the assay of alcohol dehydrogenase (alcohol-NAD^+ oxidoreductase, EC 1.1.1.1.) from yeast; Table I summarizes the pertinent literature. This note describes a procedure which in our opinion has some advantages over other assays. It also makes a direct comparison between this procedure and the assay methods of Racker (1), of Wallenfels and Sund (2) and of Hoch and Vallee (3), which have been commonly used in the past.

The reagent is prepared by taking 25.8 millimoles of NAD^+ and 5 ml of 1.66 M ethanol (10 ml of 95% ethanol diluted to 100 ml) and enough buffer (6.46×10^{-2}M sodium pyrophosphate, 6.25×10^{-4}M EDTA, pH adjusted to 8.8 with HCl) to make 25 ml. The stock enzyme solution (about 0.5 mg/ml) is prepared by centrifuging a crystalline suspension, dissolving the residue in 0.1 M phosphate-5×10^{-4}M EDTA buffer, pH 7.5 and centrifuging to remove any insoluble residue. This solution is diluted about 1000-fold with 0.01 M phosphate-5×10^{-4}M EDTA buffer pH 7.5 just before assaying.

The procedure was as follows: place 3 ml of the reagent at 25° in a 1-cm spectrophotometer cell. Add 100 µl (0.1 ml) of the diluted enzyme solution and stir vigorously. Measure the absorbance with time at 340 mµ against a blank which contains only reagent, in the following way: preset the spectrophotometer to read some convenient low value, e.g., 0.015, and start the stop-watch when this is attained

TABLE B-1

LITERATURE SURVEY OF ASSAY PROCEDURES

Author(s)	Ref.	$[NAD^+] \times 10^4$	[Ethanol]	Buffer	pH
Racker	(1)	0.5	.1	0.1 M Pyrophosphate	8.5
Wallenfels and Sund	(2)	4.5	.1	.075 Pyrophosphate .075 Semicarbazide .022 Glycine	8.6
Hoch and Vallee	(3)	83.0	.3	.033 M Pyrophosphate	8.8
Whitehead and Rabin	(4)	1.67	.01	.08 M Pyrophosphate	8.8
Keleti	(5)	1.6	.3	.16 M Pyrophosphate (+ unspecified amount of KCN)	8.5
Barron and Levine	(6)	1.8	.37	.02 M Pyrophosphate + 10^{-3} M Cysteine	8.9
Lange, et al.	(7)	11.0	.56	.017 Pyrophosphate	8.86
Romani and Tapel	(8)	2.5	.1	.01 M Pyrophosphate	8.5
This Work	(-)	10.0	.3	.05 M Pyrophosphate- 5×10^{-4}M EDTA	8.8

i.e., when the instrument comes to the null point; quickly preset again to a value, 0.01 or 0.02 higher, read the time when null point is reached and repeat the procedure as many times as desired.

When 0.1 ml of the diluted enzyme solution is added to 3 ml of the reagent, the final concentrations are: 0.05 M pyrophosphate, 5×10^{-5} M EDTA, 1.0×10^{-3} M NAD^+, 0.3 M ethanol and 1.6×10^{-5} mg/ml enzyme. Fig. 1 shows representative data obtained with five concentrations of the enzyme. The rate ($\Delta A_{340}/\Delta t$) can be calculated either as the slope of the plot of A_{340} against time or by averaging 4 or more measurements. The activity units defined according to the International Union of Biochemistry can be calculated by the equation:

$$U_{IUB} = \frac{\Delta A_{340}\,cm^{-1}}{\Delta t\ (sec)} \times \frac{60\ sec}{1\ min} \times \frac{1}{6.22 \times 10^{3}\ cm^{-1}\ 1\ mole^{-1}} \times \frac{1\ l.}{1000\ ml}$$

$$\times \frac{10^{6}\ \mu\ moles}{1\ mole} \times 3.1\ ml$$

This, of course, is the number of units in 100 µl of the sample.

Fig. 2 shows that the rates so obtained and therefore the activities are directly proportional to the enzyme concentration in the range (2-10) $\times 10^{-4}$ mg/ml. It may be pointed out that 5×10^{-4} M EDTA present does an adequate job of protecting the enzyme on dilution to these low concentrations.

Comparison was made between the present assay and those of Racker, of Wallenfels and Sund and of Hock and Vallee in parallel experiments performed at the same time with the same enzyme stock solution after affecting appropriate dilutions. One Racker Unit (R.U.) is defined as that amount of enzyme which corresponds to a change in absorbance at 340 mµ of 0.001 per min in 3 ml of assay mixture; one Wallenfels

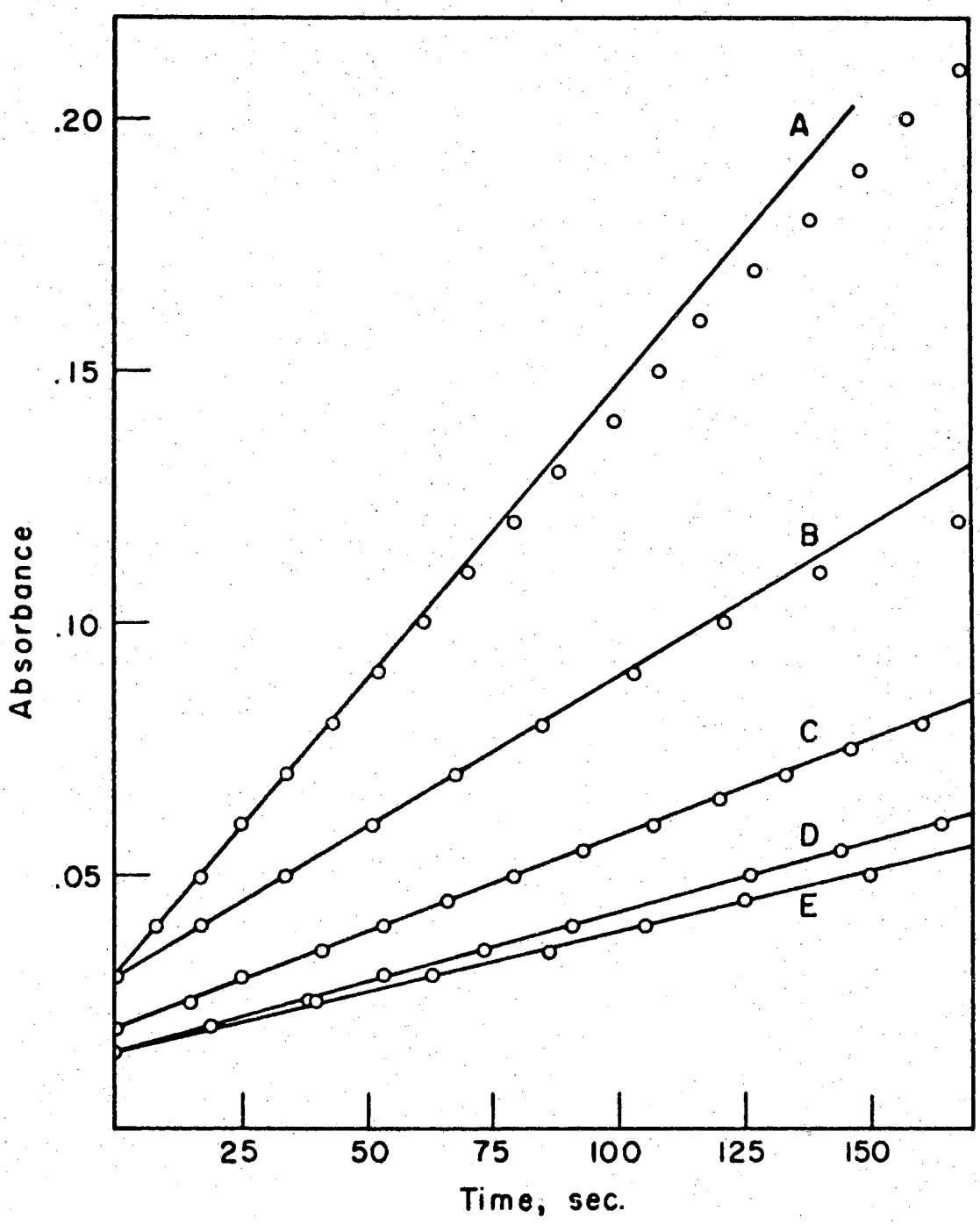


Fig. B1. Assays with different enzyme concentrations. (A) 1.12×10^{-3} mg/ml; (B) 5.64×10^{-4} mg/ml/ (C) 3.37×10^{-4} mg/ml; (D) 2.84×10^{-4} mg/ml; and (E) 2.27×10^{-4} mg/ml.

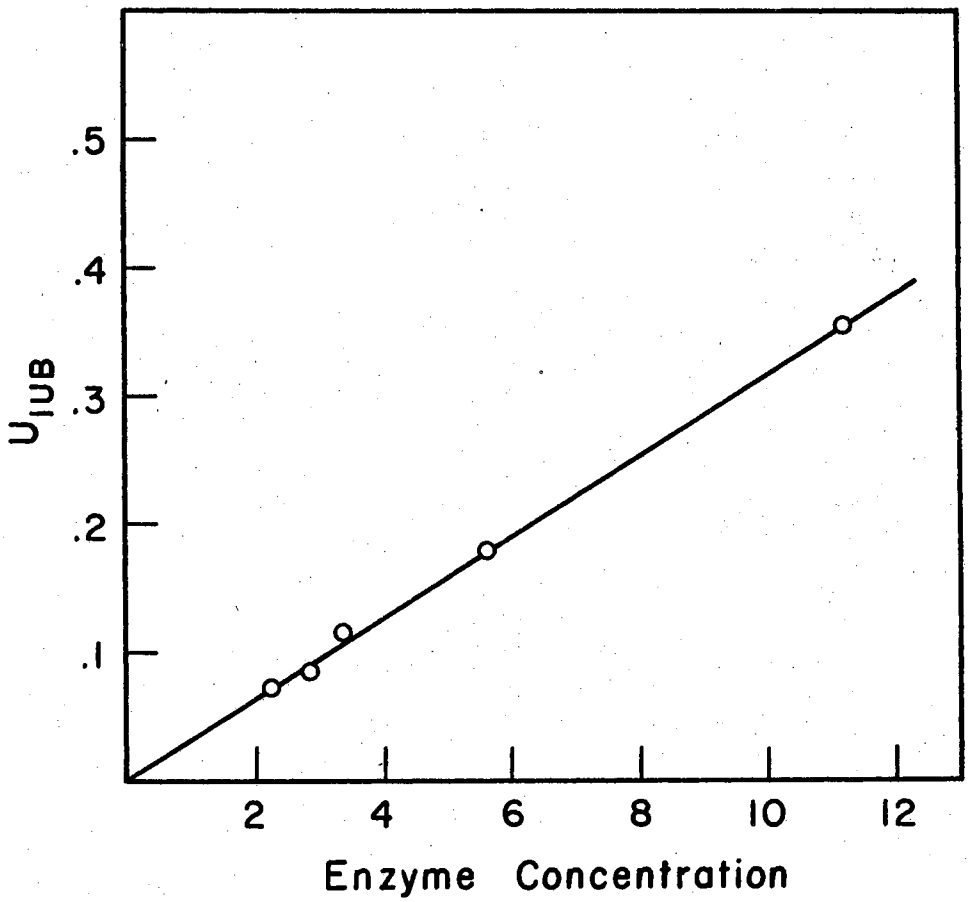


Fig. B2. Plot of activity against concentration (mg/ml).

and Sund Unit (W.S.U.) corresponds to a change of 0.100 in absorbance at 366 mμ per 1 ml of solution; and one unit (U_{IUB}) according to the International Union of Biochemistry corresponds to the reduction of one micromole of NAD^+ per min under the specified conditions at 25°C. So by definitions alone:

$$1\ U_{IUB} = 2.07 \times 10^3\ RU = 37.6\ WSU$$

When assays are made under respective conditions the rates vary in the following ratio

1 (This work): 0.16 (Racker): 0.60 (Wallenfels and Sund): 1.5_4 (Hoch and Vallee)

so that the conversion factors between various assay units are

$$1\ U_{IUB}\ \text{(This work)} = 321\ RU = 22.6\ WSU = 1.5_4\ U_{IUB}\ \text{(Hoch \& Vallee)}$$

These conversion factors are the average of three determinations on two samples of the enzyme, one a laboratory preparation and the other a commercial sample from Worthington and are reliable to 5-10%.

BIBLIOGRAPHY

1. RACKER, E., in "Methods in Enzymology," Vol. I, edited by Sidney P. Colowick and Nathan O. Kaplan, Academic Press, 1755, p. 500; J. Biol. Chem. 184, 313 (1950).

2. WALLENFELS, K., AND SUND, H., Biochem. Z. 329, 17 (1957).

3. VALLEE, B. L., AND HOCH, F. L., Proc. Nat. Acad. Sci., 41, 327 (1955).

4. WHITEHEAD, E. P., AND RABIN, B. R., Biochem. J. 90, 532, 539 (1964).

5. KELETI, T., Acta Physiol. Acad. Sci. Hung. 13, 309 (1958).

6. BARRON, E. G. S., AND LEVINE, S., Arch. Biochem. Biophys. 41, 175 (1952).

7. LANGE, R., PIHL, A., AND ELDJARN, L., Int. J. Rad. Biol. 1, 73 (1959).

8. ROMANI, ROGER J., AND TAPPEL, A. L., Arch. Biochem. Biophys. 79, 323 (1959).

APPENDIX C

Reaction of Yeast Alcohol Dehydrogenase with 5,5'-Dithiobis(2-nitrobenzoic acid) and Other Mercapto-group Reagents

The mercapto group content of yeast alcohol dehydrogenase (alcohol-NAD^+ oxidoreductase, EC 1.1.1.1.) (YADH) and the reactivity of these groups has been the subject of several studies (1,2,3,4). These have clearly shown that the enzyme contains several mercapto groups, that they are reactive and that their combination affects the activity. There is, however, considerable controversy about the quantitative findings. According to Wallenfels and Sund (2), the enzyme preparation with the highest activity contains 36 groups per 150,000 M.W.; however, the mercapto group content and the activity decreases on ageing, so that most preparations used by Wallenfels and Sund and by other investigators contained a smaller number of mercapto groups.

This note reports the results obtained with some other mercapto group reagents. For comparison purposes, the enzyme sample was tested with PCMB also. The results are summarized in Table I. N-ethylmaleimide (NEM) and iodine precipitated the native enzyme while ferricyanide reacted to a negligible extent. In the presence of 1% SDS or 6 M guanidine hydrochloride, NEM reacted without precipitation, but different results were obtained.

5,5'-Dithiobis(2-nitrobenzoic acid) (nbSSbn) gave the most satisfactory results. The same mercapto group content was found for native and denatured enzyme, albeit reaction with the native enzyme was quite

TABLE C-1

DETERMINATION OF -SH GROUPS IN YADH[a] WITH VARIOUS REAGENTS AND CONDITIONS

Reagent	Denaturant	No. of -SH Groups	Remarks
nbSSbn	none	16.6 ± 1.8	Equil. value reached in ~2 hrs. $t_{\frac{1}{2}}$ = ~30 min.
nbSSbn	1% SDS	16.4 ± 2.3	Equil. value reached in ~1½ hrs. $t_{\frac{1}{2}}$ = ~4 min.
nbSSbn	6 M G-HCl	16.7	Reaction was over in ~3 min. Color faded away fast unless EDTA was used.
PCMB	none	16.1 ± 1.2[b]	
Ferricyanide	none	negligible reaction	
Ferricyanide	1% SDS	10-20	Stoichiometry uncertain
Ferricyanide	6 M G-HCl	20.5	
NEM	none	---	Instantaneous Coagulation
NEM	1% SDS	10-20	
NEM	6 M G-HCl	18-19	
I_2	none	---	Coagulation after 10-20 equiv. of I_2 added
I_2	1% SDS	---	

[a] Sample W-1, activity 167 ± 4 U_{IUB}/mg

[b] In some observations, which were not used in figuring out the average, values as high as 27.0 (2 obs.) and as low as 11.0 were obtained.

slow.

Table II gives values of the activity remaining at various points during the reaction with nbSSbn. In the first part of the Table a large excess of nbSSbn was used, and it can be seen that the activity and the -SH content do not decrease in quite parallel fashion. Particularly noticeable is the fact that, at the beginning, the decrease in -SH content is greater. This effect would be more clearly demonstrated by adding a small amount of nbSSbn. With a 1:2 enzyme/nbSSbn ratio, 3 -SH groups react without causing any loss in activity, and on longer incubation ($\sim$12 hrs) <6% (average) loss of activity occurs. It should be noted that 2 moles of nbS^- are released, corresponding to the reaction of 2 -SH groups, per mole of nbSSbn, i.e., the stoichiometry is

$$P(SH)_2 + nbSSbn \rightleftharpoons P(S)_2 + 2nbSH$$

where P represents the protein molecule.

This indicates that not all the mercapto groups are essential, and that nbSSbn has some selectivity for the non-essential groups which react to give intramolecular disulfide linkages as opposed to mixed disulfides.

Experimental

YADH samples W-1 and W-2 were obtained from the Worthington Biochemical Corporation, Freehold, N. J. (Lot #6078 and 6115 respectively) and L-1 and L-2 were prepared in the laboratory by Racker's procedure (5). Activities of these samples were: W-1, 167 U/mg; W-2, 260 U/mg; L-1, 200 U/mg; and L-2 (aged), 111 U/mg. The corresponding numbers of mercapto groups were: 16-17, 18, 19 and 13 respectively.

TABLE C-2

RELATIONSHIP BETWEEN ACTIVITY AND NUMBER OF -SH GROUPS REACTED

Time (min)	No. of -SH reacted	% Activity remaining
	Sample W-1, (nbSSbn/YADH) = 55	
1	2.5	99
4	4.1	83
14	6.1	74
28	8.2	45
54	12.2	24
80	14.7	14
100	15.9	8
125	16.7	2
	(nbSSbn/YADH) = 61	
1	3.1	96
5	4.7	83
11	5.8	67
19	7.3	56
25	8.5	46
32	10.0	36
40	11.8	28
48	13.0	20
58	14.3	14
73	15.8	8
	(nbSSbn/YADH) = 2	
2	2.1	106
7	2.8	106
22	3.0	102
34	3.2	102
47	3.3	101
65	3.5	101
11x60	3.7	97
	Sample W-2, (nbSSbn/YADH) = 2	
10	2.2	99
90	2.9	99
180	3.4	91

TABLE C-2 (Continued)

Time (min)	No. of -SH reacted	% Activity remaining
	Sample L-1, (nbSSbn/YADH) = 2	
2	1.6	104
9	1.8	108
19	2.0	104
35	2.3	104
60	2.7	104
12x60	3.9	91
	Sample L-2, (nbSSbn/YADH) = 2	
3	2.8	98
15	3.2	100
60	3.6	98
170	4.0	94

The procedure of Leslie et al. (6) was used for the reaction with NEM; the method of Boyer (7) with PCMB and that of Cunningham and Neunke (8) with iodine. The procedure with nbSSbn (9) was to mix appropriate amounts of enzyme solution, nbSSbn and SDS (if any) in a curvette and to measure the absorbance at 410 mμ versus a reference solution containing the same reagents except the protein. For example, 1 ml of 3% SDS, 1 ml of YADH solution (~1 mg/ml) and 1 ml of 2.5×10^{-3} M nbSSbn (10 mg/ml) were mixed; the reference solution contained the same amounts of above reagents except that 1 ml of buffer was substituted for the protein solution. When guanidine hydrochloride was used as a denaturant, 7.2 M solution was prepared in buffer and the pH readjusted to 7.0; 10^{-3} M nbSSbn solution was prepared in this medium. In the sample curvette, 2.5 ml of this solution and 0.5 ml of enzyme solution were mixed; in the blank, 2.5 ml of nbSSbn solution and 0.5 ml of buffer.

When measuring activity as well as the progress of reaction with nbSSbn, 0.1 ml of aliquot portions were withdrawn and diluted about 100 times; this nearly stopped the reaction. The activity was then determined.

Activity measurements were made in assay mediums containing 10^{-3} M NAD^+, 0.3 M ethanol, 0.05 M pyrophosphate-5×10^{-4} M EDTA buffer, pH 8.8 as described elsewhere (10).

BIBLIOGRAPHY

1. HOCH, FREDRICH, AND VALLEE, BERT D., in "Sulfur in Proteins" edited by Benesch et al., Academic Press, N. Y., 1959, p. 245.

2. WALLENFELS, KURT, AND SUND, HORST, Biochem. Z. 329, 17 (1957).

3. WHITEHEAD, E. P., AND RABIN, B. R., Biochem. J. 90, 532 (1964).

4. HARRIS, IEUAN, Nature 203, 30 (1964).

5. RACKER, E., J. Biol. Chem. 184, 313 (1950).

6. LESLIE, J., WILLIAMS, D. L., AND GORIN, GEORGE, Anal. Biochem. 3 257 (1962).

7. CUNNINGHAM, LEON W., AND NUENKE, B. JOANNE, J. Biol. Chem. 234, 1447 (1959).

8. BOYER, P. D., J. Am. Chem. Soc. 76, 4431 (1954).

9. ELLMAN, GEORGE L., Arch. Biochem. Biophys. 82, 70 (1959).

10. GORIN, GEORGE, AND AIREE, SHAKTI K., to be published.

VITA

Shakti Kumar Airee

Candidate for the Degree of

Doctor of Philosophy

Thesis: REACTION OF 5,5'-DITHIOBIS(2-NITROBENZOIC ACID) WITH MERCAPTANS AND PROTEIN MERCAPTO GROUPS

Major Field: Chemistry

Biographical:

Personal Data: Born in Hosiarpur, Panjab, India, on November 12, 1934, the son of Durga Dass and Devki Devi. Married Shashi Kalia, July 7, 1963. Two children, Hans Naveen, born October 12, 1964, and Rita, born September 7, 1965.

Education: Graduated from S. D. High School Hoshiarpur, in 1951. Attended Panjab University College from 1951 to 1953; D. A. V. College and Panjab University Chemistry Department, from 1953 to 1958; University of Oregon, 1958 to 1962; and Oklahoma State University, from 1962 to 1966. Received the Bachelor of Science (Honours School) degree in 1956 and Master of Science (Honours School) degree in 1958. Completed requirements for a Doctor of Philosophy degree in Physical Chemistry in September, 1966.

Professional Experience: Assistant Professor of Chemistry at the University of Tennessee Martin Branch, Martin, Tennessee, since January 1, 1966. Member of Phi Lambda Upsilon, American Chemical Society and Tennessee Academy of Sciences. Associate Member of Sigma Xi.