IDENTIFICATION AND METABOLISM OF COENZYME A

GLUTATHIONE DISULFIDE

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May, 1966

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ACKNOWLEDGMENTS

The author gratefully acknowledges the continual guidance and counsel by his major professor Dr. D. R. Wilken.

Special thanks are due to Dr. F. R. Leach for his valuable advice and his stimulating discussions in the course of this research.

Sincere appreciation is extended to Dr. R. E. Koeppe, Dr. N. N. Durham, Dr. R. K. Gholson and Dr. K. D. Berlin for their suggestions and encouragements.

The author would like to thank Mrs. Norma Best for her valuable technical help in some of the experiments described.

Finally he is thankful for the support from the National Institutes of Health (grant GM 10524) and the Oklahoma State University Biochemistry Department which was in the form of facilities and a research assistantship.

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CHAPTER I

INTRODUCTION

A nucleotide-peptide is a compound containing both a nucleotidyl moiety and a peptidyl moiety connected by a covalent bond. A variety of compounds of this nature have been isolated from or detected in different tissues. Much evidence indicates that most of the nucleotide-peptides are activated intermediates in the biosynthesis of large molecules, such as protein and bacterial cell walls, or are activated intermediates in group transfer reactions. Many reports have appeared in the literature noting the detection of nucleotide-peptide complexes but contain incomplete information on the structure and function of the detected compounds.

The first part of this thesis presents evidence which completes the identification of a bovine liver nucleotide-peptide as the unsymmetrical disulfide of coenzyme A and glutathione (CoASSG)[1].

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[1]The following abbreviations are used: DPN, DPNH, diphosphopyridine nucleotide and its reduced form, respectively; TPN, TPNH, triphosphopyridine nucleotide and its reduced form, respectively; FAD, $FADH_2$, flavin adenine dinucleotide and its reduced form, respectively; FMN, flavin mononucleotide; GSH, GSSG, glutathione and its oxidized form, respectively; TPP, thiamine pyrophosphate; CoASH or CoA, coenzyme A; acyl CoA, acyl derivatives of coenzyme A; CoASSG, TSSG, PSSG, CSSG, HSSG, mixed disulfides of glutathione and coenzyme A, thiolethanolamine, pantotheine, cysteine, and homocysteine, respectively; HSSH, homocystine; CoASSCoA, coenzyme A disulfide; CSST, disulfide of cysteine and thiolethanolamine; RNA, ribonucleic acid; sRNA, soluble ribonucleic acid; RNase, ribonuclease; UDPG, uridine diphosphate glucose; UDPGNAc, uridine diphosphate N-acetylglucosamine; E, enzyme; EDTA, ethylenediamine-

The second part of the thesis will describe the detection and partial purification of a bovine kidney enzyme which may be important in the metabolism of CoASSG. The enzyme catalyzes the following sulfhydryl-disulfide interchange reaction:

$$GSH + CoASSG \rightleftharpoons CoASH + GSSG \qquad (1)$$

Several of the chemical and physical properties of the enzyme also will be presented.

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tracetic acid; Tris, tris(hydroxymethyl)aminomethane; NEM, N-ethylmaleimide; TCA, trichloroacetic acid; DNP, dinitrophenyl-; PPO, 2,5-diphenyloxazole; POPOP, 1,4-bis-2-(5-phenyloxazolyl)-benzene; DEAE-cellulose, diethylaminoethyl cellulose; CMC, carboxymethyl cellulose. Other abbreviations used are consistent with the abbreviations commonly accepted by the Journal of Biological Chemistry.

CHAPTER II

LITERATURE REVIEW

An attempt will be made to briefly review subject areas which directly and indirectly bear on various aspects of the thesis subject matter. Since part of the thesis will be concerned with the identification of a compound having some characteristics of a nucleotide-peptide, the structures and functions of various kinds of nucleotide-peptides will be briefly reviewed. The nucleotide-peptide which will be shown to be an unsymmetrical disulfide of coenzyme A and glutathione is cleaved as shown in equation 1 to the sulfhydryl form of coenzyme A and oxidized glutathione in the presence of glutathione and an enzyme to be described. The nature of the reactants and products of the reaction suggest that a review of important naturally occurring sulfhydryl and disulfide compounds and some of their more important chemical reactions should be undertaken. This will include a brief review of the function of sulfur in proteins. Since the above reaction is a sulfhydryl-disulfide interchange reaction and can be viewed as an oxidation-reduction reaction it was felt desirable to review, sometimes briefly, enzymes which catalyze the oxidation of sulfhydryl groups or reduction of disulfide bonds, and in more detail those enzymes which have been shown to catalyze sulfhydryl-disulfide interchange reactions.

Nucleotide-Peptides

Small molecules are frequently found to be activated by biological systems to form intermediates which subsequently either are incorporated into macromolecules or are utilized in other group transfer reactions. Nucleoside monophosphate acid anhydrides are important carriers of small molecules for incorporation into polymers, while other activated molecules such as S-adenosyl methionine exemplify the group transfer category. Thus, amino acid derivatives of adenylic acid are activated intermediates in protein synthesis (equations 2, 3 and 4).

$$\text{Amino acid} + \text{ATP} + \text{Enzyme} \rightleftharpoons \text{Aminoacyl} - \text{AMP} - \text{Enzyme} + \text{PPi} \quad (2)$$

$$\text{Aminoacyl} - \text{AMP} - \text{Enzyme} + \text{sRNA} \rightleftharpoons \text{Aminoacyl} - \text{sRNA} + \text{Enzyme} \quad (3)$$

$$\text{Aminoacyl} - \text{sRNA} \longrightarrow \longrightarrow \longrightarrow \text{Protein} \quad (4)$$

Sugar derivatives of uridine, guanosine, or thymidine diphosphate are activated intermediates in the biosynthesis of polysaccharides (1) (equations 5 and 6). An example of activation and transfer to polysaccharide is shown by equations 5 and 6, respectively.

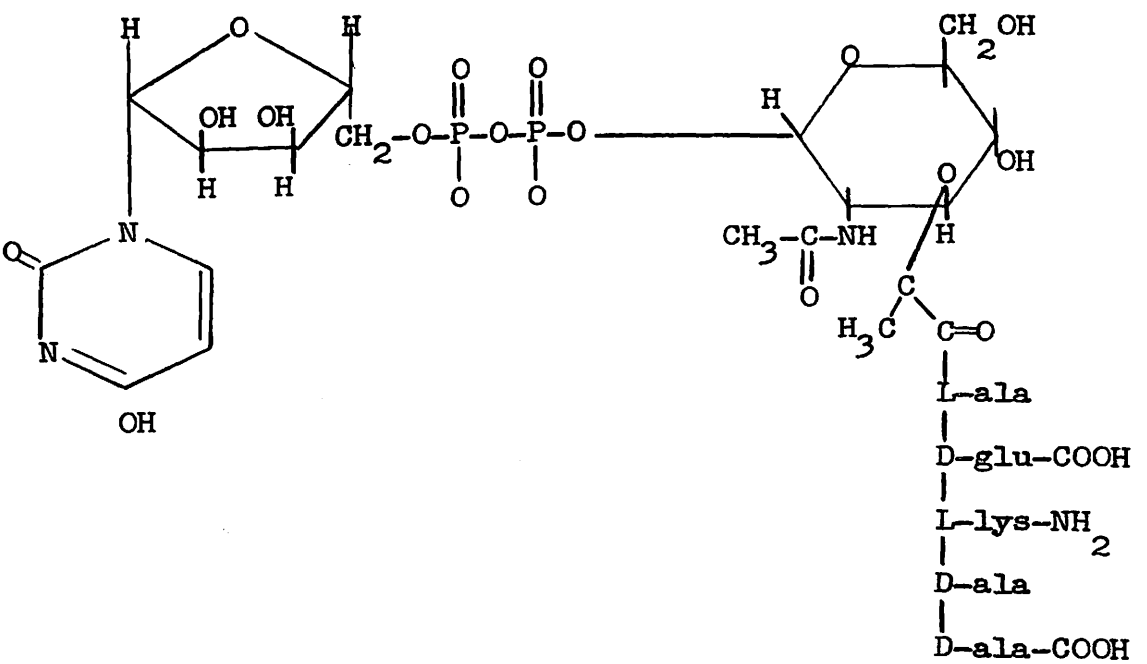
$$\text{UTP} + \text{Sugar-1-phosphate} \rightleftharpoons \text{UDP} - \text{Sugar} + \text{PP} \quad (5)$$

$$\text{UDP} - \text{Sugar} + \text{Acceptor} \rightleftharpoons \text{Acceptor} - \text{Sugar} + \text{UDP} \quad (6)$$

In addition to these relatively simple substances, more complex types of molecules can also be activated as nucleotide derivatives, for example, UDP-muramyl-peptides (2). It is currently believed that nucleotide derivatives of this type are precursors of the bacterial cell wall (3) which is a crosslinked polymer of glycopeptide, polyglycine and polyribilophosphate (4).

UDP-Muramyl-Peptides

UDP-Muramyl peptides were first isolated by Park (5) from penicillin treated Staphylococcus aureus. The chemically most complicated of those isolated was a UDP-muramyl-pentapeptide, uridine-diphospho-N-acetyl-muramyl-L-alanyl-D-glutamyl-L-lysyl-D-alanyl-D-alanine. Its structure is shown in Figure 1 (2). UDP-GNAc-lactyl-L-alanine was also observed in penicillin treated S. aureus. When the organisms were treated with bacitracin or novobiocin, instead of penicillin, the same uridine nucleotides accumulated. Oxamycin treated S. aureus accumulated UDP-GNAc-lactyl-L-ala-D-glu-L-lys. The nucleotide which accumulated in experiments with lysine deprivation is UDP-GNAc-lactyl-L-ala-D-glu (6). Recently, Wishnow and Strominger et al. (7) reported that novobiocin also induced accumulation of the uridine nucleotide which has the same structure as the UDP-muramyl-pentapeptide formed in penicillin treated S. aureus. Strominger also showed (8) that glycine induced the accumulation of four uridine nucleotides in S. aureus. These are: UDP-GNAc-lactate, UDP-GNAc-lactyl-gly-D-glu-L-lys, UDP-GNAc-lactyl-L-ala-D-glu-gly and UDP-GNAc-lactyl-gly-D-glu-L-lys-D-ala-D-alanine. The biosynthesis of these UDP-muramyl-peptides in penicillin treated S. aureus was elucidated by Ito and Strominger (9). They demonstrated the sequential incorporation of ^{14}C amino acids into different UDP-muramyl-peptide acceptor molecules as summarized in the following series of reactions designated as equation 7.


H
O
H
OH OH
H H
CH2-O-P-O-P-O
O O
O O
N
O
N
OH
CH2 OH
O
H
OH
O
CH3-C-NH
O
H
C
H3C
C=O
L-ala
D-glu-COOH
L-lys-NH2
D-ala
D-ala-COOH

$$\text{UDP-GNAc-lactate (I)} + \text{L-alanine} \xrightarrow[Mn^{++}]{ATP} \text{UDP-GNAc-lactyl-L-ala (II)}$$

$$\text{(II)} \xrightarrow[ATP,\ Mn^{++}]{\text{D-glutamic acid}} \text{UDP-GNAc-lactyl-L-ala-D-glu (III)} \xrightarrow[ATP,\ Mn^{++}]{\text{L-lysine}}$$

$$\text{UDP-GNAc-lactyl-L-ala-D-glu-L-lys (IV)} \qquad (7)$$

$$\text{2-D-alanine} \xrightarrow[Mn^{++}]{ATP} \text{D-ala-D-ala (V)}$$

$$\text{(IV)} + \text{(V)} \xrightarrow[Mn^{++}]{ATP} \text{UDP-GNAc-lactyl-L-ala-D-glu-L-lys-D-ala-D-ala (VI)}$$

The assay for the enzymatic reactions represented by the above equations was based on the conversion of radioactive amino acids to a charcoal absorbable form as a result of their addition to the appropriate nucleotides (9). Since the various transferring enzymes were insensitive to RNase, the participation of RNA in the synthesis of the UDP-muramyl-pentapeptide was excluded (10).

The function of UDP-muramyl-peptides as precursors in cell wall biosynthesis has been proposed by several workers (3, 11, 12). Chatterjii and Park (13) presented evidence that the role of UDP-muramyl-peptide was to synthesize a mucopeptide backbone in the presence of UDP-GNAc, and that this mucopeptide backbone acts as an acceptor into which polyglycine is incorporated.

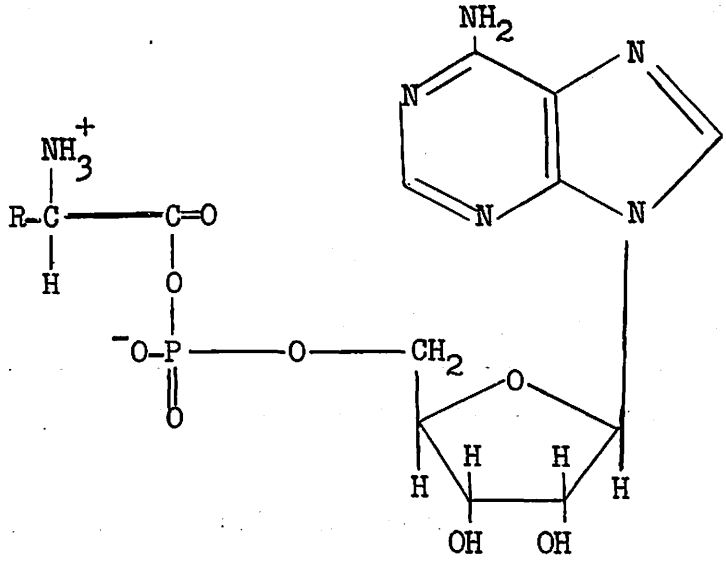
More recently, Anderson and Strominger (14) found that the initial reaction in glycopeptide synthesis in S. aureus and Micrococcus lysodeikticus is the transfer of phosphoacetyl-muramyl-pentapeptide from UDP-muramyl-peptide to a lipid fraction with liberation of UMP. The GNAc moiety of UDP-GNAc is then transferred to the lipid fraction with

liberation of UDP. Finally the disaccharide, GNAc-MurNAc-pentapeptide is transferred to an acceptor (presumably an incomplete glycopeptide) with the release of inorganic phosphate and lipid. This reaction sequence represents a relatively late step in over-all cell wall biosynthesis but appears to precede the addition of polyglycine residues.

Reviews on the history and development of this subject have been presented by Strominger (15), Perkins (16) and Ashwell (10).

Aminoacyl-AMP

Aminoacyl adenylates which function as intermediates in protein synthesis can be prepared chemically (17, 18). Their general structure is shown in Figure 2. The acyl adenylates of α-amino acids are rapidly hydrolyzed at a pH close to 7.0, but exhibit much greater stability at a pH of less than 5.5. On the other hand, β-alanyl-adenylate (like acyl adenylate or benzoyl adenylate) is relatively stable at neutral pH. Under certain conditions, α-aminoacyl adenylates are spontaneously converted to products that appear to be the corresponding isomeric derivative in which the amino acid is esterified to the 2',(3') of adenosine 5'-phosphate. Aminoacyl adenylates also react rapidly with ammonia, hydroxylamine and free amino acids. As early as 1941, Lipmann (14) suggested that high energy phosphate was involved in the activation of amino acids for protein synthesis. Zamecnik and Keller (20) showed that ATP and a rat liver protein from the soluble fraction of the cell were essential for the incorporation of ^{14}C-amino acids into the protein of microsomes. Hoagland (21) showed that a 100 x g rat liver supernatant fraction catalyzed the exchange of pyrophosphate and ATP and that the exchange was stimulated approximately three fold when all 20


NH2
N
N
N
N
NH3+
R–C
C=O
H
O
–O–P
O
O
CH2
O
H
H
H
H
OH
OH

amino acids were added. In the presence of hydroxylamine, amino acid hydroxamates were formed and AMP and pyrophosphate accumulated. These results provided the evidence for the enzymatic activation of amino acids and suggested the formation of a tightly enzyme-bound form of the activated amino acid. The direct formation of amino acid adenylate intermediates was demonstrated by Karasek et al. (22) and by Kingdon et al. (23). Recently direct evidence was obtained for the formation of enzyme bound isoleucyl adenylate, valyl adenylate (24) and threonyl adenylate (25). In these studies, the enzyme-linked intermediates were separated from reaction mixtures by passage through a column of sephadex.

The formation of an enzyme bound amino acyl-AMP is depicted in equations 8, 9 and 10.

$$\text{Enzyme} + \text{ATP} \rightleftharpoons \text{Enzyme} - \text{AMP} - \text{PP} \qquad (8)$$

$$\text{Enzyme} - \text{AMP} - \text{PP} + \text{AA} \rightleftharpoons \text{Enzyme} - \text{AMP} - \text{AA} + \text{PP} \qquad (9)$$

$$\text{Sum:}\quad \text{Enzyme} + \text{ATP} + \text{AA} \rightleftharpoons \text{PP} + \text{Enzyme} - \text{AMP} - \text{AA} \qquad (10)$$

Activating enzymes for most natural α-amino acids have been detected and some have been purified. Thus far, there has been no clear cut demonstration of the existence of either a hydroxyproline activating enzyme or a 5-hydroxyl-lysine activating enzyme (26).

Aminoacyl Soluble RNA

There is evidence that the amino acid activating enzymes also catalyze the transfer of the aminoacyl moiety of the enzyme-bound amino-acyl adenylate to specific soluble RNA acceptors (27, 28) (equation 11).

Enzyme-aminoacyl adenylate + sRNA $\rightleftharpoons$ Aminoacyl-sRNA + Enzyme + AMP (11)

The first indication that RNA was involved in the activation of amino acids came from studies of Holley (27). He observed an RNase sensitive alanine stimulated exchange of radioactive AMP and ATP which was catalyzed by a soluble protein fraction obtained from rat liver which contained sRNA. Hoagland et al. (28, 29) and Ogata et al. (30-33) showed that the fraction obtained from the soluble fraction of a rat liver homogenate by precipitation at pH 5 contained low molecular weight sRNA as well as amino acid activating enzymes, and that the sRNA became labeled when the enzyme preparation was incubated with labeled amino acids and ATP. The formation of aminoacyl hydroxamates catalyzed by amino acid activating enzyme is inhibited by RNA (34); similarly the transfer of the aminoacyl moiety of aminoacyl adenylate to RNA is inhibited by hydroxylamine and by inorganic pyrophosphate. These results support the reaction sequence shown in equation 11. Wong and Meister (35) found that the transfer reaction catalyzed by the activating enzyme is specific with respect to the aminoacyl moiety.

A number of studies have been carried out to determine the nature of the chemical linkage between the amino acid and sRNA. Evidence from studies on ^{14}C-leucyl RNA (26) showed that ^{14}C-leucine can be readily hydrolyzed from ^{14}C-leucyl-RNA by dilute alkali. When ^{14}C-leucyl-RNA was treated with pancreatic RNase, the radioactivity was liberated in the form of a low molecular weight, positively charged ^{14}C compound that gave adenosine and ^{14}C-leucine on alkaline hydrolysis. It did not react with periodate or ninhydrin prior to hydrolysis. These findings indicated that the compound is the leucyl 2'(3') ester of adenosine.

More recent studies have indicated that the amino acid is probably attached to the 3' hydroxyl group of the terminal adenosine residue of the different sRNA molecules (36-38). A partial representation of an aminoacyl sRNA is shown in Figure 3. Holley's recent papers (39, 40) may be consulted for the complete primary structure of alanyl sRNA.

Cohen and Gros (41), Berg (42), Simpson (43) and Moldave (44) have published review articles on the biosynthesis of peptides and proteins, which cover in more detail the two sections on the aminoacyl adenylates and aminoacyl sRNA complexes described above.

Other Nucleotide Peptides Which May be Intermediates in Protein Synthesis

Many papers in the literature have reported the presence of a variety of nucleotide-peptides in either the cell sap or nuclei. Koningsberger (45) found nucleotide-bound carboxyl-activated peptide compounds in yeast extracts. These compounds were reported to form hydroxamates with hydroxylamine. Szafranski reported the observation of nucleotide-peptides of similar nature from guinea pig liver cytoplasm (46) as well as in the nuclei (47). Harris and Davies (48) showed the chemical structure of one of the nucleotide-peptides which they isolated from yeast (Figure 4).

The nucleotide-peptides obtained from yeast appear to have either a 5'-phosphoanhydride or a 2' or 3'-ribose ester bonds to link the nucleotide moiety to peptide moiety (50). Harris compared these structures to the structures of the compounds involved in protein synthesis (described above) and suggested that similar steps were required for the activation and transfer of peptides as are required for the activation and transfer of amino acid units. His group reported (51) the

Figure 3

A Portion of an Aminoacyl sRNA

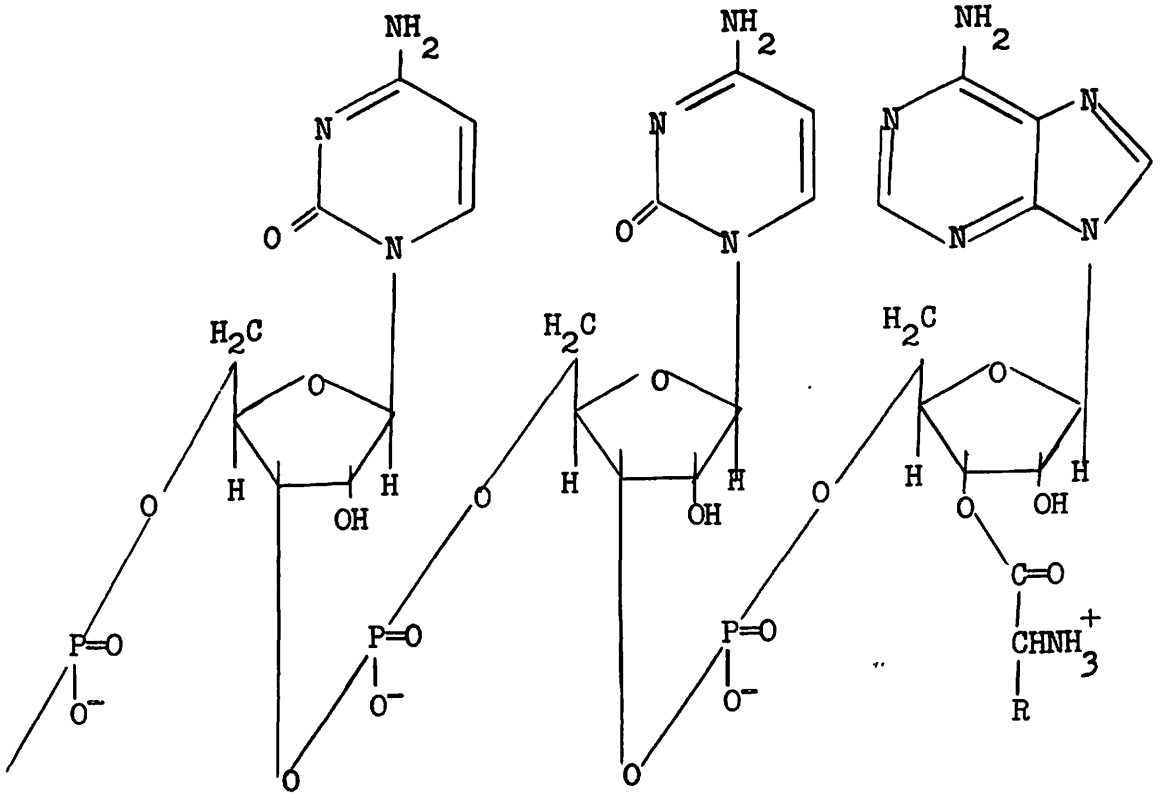
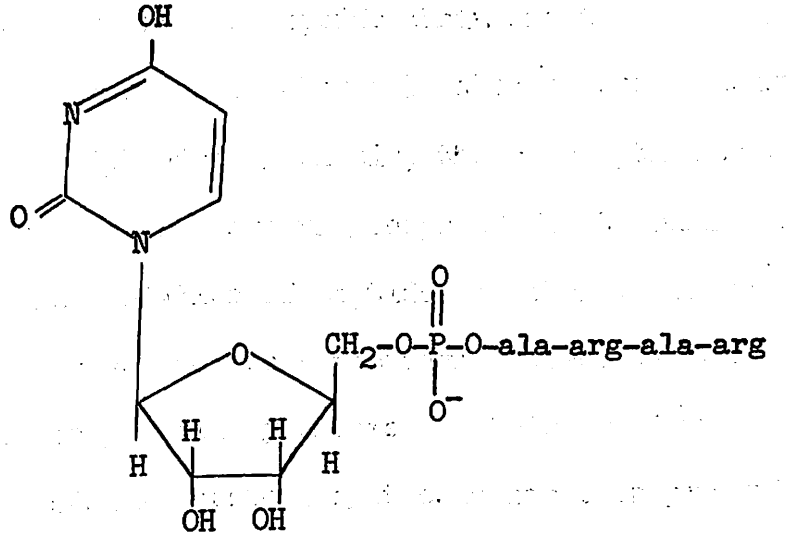


Figure 4

Arginylalanylarginylalanyl-5'-Uridylate (48)


OH
N
O
N
O
CH2-O-P-O-ala-arg-ala-arg
O-
O
H
H
H
H
OH
OH

partial fractionation of a yeast enzyme which is capable of activating peptides to form nucleotide-peptides. Harris suggested that these compounds functioned as activated intermediates of de novo protein synthesis, products of an energy dependent process of protein degradation, or intermediates in the cellular recyclization of proteins.

Gilbert (52) recently presented evidence that the polyphenylalanine chain, made by a polyuridylic-directed synthesis in a cell-free system from Escherichia coli, was covalently linked to an sRNA molecule. The bond between the polypeptide chain and the sRNA is similar to that in aminoacyl sRNA. Bretscher (53) also showed that in polyadenylic acid-directed polylysine synthesis, the polypeptide was associated with one (or both) of the hydroxyl groups of the terminal adenosine residue of sRNA. The isolation of peptides covalently linked to sRNA is consistent with the suggestion that peptidyl nucleotide compounds may be intermediates in protein synthesis. A brief review on the participation of peptidyl sRNA in protein synthesis has been presented by Moldave (44).

S-Adenosyl Methionine, S-Adenosyl Homocysteine and S-Adenosyl Ethionine

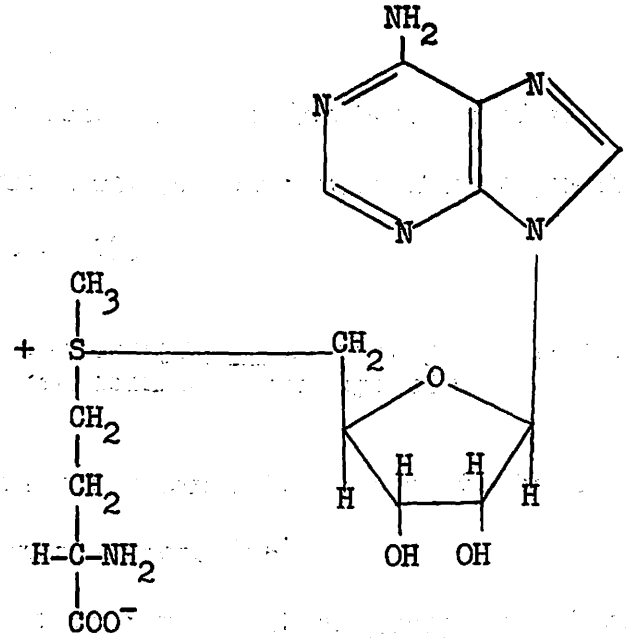
The observation that transmethylation reactions involving methionine required ATP suggested that transfer of the methyl group of methionine required prior activation (54). The activation of methionine was shown by Cantoni to proceed as shown in equation 12 (55).

$$\text{L-methionine} + \text{ATP} \longrightarrow \text{"active methionine"} + \text{PPi} + \text{Pi} \qquad (12)$$

The structure of the "active methionine" (S-adenosyl methionine; AMe) is shown in Figure 5.

Figure 5

S-Adenosyl Methionine



The evidence for this structure was summarized by Cantoni (58) as follows: (a) when ^{35}S-methionine was used in the above reaction radioactivity was incorporated into AMe, (b) the absorption spectra of AMe was identical to AMP, (c) AMe contained about one mole of pentose and one mole of labile methyl group per mole of base, and (d) adenine, homoserine and an unidentified sulfur containing fragment were obtained from AMe by acid hydrolysis. The structure was proved by total chemical synthesis by Baddiley and Jamieson (57).

S-Adenosyl methionine is present in a number of rat tissues at a level of 10 to 50 μg/gm (58).

The first transfer reaction in which S-adenosyl methionine was found to participate was a methyl group transfer to guanidinoacetic acid to give creatine (equation 13).

$$\underset{\text{acid}}{\text{Guanidinoacetic}} + \underset{\text{methionine}}{\text{S-adenosyl}} \longrightarrow \underset{\text{homocysteine}}{\text{S-adenosyl}} + \text{creatine} \qquad (13)$$

S-Adenosyl methionine has since been found to be a methyl donor in many transmethylation reactions (26).

In addition to its function in methyl group transfer, S-adenosyl methionine is an intermediate in the biosynthesis of spermine and spermidine in some microorganisms (59). In the series of reactions leading to spermine synthesis S-adenosyl methionine is first decarboxylated and the residual propylamine group is then transferred to putrescine to form spermidine. Addition of a mole of propylamine to spermidine from decarboxylated S-adenosyl methionine then produces spermine. In this series of reactions it is a propylamine group rather than a methyl group which is transferred.

S-Adenosyl homocysteine is one of the products in methyl group transfer reactions from S-adenosyl methionine. It can also be formed from adenosine and homocysteine in a reversible manner by either rat liver (60) or yeast enzymes (61) (equation 14).

$$\text{Adenosine} + \text{L-homocysteine} \longrightarrow \text{S-adenosyl-L-homocysteine} \quad (14)$$

It can also be hydrolyzed to adenine and S-ribosyl homocysteine by an E. coli nucleosidase (62).

S-Adenosyl homocysteine is a precursor in a second route leading to S-adenosyl methionine formation by addition of a one carbon unit from ^{14}C labeled formate or serine to the sulfur atom of S-adenosyl homocysteine and subsequent reduction of this carbon unit to a methyl group (cited in 63) (equation 15).

$$\text{S-adenosyl-L-homocysteine} + (C_1 \text{ unit}) \longrightarrow \text{S-adenosyl-L-methionine} \quad (15)$$

When Torulopsis utilis is cultured in a medium containing ethionine, a compound, S-adenosyl ethionine, is formed. Transethylation reactions involving S-adenosyl ethionine have been observed (63). Formation of S-adenosyl ethionine and transethylation may be due to incomplete specificity of the enzyme responsible for the analogous reactions involving methionine. A review of the structure and function of S-adenosyl methionine has been presented by Shapiro (63).

A Nucleotide-Peptide from Bovine Liver

In 1961, Wilken and Hansen (64) reported the isolation and partial characterization of a nucleotide-peptide. This compound was isolated from perchloric acid extracts of bovine liver by purification on Dowex-1

formate resin columns followed by paper chromatography. The purified compound was found to absorb ultraviolet light and to react with ninhydrin. The compound had an absorption spectrum similar to that of adenosine at pH 2 and pH 7. After acid hydrolysis and paper chromatography, only one ultraviolet absorbing compound could be detected. It had the mobility of adenine. The original compound contained one mole of pentose and three moles of organic phosphate per mole of adenosine. The organic phosphate was relatively stable to acid hydrolysis. The number of ninhydrin amino equivalents before acid hydrolysis was found to be one mole per mole of base and after hydrolysis, this number increased to five to six. Two dimensional paper chromatography of an HCl hydrolysate of the compound showed that the compound contained glycine, glutamic acid, β-alanine, cysteic acid and taurine. An unidentified ninhydrin positive component with a mobility similar to but not identical with serine was also detected. A DNP derivative of the compound was prepared which gave DNP-glutamic acid after acid hydrolysis and chromatography, thereby showing that the amino terminal residue was glutamic acid.

Rye grass 3'-nucleotidase released one of the three phosphate moieties from the molecule. When the nucleotide-peptide was preincubated with 3'-nucleotidase followed by incubation with Crotalus atrox venom which contained 5'-nucleotidase, two moles of inorganic phosphate were released per mole of the compound. Paper chromatography of a mildly alkaline hydrolysate of the compound gave a component which had the mobility of 3',5'-adenosine diphosphate. When the products of 3'-nucleotidase and 3'-nucleotidase plus 5'-nucleotidase treatments were hydrolyzed in 0.01 M KOH and chromatographed, components having the

mobility of 5' AMP and adenosine, respectively, were detected. Furthermore, when the nucleotide-peptide was treated with 3'-nucleotidase and chromatographed, only inorganic phosphate and 3'-dephospho-nucleotide-peptide were detected. The above evidence indicated that the bovine liver nucleotide-peptide contained an adenosine 3',5'-diphosphate moiety and six amino acids in the peptide moiety with glutamic acid as the amino-terminal amino acid. Although the compound was not completely characterized, the possible types bond between the nucleotide moiety and the peptide moiety, the location of the third phosphate group, and the nature of the unidentified ninhydrin positive component were discussed.

More recently, Okahara and Hansen (65) have reinvestigated the nucleotide-peptide. Their results confirmed the previous findings (64) with one exception. They did not observe an "unidentified ninhydrin positive component." This point will be discussed in the chapter on "Results" later in this thesis. This compound appears to be present in trichloroacetic acid filtrates prepared from guinea pig liver.[2] A compound with some properties similar to the bovine liver nucleotide-peptide has been reported to occur in rat liver (66).

Compounds Containing Sulfhydryl and Disulfide Groups

This portion of the literature review will be concerned with sulfhydryl and disulfide compounds. The low molecular weight compounds of this type will be discussed first followed by a discussion of the sulfur containing peptides and proteins. Enzymes involved in sulfhydryl-

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[2] G. C. Mill, Personal communication.

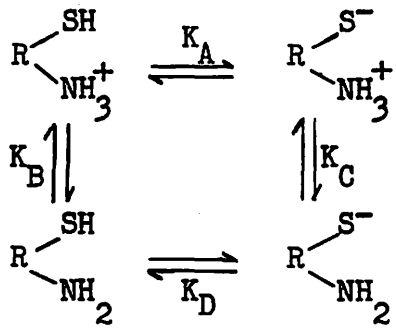
disulfide exchange reactions will be discussed last. The chemistry of sulfhydryl and disulfide compounds will be described with cysteine. Coenzyme A and glutathione and sulfhydryl-disulfide exchange reaction catalyzing enzymes will be covered in the greatest detail due to their relation to the current problem.

Sulfhydryl and Disulfide Groups in Low Molecular Weight Compounds

Cysteine and Cystine. Greenstein and Winitz have presented a historical review on cysteine and cystine (67). Cystine was first isolated by Wollaston (68) in 1810 from urinary calculi. Baumann (69) in 1884 first reduced cystine to cysteine by using tin and hydrochloric acid. Mörner (70) in 1899 isolated cystine from protein. The structure of cystine and cysteine were proved by synthesis by Erlenmeyer (71). Cystine is not very soluble in neutral solution and little or no free cystine is present in cells. Cystine does occur as one of the components of proteins. For example, it accounts for 12 percent of the protein of human hair, keratin. Only cystine and not cysteine is found after acid hydrolysis of proteins. Cysteine is known to occur in proteins, however, since many proteins show a distinctive red color with sodium nitroprusside, a sensitive reagent for sulfhydryl groups. In addition S-carboxymethyl cysteine has been isolated from several proteins by acid hydrolysis following treatment with iodoacetate. There is evidence that the cystine of protein is formed by oxidation of cysteine after its incorporation into the polypeptide chain as will be discussed in a later section of this chapter.

The presence of both -SH and $-NH_3^+$ groups in cysteine has caused difficulty in determining the dissociation constants of these groups

because of the overlap of the acid strength of the ammonium and mercapto groups (72). At pH values affecting the -SH and $-NH_3^+$ groups, the carboxyl group is fully ionized. The dissociations of cysteine are presented by the scheme shown as equation 16.



(16)

Where R = $-CH_2-CH(-)-COO^-$

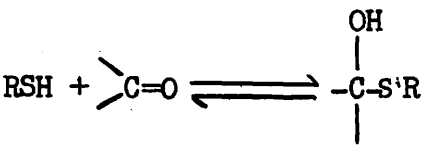
Noda et al. (73) showed that RS^- exhibited an appreciable absorption in the 230 mμ region which was not exhibited by RSH. Benesch and Benesch (74) used this observation to determine the various pK values for cysteine. The values they obtained at 23° C were pK_A, 8.53; pK_B, 8.86; pK_C, 10.36; and pK_D, 10.03 corresponding to the various equilibria shown in equation 16. These values are in agreement with the titration data of S-methyl cysteine and cysteine betaine by Grafins and Nielands (75).

The relationship between cysteine and cystine was first indicated by Baumann (69) except that the structure of these amino acids was not fully known at that time. Oxidation of the -SH group of cysteine to the -S-S- of cystine is readily effected by atmospheric oxygen if trace amounts of metal ions, particularly those of iron and copper, are present. Metal catalyzed oxidation is suppressed by agents such

as cyanide and pyrophosphate. Cysteine is much more readily oxidized by atmospheric oxygen than is glutathione, a tripeptide containing cysteine.

The oxidation of cysteine to cystine is also effected by iodine in acetic acid, ferric cyanide and o-iodosobenzoic acid (72). The oxidation of cysteine or glutathione by H_2O_2 is considerably slower at acidic pH than at neutrality, and the oxidation in acidic solution is catalyzed by copper ions (72). The oxidation of cysteine with bromine water goes beyond the disulfide stage, and the sulfhydryl group is converted to a sulfonic acid group with the formation of cysteic acid (72).

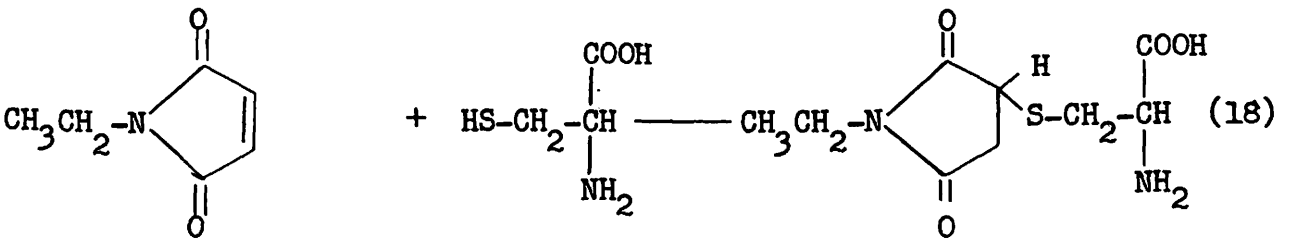
In addition to oxidation the sulfhydryl group in cysteine or other sulfhydryl compounds also undergoes addition and alkylation reactions. Sulfhydryl groups add to carbonyls as is shown in equation 17 to give



(17)

thiohemiacetals or thiohemiketals (84). Rothschild and Barron (143) have reported spectrophotometric evidence for the formation of an addition compound of cysteine and betaine aldehyde. Cysteine reacts with glyceraldehyde-3-phosphate to give a product which is inactive with glyceraldehyde-3-phosphate dehydrogenase (76).

Sulfhydryl groups also add across double bonds. The addition reaction of cysteine to N-ethylmaleimide is shown in equation 18.



(18)

This reaction is useful in the inhibition of a number of enzymes containing essential -SH groups by N-ethylmaleimide and other compounds with a reactive double bond (72). Maleic acid reacts similarly but much less readily (77).

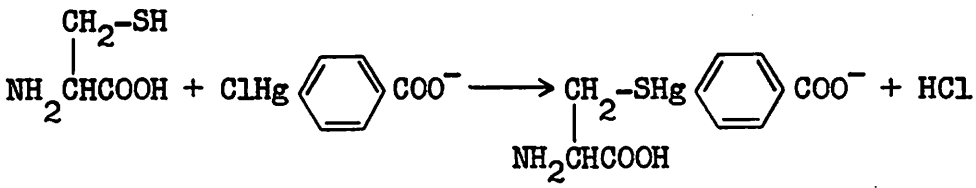
Alkylation of sulfhydryl groups with iodoacetate occurs readily, particularly at an elevated pH, and is frequently used to test for essential -SH groups in enzymes. The reaction is shown in equation 19.

$$RS^- + ICH_2COO^- \longrightarrow RS\text{-}CH_2COO^- + I \qquad (19)$$

With chloroacetate which contains a more firmly bound chlorine atom, the reaction proceeds more slowly and with fluoroacetate, no significant alkylation is observed. Cysteine and glutathione are also readily alkylated in methyl bromide vapor. The reaction of fluoropyruvate with cysteine produces an intensive absorption at 300 mμ (72).

The formation of mercaptides (heavy metal salts of sulfhydryl compounds) is relatively specific for sulfhydryl groups in biological materials and is useful as an analytical tool. Boyer (72) has presented a review covering mercaptide formation. Trivalent arsenic and antimony, bivalent lead, mercury, copper, cadmium, zinc and monovalent mercury, copper, silver and gold readily form slightly dissociable mercaptides (72). Mercuric salts also cleave thioesters and disulfides with resultant mercaptide formation.

The reaction of cysteine with p-chloromercuribenzoic acid, another widely used sulfhydryl reagent is shown in equation 20 (72).



(20)

This reaction is important in enzyme investigations. Mercaptide formation by p-chloromercuribenzoate is accomplished by a considerable increase in the ultraviolet absorption at 250 mμ which can be quantitatively related to the sulfhydryl content. This provides a useful analytical tool (78).

The disulfide bond in cystine and other disulfides is readily cleaved by sulfhydryl groups, sulfite, and cyanide and oxidatively cleaved by performic acid (72). The cleavage by sulfhydryl groups represents a sulfhydryl-disulfide interchange reaction as shown in equations 21 to 23.

$$RSH + R'SSR' \rightleftharpoons R'SH + R'SSR \qquad (21)$$

$$RSH + R'SSR \rightleftharpoons R'SH + RSSR \qquad (22)$$

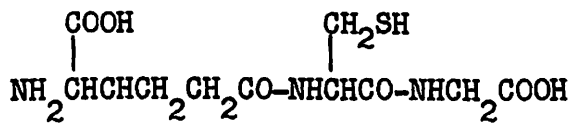
$$\text{Sum:}\quad 2RSH + R'SSR' \rightleftharpoons 2R'SH + RSSR \qquad (23)$$

The reaction with cyanide as well as that with HCN appears to involve cleavage of the disulfide bond with addition, the products being -SH and -SCN (79).

Homocysteine and Homocystine (26). Homocysteine is the next higher homolog of cysteine and thus undergoes many of the same reactions as does cysteine. It is not a constituent of protein; however, it is an important participant in metabolic reactions involving methionine as has been discussed under reactions of S-adenosyl methionine. The oxidation

of homocysteine leads to the formation of the corresponding disulfide homocystine.

Glutathione. This widely distributed sulfur containing peptide was discovered in 1921 by Hopkins (80) and was shown to be a tripeptide of cysteine, glutamic acid and glycine (81). The structure (shown below) was finally established as γ-L-glutamyl-L-cysteinyl-glycine by Harrington and Mead (82).



One functional group in the molecule is the thiol group which is present in the cysteinyl moiety. The chemistry of GSH therefore is similar to that of other sulfhydryl and disulfide compounds which have been described in the section on cysteine and cystine in this chapter.

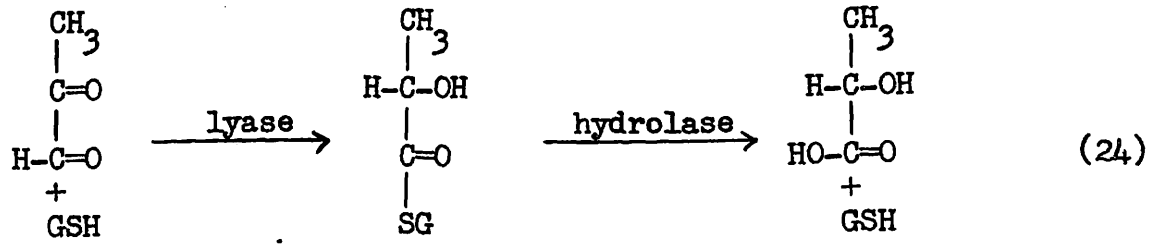
A list of the distribution of glutathione in various tissues has been reported (83).

Although glutathione has been known for so many years, and has given rise to a very extensive literature, its function in biological systems is still somewhat obscure. It can provide a path for the oxidation of the coenzymes through ascorbate and either ascorbate oxidase in plants or cytochrome oxidase in animals. There is no evidence as yet as to the physiological importance of these pathways. Many enzymes are -SH enzymes which are active only in thiol state. Thus, an "euphoristic theory" has grown from the observations of the non-specificity of glutathione in keeping these enzymes in the reduced form. As Racker has stated (92) "GSH is present in cells to keep the

enzymes in a happy state either by preventing their oxidation or by protecting them against toxic heavy metals."

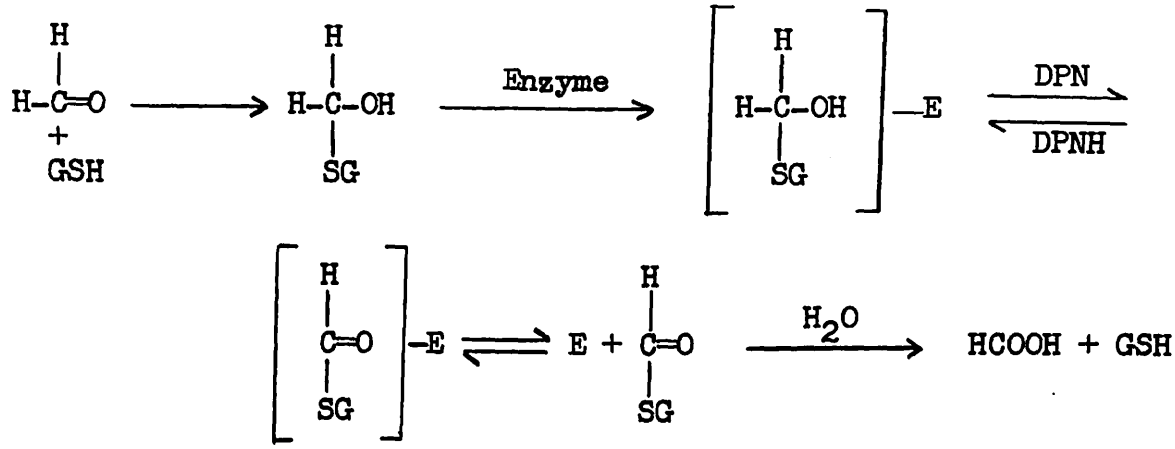
Glutathione acts as a specific coenzyme for a few enzymes or enzyme systems (85). In these cases, cysteine can not replace GSH. The GSH probably forms a covalent compound with the substrates in all these cases.

Lohmann identified GSH as a coenzyme of glyoxalase (86). Hopkins and Morgan (87) showed that this enzyme system contains two enzymes and called them "enzyme" and "factor." The mechanism of the conversion of methylglyoxal to lactic acid was elucidated by Racker (88). The "enzyme" and "factor" of Hopkins and Morgan are now known as lactoylglutathione lyase and hydroxyacyl glutathione hydrolase, respectively. The second enzyme hydrolyzes a number of glutathione thiol esters of α-hydroxy acids. These reactions are shown in equation 24.



(24)

Another case in which the true substrate of an enzyme is a compound formed between the -SH group of GSH and an aldehyde is that of formaldehyde dehydrogenase of liver. Strittmater and Ball (89) found no other thiol which would act. They suggested that the reaction catalyzed by the enzyme is:



(25)

The final hydrolysis is thought to be catalyzed by an enzyme in the preparation, since the preparation also rapidly hydrolyzes S-acetyl-glutathione.

GSH is also the specific coenzyme of some of the cis-trans isomerizations (90). These enzymes appear to catalyze a shift in the disposition of groups in planar molecules. The addition of glutathione across the double bond would produce a saturated compound and allow free rotation. If the elimination of glutathione then takes place with removal of the hydrogen other than which was was added, isomerization would result.

Conflicting reports have been published concerning the involvement of GSH in the enzyme reaction catalyzed by glyceraldehyde-3-phosphate dehydrogenase. Krimsky and Racker (91-94) presented the following evidence which suggested that glutathione is a firmly bound prosthetic group of glyceraldehyde-3-phosphate dehydrogenase from rabbit muscle: a) the enzyme liberated glutathione when treated with trypsin, b) tryptic digestion of the enzyme after reaction with ^{14}C acetyl phosphate liberated a radioactive component which moved with carrier acetyl glutathione on Dowex 50 resin and on paper chromatography. Based on their quantitative studies on the effect of iodoacetate

on the release of glutathione by proteolytic digestion, these authors also suggested that the bound glutathione is the binding site for DPN (93). Recent results of Perham and Harris (95) showed that the amino acid sequence around the reactive cysteine residues in glyceraldehyde-3-phosphate dehydrogenase from yeast and pig muscle and rabbit muscle are identical. The sulfhydryl derivatives of $1^{14}C$ iodoacetate and $1^{14}C$-p-nitrophenyl acetate occurred exclusively in the same unique octadecaptide sequence of amino acids in the primary structure of the enzyme isolated from the three different sources. No evidence was obtained that a glutathione moiety is involved in the reactive site of the enzyme.

Coenzyme A. Coenzyme A is the coenzyme form of the vitamin pantothenic acid (96). It was discovered as a cofactor for acetylation in liver and microorganisms by Lipmann (97). Its structure was established by Lipmann and his coworkers by a series of selective enzyme degradations (98). (The structure of coenzyme A is shown as part of the structure of CoASSG in Figure 12.) Crystalline coenzyme A has so far not been obtained. The best preparations are of about 95 percent purity. They are of white powdery, amorphous consistency, exhibit a typical odor, and are readily soluble in water but insoluble in acetone, ether and ethanol. Coenzyme A has the properties of a nucleotide and those of a mercaptan. It is a strong acid. It forms insoluble mercuric, silver and cuprous mercaptides (99). Electrometric titration shows the following pK values: pK 9.6 (thiol), pK 6.4 (secondary phosphate) and pK 4.0 (adenine NH_3^+) (100). Coenzyme A, like other thiols (see cysteine section this chapter) is readily oxidized to a biologically inactive

disulfide. Reagents which alkylate thiol groups such as iodoacetate, N-ethylmaleimide, p-chloromercuribenzoate and arsenite also inactivate coenzyme A. Free coenzyme A is stable if kept dry. In solution it is fairly stable between pH 2 to 6 and between 0° to 40° and not stable at pH 8.0 or higher (101). In more acid solutions, the molecule is broken down especially at elevated temperature. Among the breakdown products, dephospho-CoA, 3',5'-ADP, pantotheine 4'-phosphate and adenine were found (102).

Usually coenzyme A is isolated from yeast and purified by charcoal absorption (102), mercaptide formation with cuprous ion (100) and by ion exchange chromatography preceded by protective acylation of the thiol group (104). The coenzyme A prepared in this manner is of poor purity.

Impurities result because coenzyme A, like other thiols, is readily oxidized to the catalytically inactive disulfide by air expecially in the presence of trace amounts of heavy metals (105). If other thiol compounds are present, as is generally the case in biological materials, mixed disulfides may be formed (106) for example with glutathione or cysteine (98, 104, 107, 108). The disulfide bond may be reduced by zinc-hydrochloric acid (103), sodium amalgam (106, 109), H_2S, mercaptans (110), or alkali metal borohydride (111). Protection of the thiol group during purification by benzolation (112) or introduction of a reductive step in the purification procedure increases the purity of the product (101). Small samples of coenzyme A can be further purified by paper chromatography (107, 113, 114) or by paper electrophoresis in weak acid medium (115, 116).

Coenzyme A occurs in a wide variety of animal and plant tissues

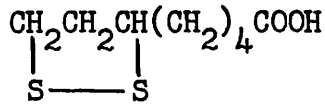
and in microorganisms (110, 112). More than 50 percent of the coenzyme A in liver is present in the mitochondria. Tables listing the distribution of CoA in animal tissues, plants and microorganisms have been compiled (110).

Since coenzyme A is the coenzyme form of the vitamin pantothenic acid the first portion of de novo synthesis of this coenzyme in microorganisms is identical to the pathway of pantothenic acid biosynthesis. Formation of coenzyme A from pantothenic acid in microorganisms and in mammalian tissue was established by Brown (117-119) and has been reviewed by Jaenike and Lynen (112) and by Brown and Reynolds (117). Coenzyme A prepared chemically contains two isomers, the natural form (3'-phosphate) and iso-coenzyme A (2'-phosphate). Iso-coenzyme A has no coenzyme A activity in biochemical reactions which require the natural isomer (120).

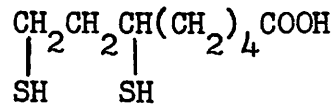
The major metabolic role of coenzyme A is its function in acyl group transfer. Its thioester compounds, acyl-CoA derivatives, are important activated intermediates in metabolism. This is shown by the fact that over 60 enzymes act on acyl-CoA compounds (112). Thioesters contain a carbonyl function which is linked to sulfur. The enzymatic activation process may occur at both the carbonyl function and the neighboring carbon atom. The activated carbonyl group is relatively positively charged while the α-methylene (or methyl) carbon is relatively negatively charged having carbonion character. Thus reactions of acyl-CoA derivatives, that is, acylation, condensation, substitution and elimination can take place nucleophilically or electrophilically at the respective activated locus. Detailed reviews on this subject have been presented by Jaenike and Lynen (112) and by Wagner and

Folkers (96).

Lipoic Acid (121). Dewey first reported this coenzyme in 1941 to be a growth factor, "Factor II," for the protozoan Tetrahymena. This factor was concentrated by Stokstad (122) who called it "Protogen" or "Protogen A." Meanwhile Guirard (123) showed the existence of a factor which could replace the requirement for acetate in the growth of certain bacteria, which they called "acetate-replacing factor" and O'Kane and Gunsalus (124) had discovered a "pyruvate oxidation factor" for Streptococcus faecalis. These factors were eventually shown to be identical with protogen. The substance was crystallized in 1951 by Reed, DeBush, Gunsalus and Hornberger (125) who proposed the name "α-lipoic acid" because of its solubility in organic solvents. It has also been called "thioctic acid." The name "lipoic acid" is now most commonly used for both oxidized and reduced forms shown below (126).



Oxidized lipoic acid



Reduced lipoic acid

The essential biological change in lipoic acid is the reduction of the disulfide group into two thiol groups and their reoxidation. This is shown later in equations 26-30 in the section on the function of -SH and -S-S- in enzyme activity. One of the carbons is assymmetric, so that two optical isomers exist. The natural isomer of the oxidized form is dextrorotatory, but gives rise on reduction to the levorotatory reduced form. Only the natural isomer is active in biological systems

such as the pyruvate dehydrogenase system. A considerable part of the lipoate in cells appears to exist bound to protein, attached by a peptide bond to the ϵ-amino group of a lysine group and can be removed by an enzyme from yeast or bacteria (126).

Disulfide Compounds in Amino Aciduria (127). The first discovered amino aciduria, cystinuria, was believed to be an inborn error of metabolism (128). Subsequent work has shown that cystinuria is associated with a defect in the tubular reabsorption system for this amino acid as well as ornithine, arginine and lysine (129). The daily excretion of cystine in cystinuria is about one gram. Similar amounts of basic amino acids may be excreted (127). Humans and dogs having cystinuria have been found to excrete the unsymmetrical disulfide of L-cysteine and L-homocysteine (130). This disulfide has also been found in the urine of patients with Wilson's disease. Recently, it was found that the administration of D-penicillamine leads to excretion of L-cysteine-D-penicillamine disulfide and a reduction of cystine excretion in cystinuria (131).

Sulfhydryl and Disulfide Groups in Macromolecules

Function of Sulfhydryl and Disulfide Groups in Protein Structure. An important role of disulfide groups in proteins is the maintenance of their three dimensional structure or conformation. Markus and Karush (132) found that complete reduction of the disulfide bonds of serum albumin in the presence of detergent increased the viscosity of the solution, indicating disorganization of the tertiary structure.

The necessity of disulfide bonds for activity in trypsin, chymo-

trypsin and chymotrypsinogen was shown by Peter et al. (133). Mercaptoethanol, cysteine, glutathione, 2,3-dithioglycerol, thiosalicylate and Na_2S inactivated crystalline trypsin. The inactivation which was accompanied by the appearance of sulfhydryl groups (134) demonstrated the essentiality of the disulfide linkage in trypsin. The sedimentation constant of trypsin in urea (1.585) compared to native trypsin (2.55) indicated that trypsin in urea exists in an unfolded form. Rupture of the disulfide bonds led to a further lowering of the sedimentation constant (0.985) which was interpreted as more extensive unfolding. Complete reduction of chymotrypsin by sulfite in the presence of p-mercuribenzoate gave small fragments (134). This suggested that the protein contains chains held together by disulfide linkages (134). In addition to data implicating the importance of disulfide bonds in protein structure by physical techniques many experiments have been performed with enzymes in which alteration of the number of disulfide bonds has led to a decrease in enzyme activity suggesting structural changes have occurred.

Boyer et al. (135) have suggested that sulfhydryl groups as well as disulfide groups may be involved in the maintenance of tertiary structure of proteins. The hydrogen of sulfhydryl groups might act as a donor for weak hydrogen bonds and such bonds might contribute critical extra stabilization. They also pointed out that the possibility of charge transfer complexes between the sulfhydryl groups and tyrosine residues should not be overlooked. Ionic attractions from sulfhydryl groups or even covalent bonds other than disulfide bonds are other possible contributing factors to protein conformation.

Function of Sulfhydryl and Disulfide Groups in Enzyme Activity (72). The literature contains numerous reports indicating the necessity for free sulfhydryl groups for enzyme activity; however, little basic information is available to indicate how sulfhydryl groups function in enzyme catalysis. Most postulates remain unproved or untested. Many studies of a possible relationship between a sulfhydryl group and enzyme catalysis consist only of a report on the effect of various reagents that react with sulfhydryl groups on the catalysis, the possible prevention or reversal of any observed effect by low-molecular weight sulfhydryl compounds, and less often, evaluation of the presence of substrate on any effect noted. p-Chloromercuribenzoate, iodoacetamide and N-ethylmaleimide are the most frequently used sulfhydryl reagents which often cause inhibition of enzyme catalysis. GSH, cysteine, mercaptoethanol and 2,3-dimercapto-1-propanol (BAL) are commonly used to attempt to reverse the inhibitory effects of the sulfhydryl reagents listed above.

Evidence has been obtained to indicate an involvement of specific sulfur groups in enzyme catalysis in some cases. The studies of Hellerman on urease (136) demonstrated that some sulfhydryl groups may be blocked without much loss in activity. With urease (137), muscle aldolase (138) and yeast alcohol dehydrogenase (72), a considerable portion of the total sulfhydryl groups react with p-chloromercuribenzoate before activity loss occurs. Limited reaction of myosin actually increases its ATPase activity with Ca^{++} as an activator (139). Hartshorne and Morales (140) have recently shown that a β-state myosin (modified form) has three to four times greater ATPase activity than the α-state (native form). Further modification of myosin to the γ-state yielded a protein form having no activity at all. Glyceraldehyde-3-phosphate

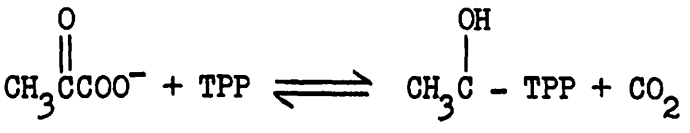
dehydrogenase (72) has some sulfhydryl groups that do not participate in catalysis. Inactivation of lactic dehydrogenase occurred slowly and roughly correlated with the reaction of the sulfhydryl groups with p-mercuribenzoate (141). Kaplan (142) recently showed that lactic dehydrogenase is partially inactivated as a result of freezing and thawing and that this is caused by oxidation of the sulfhydryl groups in the enzyme. Black (183) has shown that two out of the three sulfhydryl groups in glutathione reductase are not essential to its catalytic activity. Only that sulfhydryl group which reacts with N-ethylmaleimide after TPNH reduction is essential for activity. Koshland (144) has shown that phosphoglucomutase undergoes a conformation change induced by its substrate glucose-6-phosphate. This change exposes certain sulfhydryl groups of the enzyme and increases the rate of inactivation of the enzyme by iodoacetamide.

Several different methods of involvement of sulfur in a catalytic role in different enzymes have been proposed. They include the following four types:

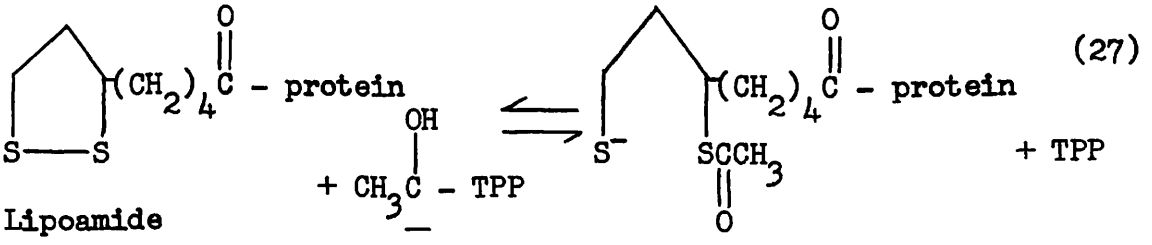
(1) Interconversion of a sulfhydryl and a disulfide in an enzyme bound cofactor.

Gunsalus (145) first proposed the mechanism of α-keto acid oxidation in the Krebs cycle. His work on the oxidation of α-ketoglutarate by preparations from Crookes strain of E. coli lent support to the role of the lipoyl group in the enzyme complex of α-ketoglutarate dehydrogenase and in the pyruvate dehydrogenase complex. The role of lipoate in oxidative decarboxylation in which the disulfide of lipoic acid is reduced and then reoxidized is shown in the following series of reactions taken

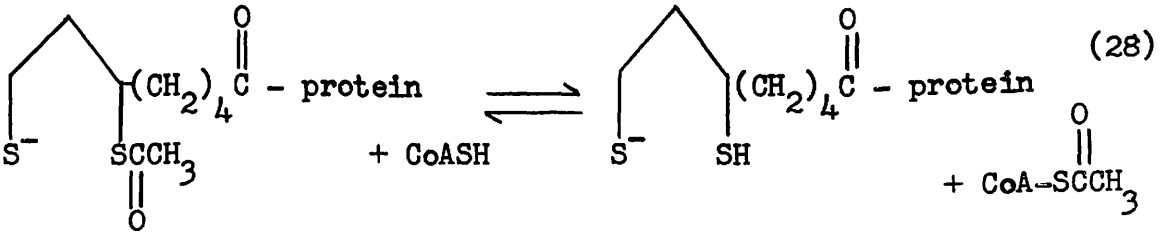
from Wagner (96) (equations 26-30).



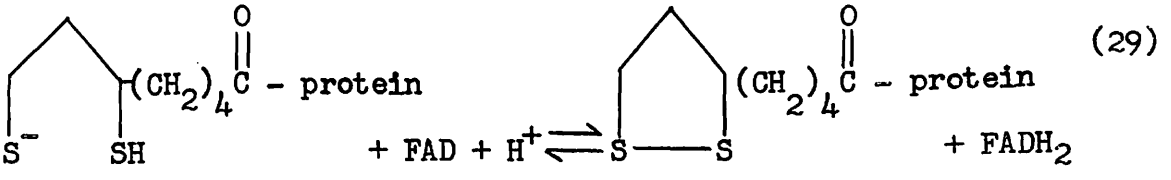
Pyruvate (26)



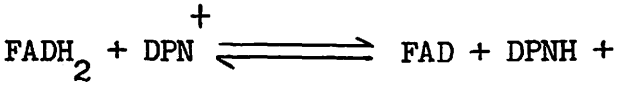
Lipoamide (27)



(28)



Reduced Lipoamide (29)



(30)

(2) Possible free radical formation.

Barron (146) indicated the possibility of the existence of a thiol radical in the oxidation of alcohol catalyzed by alcohol dehydrogenase. He suggested that the oxidation reduction is mediated by a one electron oxidation-reduction system in which a free radical protein, P-$\dot{S}$, formed as follows could participate.

$$P\text{-}SH \rightleftharpoons P\text{-}\dot{S} + H^+ + e \qquad (31)$$

The possible participation of a free radical protein is shown in the following series of reactions:

$$RCH_2OH + P\text{-}\dot{S} \rightleftharpoons R\dot{C}HOH + P\text{-}SH \qquad (32)$$

$$P\text{-}SH + DPN^+ \rightleftharpoons P\text{-}\dot{S} + DP\dot{N} + H^+ \qquad (33)$$

$$R\dot{C}HOH + P\text{-}\dot{S} \rightleftharpoons RCHO + PSH \qquad (34)$$

$$P\text{-}SH + DP\dot{N} \rightleftharpoons P\text{-}\dot{S} + DPNH \qquad (35)$$

$$\text{Sum: } RCH_2OH + DPN^+ \rightleftharpoons RCHO + DPNH + H^+ \qquad (36)$$

Evidence which supports the possible involvement of free radicals was obtained by Commoner et al. (147) who reported the detection of a small concentration of free radical during alcohol dehydrogenase reactions by use of very sensitive electron paramagnetic resonance techniques.

(3) Binding of substrates (72)

A commonly used type of evidence that sulfhydryl groups are located at or near the binding site for substrates is the protection which substrates afford against reaction of essential sulfhydryl groups with various reagents. Unfortunately, substrate protection by itself falls far short of establishing that the sulfhydryl group protected is at or near the actual binding site. Substrates may induce a conformational change in the enzyme (144). Therefore, the substrate could conceivably prevent the reaction of a sulfhydryl group some distance from the actual reaction site by causing it to assume a position inaccessible to the reagent.

The binding of substrate by sulfhydryl groups might occur by hydrogen bond formation using the hydrogen on the sulfur or by electro-

static attraction between a negatively charged sulfur atom and a positive center of the substrate. In some cases a covalent bond can be formed. For example, during fatty acid biosynthesis in E. coli, the substrates are bound to the acyl carrier protein through a thioester linkage (148-155). Majerus, Alberts and Vagelos (156) have presented evidence that the sulfhydryl group of 4'-phosphopantotheine is the binding site. This prosthetic group is bound by a phosphodiester linkage to the hydroxyl group of a serine residue of the protein.

(4) Binding of cofactors

The most pertinent evidence suggesting possible sulfhydryl group participation in the binding of cofactors comes from studies on the interaction of enzymes with DPN. Theorell and Bonnichsen (157) reported a shift in the spectral maximum of DPNH from 340 to 325 mμ by binding to liver alcohol dehydrogenase. The spectral shift was abolished by reaction of the enzyme with p-mercuribenzoate. Racker and Krimsky (158) observed that DPN bound to muscle glyceraldehyde-3-phosphate dehydrogenase shows a broad weak increase in absorption in the 360 mμ region and that the increased absorption is abolished by iodoacetate. Slater et al. (159) recently studied this relationship and suggested that a sulfhydryl group on the enzyme has a dual function. It binds with the DPN molecule and in a later step also with the substrate, an aldehyde. A mechanism of charge transfer complex was proposed to account for these actions.

Functions in Peptide Hormones. Schwartz (196) formulated a thiol-disulfide interchange hypothesis for disulfide hormone action. Cadenas (161) found that 10^{-3} M N-ethylmaleimide strongly suppressed the insulin

dependent ability of the perfused rat heart to accumulate free glucose, though it did not effect the capacity of the cell to utilize this sugar. The inhibitor also reduced by one-half the binding of ^{131}I-insulin by tissue. Fong (162) demonstrated that ^{131}I-insulin became bound to the insulin sensitive tissue, rat epididymal fat pads and diaphragms, and that about one-half of this bound material was released by treatment with thiols under conditions expected to cleave disulfide bonds. Carlin and Huchter (163) found that if diaphragm was pretreated with insulin the inhibitory effects of N-ethylmaleimide on glycogen synthesis were increased rather than reduced. These authors suggested that the hormone "alters the muscle fiber in such a way that the N-ethylmaleimide reactive site previously unavailable to the sulfhydryl reagent became exposed." This phenomenon does not call for the reduction in the number of sulfhydryl groups but rather for an exchange of position with disulfide groups. In its new position, the sulfhydryl group might render the permeability process more sensitive to thiol reagents than it was originally.

Lehninger and Neubert (164) have found that disulfides as well as thiols have a profound effect on the water absorption of rat liver and kidney mitochondria. Especially to be noted is the fact a mixture of sulfhydryl and disulfide compounds was far more effective in inducing such mitochondrial swelling than either was alone. The disulfide hormones, vasopressin, oxytosin and insulin were approximately 100 times more effective than a simple disulfide such as GSSG.

Cafruny *et al.* (165) found that the hormone, pitressin, reduced the quantity of histochemically detectable sulfhydryl groups in kidney tubules and suggested that this is related to the hormone's diuretic

function.

Function in Cell Division. Mazia (166), who isolated the mitotic apparatus of the sea urchin egg, proposed the possible involvement of sulfhydryl and disulfide groups in the cellular division process. The mitotic apparatus was suggested to be a "disulfide bonded structure" on the basis of its stabilization by the disulfide form of mercaptoethanol and its loss of structure in the presence of sulfhydryl compounds.

Sakai and Dan (167) suggested that the cyclic change of sulfhydryl groups, which occurs in cells during the division cycle, occurs in a protein substance which is precipitated by saturated $(NH_4)_2SO_4$ but not by TCA. Sakai (168) has prepared a thread-like precipitate from a KCl-extract of the water-insoluble portion of cell homogenates and made a study of the contraction properties of these threads. In general, contraction could be caused by metallic ions such as Mn^{++}, Cd^{++} or Al^{+++} or by oxidizing agents such as dehydroascorbic acid or cystine. A water soluble fraction of the homogenate could be substituted for metal ion or oxidizing agent in the induction of thread contraction. This effect was due to the combined action of a non-dialyzable but labile component and a dialyzable heat stable component. The heat labile component might be an enzyme having transhydrogenase-like activity.

Enzymes Catalyzing Sulfhydryl Group Oxidations, Disulfide Group Reductions and Sulfhydryl-Disulfide Interchange Reactions.

Lipoyl Dehydrogenase (E.C. 1.6.4.3. $NADH_2$:lipoamide oxidoreductase; originally known as diaphorase). The enzyme catalyzes the reaction:

$$DPNH + H^+ + \text{oxidized lipoamide} \rightleftharpoons DPN^+ + \text{dihydro lipoamide} \quad (37)$$

The enzyme activity is distributed in animal tissues, plants, yeast and in bacteria. Hager and Gunsalus (169) first demonstrated the presence of this enzyme activity in bacteria and animals. It was also found in yeast by Cutolo (170) and in plant tissue by Busu and Burma (171). Searl et al. (172) and Massey et al. (173) found that lipoyl dehydrogenase could be inhibited by arsenite only in the presence of DPNH. The DPNH exposable group is the disulfide bond of a cystine residue of the protein. Massey (174) found the reduced form of the enzyme has one-half the molecular weight of the oxidized form. This suggested that in the enzyme two peptide chains are joined by a disulfide bond between cysteine residues. The involvement of this enzyme as part of the pyruvate dehydrogenase complex has been described in an earlier section.

Glutathione Dehydrogenase (E.C. 1.8.5.1. Glutathione:dehydroascorbate oxidoreductase). The enzyme catalyzes the following reaction:

$$2\ GSH + \text{dehydroascorbate} \rightleftharpoons GSSG + \text{ascorbate} \quad (38)$$

This enzyme is found in higher plants and in yeast. Crook (175) isolated the enzyme from plant tissue and reported its kinetic properties.

Nitrate Ester Reductase (E.C. 1.8.6.1. Glutathione:polynitrate oxidoreductase).

$$2\ GSH + \text{organonitrate} \rightleftharpoons GSSG + \text{organonitrite} \quad (39)$$

Heppel et al. (176) reported the purification of this enzyme from hog

liver. The enzyme had a pH optimum between 7 and 8 and was sensitive to trace amounts of cupric salts. The enzyme did not stimulate the reaction between cysteine and nitroglycerin.

Glutathione Peroxidase (E.C. 1.11.1.a. Glutathione:H_2O_2 oxidoreductase).

$$2\ GSH + H_2O_2 \rightleftharpoons GSSG + 2H_2O \qquad (40)$$

Mills (177) partially purified the enzyme from rat liver, kidney and lung.

Cystine Reductase (E.C. 1.6.4.1. $NADH_2$:L-cystine oxidoreductase).

Romano and Nickerson (178) reported the observation of an enzyme activity in pea seeds and yeast which catalyzes the reduction of cystine by DPNH.

$$\text{Cystine} + \text{DPNH} + H^+ \longrightarrow 2\ \text{cysteine} + \text{DPN}^+ \qquad (41)$$

They reported that this enzyme is specific with respect to both substrates cystine and DPNH. Black (179) pointed out that such an activity has not been separated from or adequately distinguished from other known disulfide reducing systems. In the report of Powning and Irzykiewics (180) evidence was presented that a dialyzed extract of clothes moths could reduce the disulfide bond of cystine with the oxidation of TPNH, a property that is especially noteworthy in view of the moth's ability to digest wool which is rich in disulfide bonds. However, no evidence was presented to indicate that this reaction was not due to the coupled action of glutathione reductase and a nonspecific transhydrogenase or to some other nonspecific system.

Glutathione Reductase (E.C. 1.6.4.2. $NADPH_2$:glutathione oxidoreductase) (179, 181, 182, 183).

$$TPNH + H^+ + GSSG \longrightarrow 2\ GSH + TPN^+ \qquad (42)$$

The ubiquitous occurrence of glutathione and of glutathione reductase in cells suggested that the oxidation and reduction of this thiol compound may play a central role in cellular metabolism (179).

In 1931, Hopkins and Elliott (184) first demonstrated a heat labile system in liver which was capable of reducing oxidized glutathione. Subsequently, enzyme preparations from beef, sheep and rabbit liver (185) and erythrocytes (186) were also found to catalyze glutathione reduction. In 1952, Rall and Lehninger (187) using crude pig liver preparations, demonstrated the enzymatic oxidation of TPNH by GSSG. Their work showed that the purified liver enzyme was specific with respect to TPNH and that the reaction catalyzed was not reversible. Pyridine nucleotide-linked glutathione reductase has been observed in other mammalian tissues and also in microorganisms, yeast, and higher plants (179).

TPNH is a more active reductant than DPNH with all the glutathione reductase enzyme preparations studied; in most cases no reaction occurs with DPNH (85). Racker (86), however, found reduction of GSSG by DPNH using the yeast enzyme but the reaction rate with DPNH was only about one-hundredth that with TPNH. No disulfide except GSSG is known to be reduced by glutathione reductase (84). Several mixed disulfides, formed by exchange of GSH with other disulfides, cannot be reduced by the liver enzyme (188).

Mize et al. (189) found that rat liver glutathione reductase bound ^{14}C-glutathione when exposed to this substrate and liberated the labeled

compound on treatment with TPNH. On the basis of their results, they postulated the following mechanism of the enzyme action:

$$\text{E–SH} + \text{GSSG} \longrightarrow \text{E–SSG} + \text{GSH} \qquad (43)$$

$$\text{E–SSG} + \text{TPNH} \longrightarrow \text{E–SH} + \text{GSH} + \text{TPN} \qquad (44)$$

This pattern shows that TPNH is not required to expose the reactive sulfhydryl groups of the enzyme as it does on lipoyl dehydrogenase (190) or yeast glutathione reductase (191). Buzard and Kopko (192) showed that glutathione reductase contains FAD. The above mechanism suggested by Mize needs to be revised to account for the role of flavin.

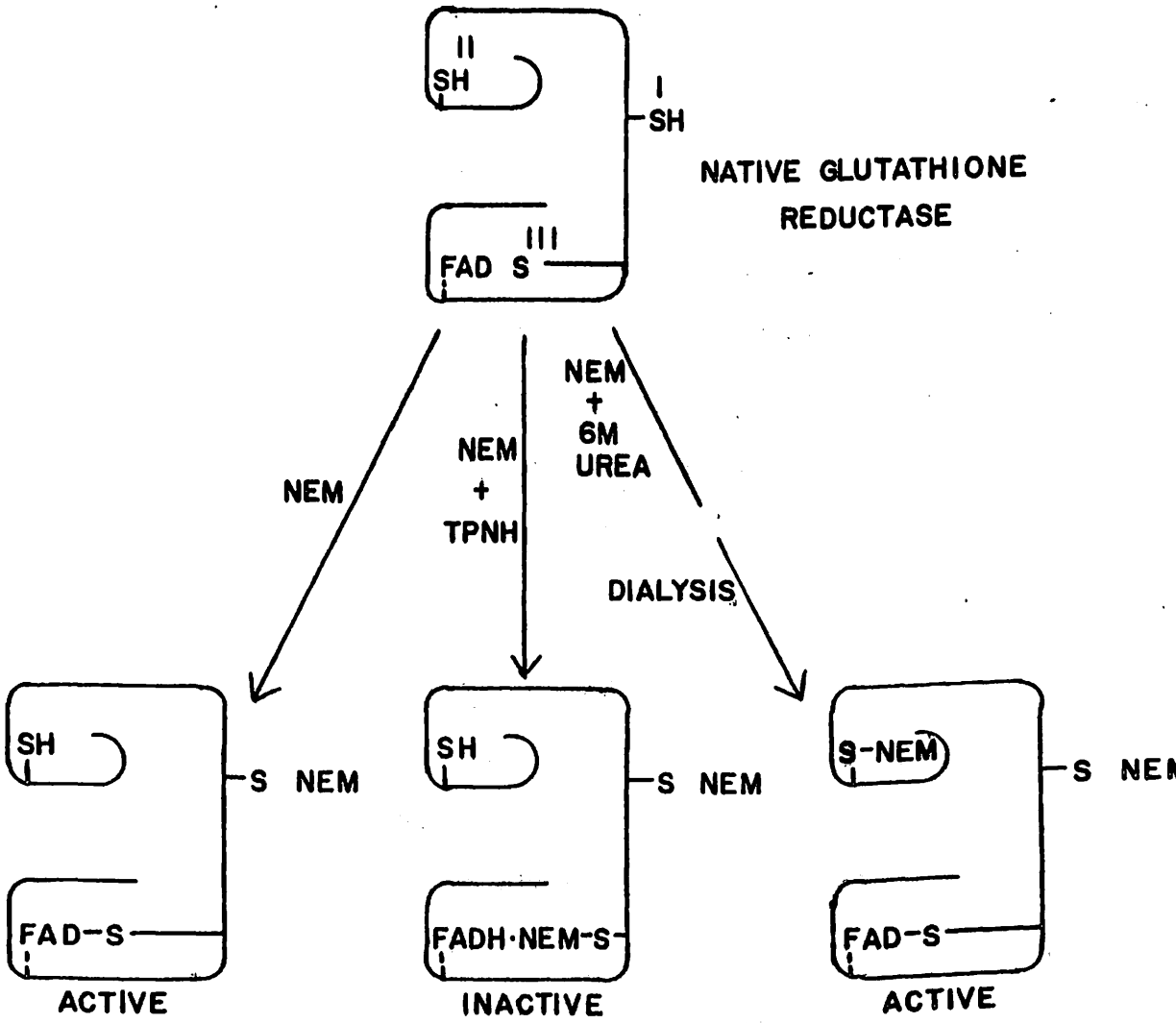
Colman and Black (183) recently studied the role of FAD and thiol groups in the catalytic mechanism of yeast glutathione reductase. Their results are shown schematically in Figure 6. On the basis of the capacity of the enzyme to bind ^{14}C-N-ethylmaleimide, the enzyme had three distinguishable sulfhydryl groups. Only the first sulfhydryl group (I) was determinable in the native enzyme. The second one (II) was revealed by dissolving the enzyme in 6 M urea. The third sulfhydryl group (III) appeared on reduction of the enzyme with TPNH. The first two sulfhydryl groups were not essential to the catalytic activity of the enzyme, whereas binding of the third one by NEM abolished the enzymatic activity.

FAD also appeared to be involved in the catalytic mechanism but its role was not established. The molecular weight of yeast glutathione reductase was determined by the same authors as 118,000 and it contained approximately one equivalent of FAD per mole of enzyme.

Glutathione-Homocystine Transhydrogenase (E.C. 1.8.4.1. Glutathione:homocystine oxidoreductase).

Figure 6

Schematic Diagram of the Yeast Glutathione Reductase and Its Reaction with N-Ethylmaleimide (NEM) (183)


II
SH
I
SH
NATIVE GLUTATHIONE
REDUCTASE
III
FAD S
NEM
NEM
+
TPNH
NEM
+
6M
UREA
DIALYSIS
SH
S NEM
FAD-S
ACTIVE
SH
S NEM
FADH·NEM-S
INACTIVE
S-NEM
S NEM
FAD-S
ACTIVE

In the course of studies on the reduction of GSSG by liver glutathione reductase, Racker (193) first found that a number of other disulfide compounds were reduced in the presence of a crude liver extract. Homocystine was found to be the most active hydrogen acceptor. A reaction scheme for this hydrogen transfer was proposed.

$$\text{homocystine} + 2\ \text{GSH} \rightleftharpoons \text{GSSG} + \text{homocysteine} \qquad (45)$$

$$\text{GSSG} + \text{TPNH} + \text{H}^+ \rightleftharpoons 2\ \text{GSH} + \text{TPN}^+ \qquad (46)$$

Racker partially purified this enzyme from beef liver, and found that it is not stable if fractionated with ammonium sulfate. Dibasic ammonium phosphate was used in his fractionation steps. Although the enzyme was reported not to have any activity with cystine (193), Racker later (179) found that at a suitable concentration, cystine also serves as a substrate.

Glutathione-Insulin Transhydrogenase (194).

Narahara and Williams (194) first detected glutathione-insulin transhydrogenase in rat liver. The enzyme catalyzed the hydrogen transfer between the sulfhydryl group of glutathione and the disulfide bonds in insulin. For the purpose of assaying the enzyme, one product, GSSG, was reduced by glutathione reductase with the concomitant oxidation of TPNH to TPN which was detected spectrophotometrically. The reactions are shown in equations 47 and 48.

$$\text{insulin} + 2\ \text{GSH} \xleftarrow{\text{GSH-insulin transhydrogenase}} \text{reduced insulin} + \text{GSSG} \qquad (47)$$

$$\text{GSSG} + \text{TPNH} + \text{H}^+ \xrightarrow{\text{glutathione reductase}} 2\ \text{GSH} + \text{TPN}^+ \qquad (48)$$

Tomizawa and Halsey (195) purified this enzyme to a high degree

from beef liver. The enzyme reaction was studied in more detail by Katzen and Stetten (196). They found that all three of the disulfide bonds in insulin were reduced to form the two insulin peptide chains with the disulfide bridge of the glycine (A) chain opened. A K_m for insulin of 4.3×10^{-5} M was determined. Oxytocin and vassopressin were also found to serve as substrates, whereas lipoic acid, homocystine, or cystine and ribonuclease were not substrates (179).

Katzen and Stetten (196) showed that the transhydrogenase also catalyzes the reformation of physiologically active insulin from its reduced components. The regeneration of physiologically active insulin was assayed by observing its stimulatory effect upon the metabolism of epididymal adipose tissue. Eleven per cent of the original activity of insulin was recovered when the reduced fragments were oxidized in the presence of the transhydrogenase, whereas only 0.6 percent was recovered on oxidation in the absence of the enzyme. The authors considered that the extent of sulfhydryl oxidation was similar in both experiments and therefore suggested that the enzyme directs the reformation of the "correct" disulfide bonds for biological activity. Tietze and Katzen (197) showed further evidence to support the earlier observation on the reversibility of the enzyme reaction. The enzyme was found to enhance the rate of regeneration of insulin according to immunochemical assay methods following reduction of the hormone by mercaptoethanol in 8 M urea. They further observed that glutathione-insulin transhydrogenase accelerated the rate of reappearance of RNase activity following reduction of the latter enzyme by mercaptoethanol in 8 M urea. This observation leaves open the possibility of a similarity between glutathione-insulin transhydrogenase and the RNase-regenerating enzyme

described below.

Recently Tomizawa and Varandan (198) reported the presence of glutathione-insulin transhydrogenase in human liver. The K_m value of human hepatic enzyme was determined to be 35 μM compared to 50 μM for the beef hepatic enzyme. The TCA soluble product produced by the human enzyme from insulin again was found to be the A chain by NH_2-terminal and complete amino acid analysis. Tomizawa (199) also has reported the isolation of glutathione-insulin transhydrogenase from beef pancreas.

An Enzyme Catalyzing the Sulfhydryl-Disulfide Interchange Within Protein Molecules. Goldberger et al. (200) and Venetianer and Straub (201) nearly simultaneously reported the detection of an enzyme in pancreas and liver microsomes, respectively, which catalyzed the reactivation of reduced RNase in the presence of soluble cofactors (200). The soluble factors could be replaced by dehydroascorbic acid (202). Further studies by both groups (203, 204) led to the conclusion that the sulfhydryl groups of reduced RNase were at least partially reoxidized by dehydroascorbate to form incorrectly paired disulfide bridges and that the microsomal enzyme catalyzed an intramolecular sulfhydryl-disulfide exchange to form correctly paired disulfide bridges to yield native RNase. The enzyme, which also reactivated reduced lysozyme (205), has been suggested to play a role at a late stage of protein synthesis to form correctly paired disulfide bonds in proteins (201, 203, 206). The location of the enzyme in the microsomes is consistent with this viewpoint.

CHAPTER II

EXPERIMENTAL PROCEDURE

Methods

The bovine liver nucleotide-peptide was usually isolated by a slight modification of the previously reported procedure (64). The modification is described in the chapter on "Results." The methods used for the purification of the nucleotide-peptide were: (a) paper electrophoresis in System 1, formic acid-acetic acid-H_2O (3:12:85 V/V) (207) and (b) paper chromatography in System 2, isobutyric acid-H_2O-NH_4OH (66:33:1 V/V) (210). The Whatman 3 MM paper used for electrophoresis was previously washed with 2 N acetic acid, water and ethanol in that order and then dried in air. Chromatography in System 3, isobutyric acid-NH_4OH-H_2O (57:4:39 V/V) (208); System 4, 95 percent ethanol-1 M ammonium acetate pH 3.5 (7.5:3 V/V) (209); or System 5, 95 percent ethanol-1 M ammonium acetate pH 7.5 (7.5:3 V/V) (209) was used in addition to the systems indicated above to test homogeneity of some nucleotide-peptide preparations.

Ultraviolet absorbing compounds were detected on paper using short wavelength (253.7 mμ) ultraviolet light (Chromato-VUE, Model C-3, Ultra-Violet Product Inc.). Ninhydrin reactive compounds were detected on paper by spraying with ninhydrin reagent A, a solution of one percent ninhydrin in ethanol, collidine, water (90:5:5 V/V), or B, the poly-

chromatic ninhydrin spray described by Moffat and Lytle (210). Sulfhydryl groups and disulfide bonds were detected with the reagents described by Toennies and Kolb (211).

A molar absorptivity of 15.4×10^3 at pH 7.0 (208) was used to estimate adenosine nucleotide concentration at 259 mμ. Phosphorus was determined by the method of Fisk and SubbaRow (212) and ninhydrin amino-equivalents were determined according to Moore and Stein (213).

CoASSG was synthesized according to the method of Eldjarn and Pihl (214) for the preparation of unsymmetrical disulfides. Details of the procedure are described in the "Results." Other mixed disulfides were prepared in the same manner using the appropriate sulfhydryl compound and disulfide compound in a one to four molar ratio, respectively. The mixed disulfides except GSSC and CSST were separated from the other reaction products by paper electrophoresis in System 1, cut from the paper and eluted with water. GSSC and CSST were separated from other reaction products by chromatography on a Dowex-1 acetate resin column under the same conditions used to separate GSH and GSSG described below. For the purpose of determining the concentration of solutions of mixed disulfides containing CoA, a molar absorptivity of 15.4×10^3 per CoA residue at 259 mμ and neutral pH was assumed. All other mixed disulfides which were prepared contained either one or two residues which could be determined after performic acid oxidation (215) using the colorimetric ninhydrin procedure (213). Appropriate known disulfides were used as standards. Since the color development of equimolar amounts of the various disulfides tested yield slightly different values, the average value of the appropriate disulfides was used in the calculation of the concentration of the appropriate mixed disulfide. For example, the

average color development obtained per mole of known HSSH and GSSG was used in determining the concentration of the synthesized mixed disulfide HSSG. In order to be certain that products isolated were actually the expected mixed disulfides, each one was submitted to performic acid oxidation (215) and the sulfonic acids produced were separated by paper electrophoresis in System 1. The products were detected with ninhydrin reagent A and their mobilities were compared to the mobilities of the sulfonic acids prepared from the appropriate known sulfhydryl or disulfide compounds by the performate treatment. All the synthesized disulfides (except one not reported in this thesis) yielded the correct products and did not appear to be contaminated with other ninhydrin reactive components.

The procedure for the fractionation of GSH-CoASSG transhydrogenase from bovine kidney is described in "Results." The percent saturation of ammonium sulfate was calculated according to Kunitz (216). The DEAE cellulose column used in the final step of the purification of the enzyme was prepared as follows: DEAE cellulose (Cellex-D, exchange capacity 0.91 meq/gm, Calbiochem) was suspended in water. After removing the "fines" by decanting, the cellulose was cycled with 0.1 M NaOH, water, 1 mM EDTA, 0.1 M HCl and finally water until the excess acid was removed. The DEAE cellulose was converted to the phosphate form using 1 M potassium phosphate pH 7.6 and then washed with water until the excess salt was removed. The cellulose suspension in water was packed into a column under 150 cm of hydrostatic pressure and then equilibrated with 1 mM potassium phosphate pH 7.6 in the cold room. Details of the chromatographic procedure are described in "Results."

Enzyme assays are described in detail under "Results." The unit

of GSH-CoASSG transhydrogenase is defined as the amount of enzyme which catalyzed the oxidation of one micromole of TPNH per minute at 25° under the conditions of the standard spectrometric assay (Results). This is equivalent to the cleavage of one micromole of CoASSG per minute. This unit is the same one suggested by the IUB Commission on enzyme nomenclature (217) and is used throughout the thesis to describe units of all enzymes used in the various experiments. Specific activity is the number of units per mg of protein. Protein was determined by the method of Lowry et al. (218).

In experiments measuring the ^{35}S exchange between ^{35}S-GSH and GSSG the reaction products were separated by paper electrophoresis in System 1, and located by spraying with ninhydrin reagent A. The purple spots were cut from the paper and decolorized in the counting vial as follows: the paper was moistened with ethanol followed by the addition of two drops of formic acid and two drops of 30 percent hydrogen peroxide, in that order. The excess ethanol, formic acid and H_2O_2 were then removed under vacuum. The purpose of this treatment was to decolorize the purple color which otherwise would cause quenching during liquid scintillation counting in a Packard, Tri-Carb Liquid Scintillation Spectrometer, Series 314A. The composition of the counting fluid was: PPO, 4 g; POPOP, 0.2 g; toluene, 400 ml; and ethanol, 600 ml.

The glutathione used in Michaelis constant experiments was purified by Dowex-1-acetate column chromatography to partially remove small amounts of GSSG present in the commercial preparation of GSH. Dowex-1 x 8 resin was converted to the acetate form by washing with 2 M sodium acetate and then with water to remove the excess salt. Two hundred micromoles of GSH were dissolved in 4 ml of water and applied to a

Dowex-1-acetate column (2 x 30 cm) and eluted by a linear gradient starting with one liter of H_2O and one liter 2 M acetic acid in the mixing flask and reservoir, respectively. Five ml fractions were collected. The GSH fractions were detected by drying a small drop from each fraction on a piece of Whatman 1 paper and spraying the paper with ninhydrin reagent A. Glutathione usually was present in fractions 90 to 105. These fractions were pooled and lyophilized. The vacuum was released under nitrogen. The purified GSH was dissolved in water and quantitated by the quantitative colorimetric ninhydrin method (213). A solution of purified GSH is stable for one to two days when it is stored in a stoppered test tube in the cold.

Materials

The enzymes used were as follows: the supernatant solution obtained from autolysis of dried Clostridium kluyveri (Worthington Biochemical Corporation) in 0.01 M phosphate buffer pH 7.5 was used as a crude preparation (219) of phosphotransacetylase (acetyl-CoA:orthophosphate acetyltransferase, E.C. 2.3.1.8). Yeast glutathione reductase (NAD(P) H_2:glutathione oxidoreductase, E.C. 1.6.4.2) was obtained from Sigma Chemical Company, alkaline phosphatase (orthophosphoric monoester phosphohydrolase, E.C. 3.1.3.1) and catalase (H_2O_2:H_2O_2 oxidoreductase, E.C. 1.11.1.6) from Worthington Biochemical Corporation and RNase (polyribonucleotide 2-oligonucleotidotransferase (cyclizing), E.C. 2.7.7.16) from Mann Research Laboratories, Inc. The enzyme which hydrolyzes pantothenic acid from pantotheine and which was used in the liberation of pantothenic acid from the nucleotide-peptide and from CoA

was prepared by extracting 200 mg of chicken liver acetone powder with one ml of 0.02 M sodium bicarbonate solution (220).

Chemicals

Pantothenate assay medium was obtained from Difco Laboratories, Inc. Acetyl phosphate was prepared according to Avison (221). Calcium pantothenate was obtained from Merck, Sharp and Dohme. Coenzyme A was obtained from P-L Biochemicals, Inc.; lipoic acid, pantothine, insulin, cytochrome c, TPNH and bovine serum albumin from Sigma Chemical Co.; GSH and GSSG from Boehringer and Soehne; cystine from Mann Research Laboratories, Inc.; homocystine from K and K Laboratories, Inc.; egg albumin from Nutritional Biochemical Corp.; ^{35}S-GSH from Schwarz Bioresearch, Inc.; DEAE cellulose from Calbiochem Corp.; and Sephadex G-100 from Pharmacia Fine Chemicals, Inc. All other chemicals used were of reagent grade.

CHAPTER III

RESULTS

Identification of Bovine Liver Nucleotide-Peptide

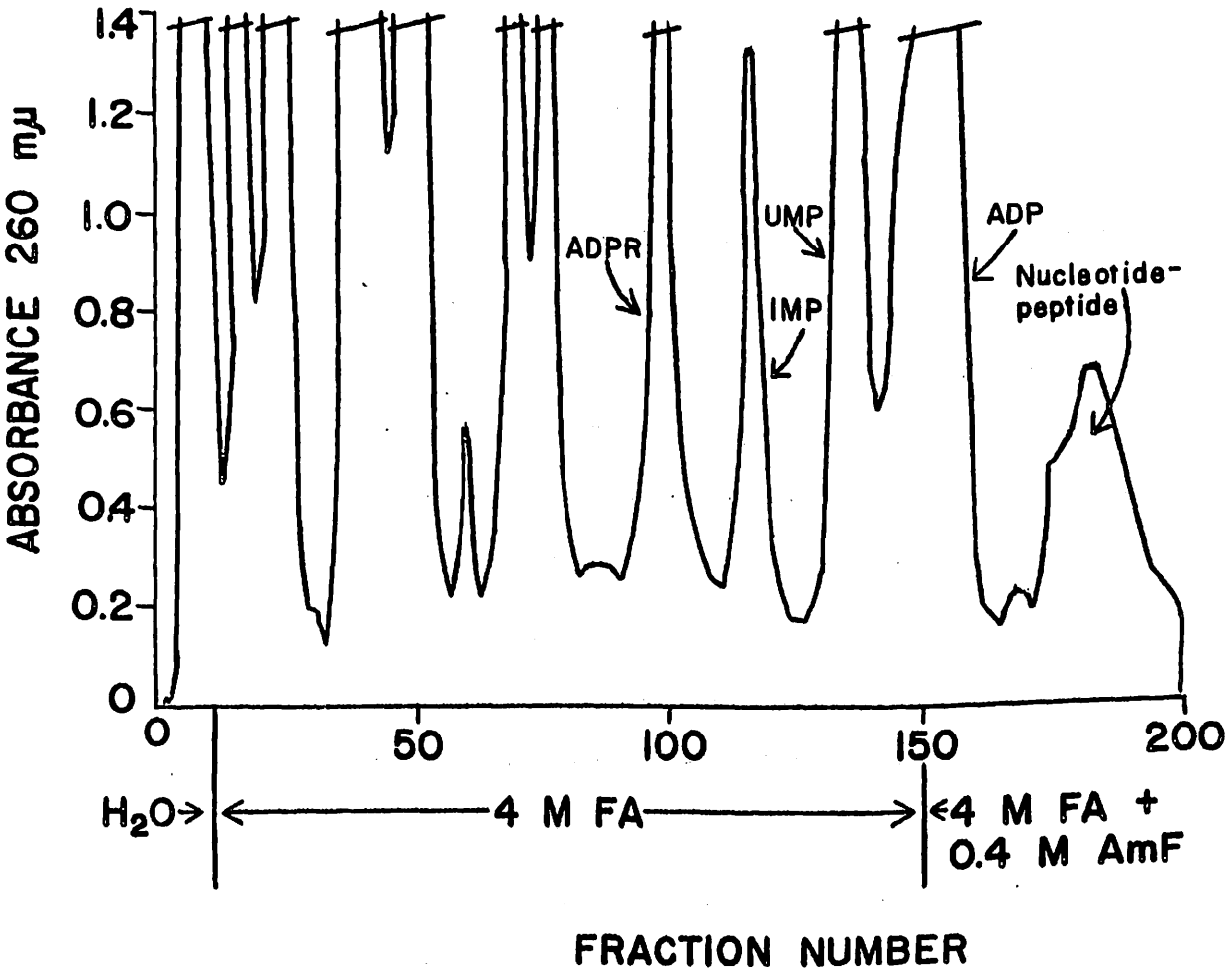
Isolation of the Nucleotide-Peptide from Bovine Liver

The procedure of isolation of the nucleotide-peptide from bovine liver was a slight modification of that previously reported by Wilken and Hansen (64). A neutralized perchloric acid extract of bovine liver (64) containing the acid soluble nucleotides was chromatographed on a 5.5 x 54 cm column of Dowex-1-formate resin as shown in Figure 7. The mixing flask originally contained 5,000 ml H_2O. The eluting solutions were (1) 1,000 ml water, (2) 4 M formic acid until the peak tube of the ADP fraction had eluted (about 14 liters), and (3) 4 M formic acid plus 0.2 M ammonium formate in that order. Fractions containing approximately 100 ml each were collected. Washing the column with water prior to elution with 4 M formic acid resulted in an improved separation of the nucleotide-peptide from other nucleotides. Further purification of the nucleotide-peptide by chromatography on a second ion-exchange column and paper chromatography was done by the previously reported procedure (64). Approximately 20 micromoles of the compound could be obtained from 1 kg of liver. It was later found that an alternate purification procedure could also be used. The nucleotide-peptide from the first Dowex-1-formate resin column was desalted by absorption and desorption

Figure 7

Partial Separation of Nucleotide-Peptide from Other Bovine Liver Acid Soluble Nucleotides on a Dowex-1-Formate Resin Column

The separation shown represents the nucleotides obtained from 500 grams of bovine liver. The fractions other than the nucleotide-peptide were identified by their characteristic 280/260 ratio and by comparison of the elution diagram with the elution patterns previously reported (64, 65) using essentially the same chromatographic procedure. FA is formic acid and AmF is ammonium formate. For details of the chromatographic procedure, see text.


ABSORBANCE 260 mμ
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
ADPR
UMP
IMP
ADP
Nucleotide-peptide
0
50
100
150
200
H2O→
←4 M FA→
←4 M FA + 0.4 M AmF
FRACTION NUMBER

from charcoal, then further purified by (a) electrophoresis on acid washed 3 MM paper for 30 minutes at 80 volts/cm in System 1 (Methods), (b) chromatography in System 2 (Methods) and (c) repetition of electrophoresis as described above.

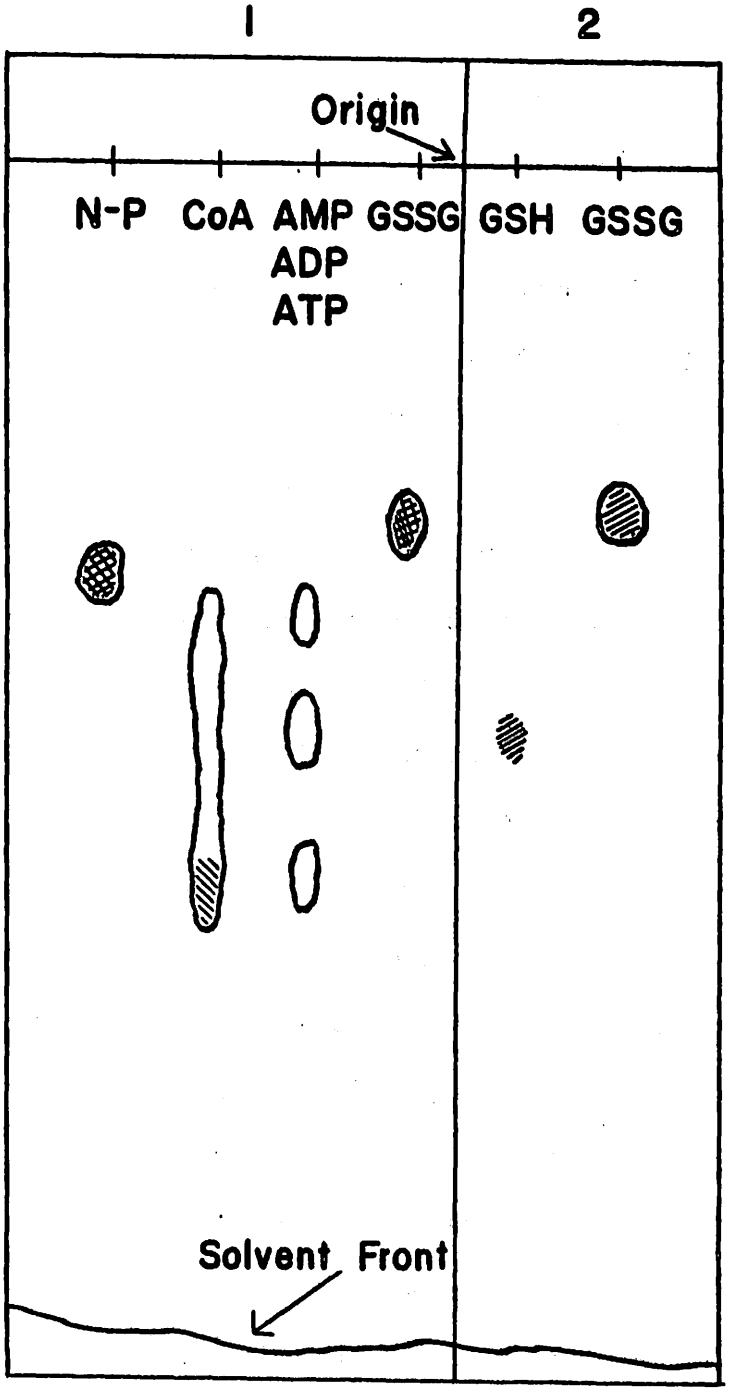
Presence of a Disulfide Bond in the Nucleotide-Peptide

Fifty mμmoles of the nucleotide-peptide purified by System 2 was spotted on Whatman No. 1 paper with 50 mμmoles of coenzyme A as a marker. After dipping the paper through a nitroprusside solution (211) the coenzyme A spot was pink, but no pink color developed in the area occupied by the ultraviolet absorbing spot corresponding to the nucleotide-peptide. Another 50 mμmoles of the purified nucleotide-peptide was spotted on Whatman No. 1 paper with 50 mμmoles of oxidized glutathione as a marker. The paper was dipped through the nitroprusside solution first; then while the paper was still damp, it was dipped through an alkaline solution of NaCN in methanol (211), and hung in a hood. A pink color was visible within five minutes at both the oxidized glutathione spot and the nucleotide-peptide spot. This color development under these conditions is characteristic of disulfide bonds. The third and fourth samples of the purified nucleotide-peptide were spotted on Whatman No. 1 paper with oxidized glutathione and a mixture of AMP, ADP, and ATP as markers. The paper was chromatographed in System 3 (Methods) which is able to resolve the nucleotide-peptide, free coenzyme A, GSH and GSSG (Figure 8). The developed chromatogram was tested both for ninhydrin positive compounds and nitroprusside reactive compounds. The nucleotide-peptide spot again showed a positive nitroprusside test only after treatment with an alkaline solution of cyanide in methanol.

Figure 8

Presence of a Disulfide Bond in the Bovine Liver Nucleotide-Peptide

This figure is a reproduction of a chromatogram which shows the mobility of the nucleotide-peptide relative to other compounds and that the nucleotide-peptide contains a disulfide bond. The nucleotide-peptide (N-P) was chromatographed in System 3 (Methods) along with standard markers of GSH, GSSG, CoA and a mixture of AMP, ADP and ATP. The position of ultraviolet absorbing compounds is indicated by a closed line (○). Strip 1 of the chromatogram was dipped through nitroprusside and cyanide reagents (211) in that order. Compounds which gave a red color before dipping through the cyanide reagent (indicating a sulfhydryl group) are shown as  and those which gave a red color after treating with the cyanide reagent (indicating a disulfide bond) are shown as  . Strip 2 of the chromatogram was sprayed with ninhydrin reagent A. A positive reaction is shown as  .


1
2
Origin
N-P
CoA
AMP
ADP
ATP
GSSG
GSH
GSSG
Solvent Front

From these results it was found that the bovine liver nucleotide-peptide contains a disulfide bond and that it does not contain sulfhydryl groups.

Presence of a Pantothenic Acid Residue in the Nucleotide-Peptide

The pantothenic acid content of the nucleotide-peptide was determined by microbiological assay with the use of Lactobacillus arabinosus 17-5 as the test organism. The procedure used was essentially the one described by Novelli, Kaplan and Lipmann (220) except that chicken liver acetone power extract (in place of pigeon liver acetone powder extract) and commercial alkaline phosphatase were used to liberate pantothenic acid from coenzyme A. In some assays, the incubation mixture responsible for liberation of pantothenic acid from the nucleotide-peptide was made 6.9 mM with cysteine. This amount of cysteine in the incubation mixture during the cleavage of coenzyme A yielded a molar ratio of cysteine tc nucleotide-peptide of 280. The significance of this ratio will be discussed later. Growth in Difco pantothenate medium supplemented with the enzymatic hydrolysates of coenzyme A or the nucleotide-peptide was compared to growth in tubes supplemented with various levels of calcium pantothenate by measuring the turbidity of the organisms at 660 mμ. The results shown in Table I, experiment 1, indicate that the nucleotide-peptide contains one mole of pantothenic acid per mole of adenosine when the enzymatic cleavage to pantothenic acid was conducted in the presence of cysteine. In the absence of cysteine, the liberation of pantothenic acid from the nucleotide-peptide was not complete within the incubation time employed. Recovery of pantothenic acid from coenzyme A was also slightly depressed in the absence of cysteine. The effect of the cysteine may have been to liberate a CoA residue from the

TABLE I

PANTOTHENIC ACID AND COENZYME A CONTENT
OF NUCLEOTIDE-PEPTIDE

Experiment and compound tested	Amount of product	
	Plus cysteine[a]	Minus cysteine[a]
	µmoles/µmole adenosine	
1. Pantothenic acid formation		
Nucleotide-peptide	0.98	0.67
CoA	1.01	0.86
2. Coenzyme A formation		
Nucleotide-peptide	0.85	0.00

[a]In experiment 1 incubation mixtures for the enzymatic cleavage of CoA or the nucleotide-peptide to liberate pantothenate were adjusted to contain 6.9 mM cysteine except as indicated. Pantothenate was subsequently determined by microbiological assay (see text and reference 220). In experiment 2 phosphotransacetylase assays (see text and reference 222) for the estimation of CoA were conducted in the presence of 11 mM cysteine except as indicated.

nucleotide-peptide by a sulfhydryl-disulfide interchange. The CoA then might liberate a pantothenic acid residue at a faster rate than the intact nucleotide-peptide.

Presence of Coenzyme A Residue in the Nucleotide-Peptide

The coenzyme A content of the nucleotide-peptide was determined by comparing its ability to replace a limiting amount of coenzyme A in the hydrolysis of acetyl phosphate in the presence of excess phosphotransacetylase (222). The residual acetyl phosphate was estimated by a hydroxamic acid procedure (223). Although the assay mixture normally contained cysteine (11 mM) during the incubation prior to the determination of residual acetyl phosphate, one set of experiments was conducted in which cysteine was omitted. Since the amount of nucleotide-peptide was varied in different experiments, the ratio of cysteine to nucleotide-peptide varied between 1180 and 2200. The results shown in Table I, experiment 2, indicate that in the presence of a large excess of cysteine, the nucleotide-peptide yielded nearly one mole of coenzyme A per mole of adenosine. In the absence of added cysteine no coenzyme A activity was observed. This finding is consistent with data presented above which indicated the presence of a disulfide group in the nucleotide-peptide rather than a free sulfhydryl group. A disulfide derivative of coenzyme A would be expected to show coenzyme A activity in this assay only after cleavage to free coenzyme A. This presumably occurred in the experimental tubes containing a large excess of cysteine compared to the nucleotide-peptide by a sulfhydryl-disulfide interchange reaction. Lack of coenzyme A activity in the phosphotransacetylase assay system in the absence of added cysteine has previously been observed in the

studies of disulfide forms of coenzyme A (107).

Presence of a Glutathione Residue in the Nucleotide-Peptide

The presence of a glutathione residue in the nucleotide-peptide was detected qualitatively by hydrogen sulfide reduction and electrophoresis. The nucleotide-peptide was reduced by bubbling hydrogen sulfide through a solution of the nucleotide-peptide for three hours at neutral pH (occasional adjustment of the pH to neutrality) and the excess hydrogen sulfide was removed by a stream of nitrogen after adjustment of the pH value to 2.5. The solution was lyophilized and the residue was dissolved in a small volume of the electrophoretic buffer and subjected to electrophoresis in System 1 for 45 minutes at 80 volts per cm. After the paper had been sprayed with ninhydrin reagent A, a ninhydrin positive component which had the same mobility as standard glutathione was detected as a reduction product of the nucleotide-peptide. This indicates that a glutathione residue is reductively cleaved from the nucleotide-peptide molecule. Figure 9 shows the results of this experiment.

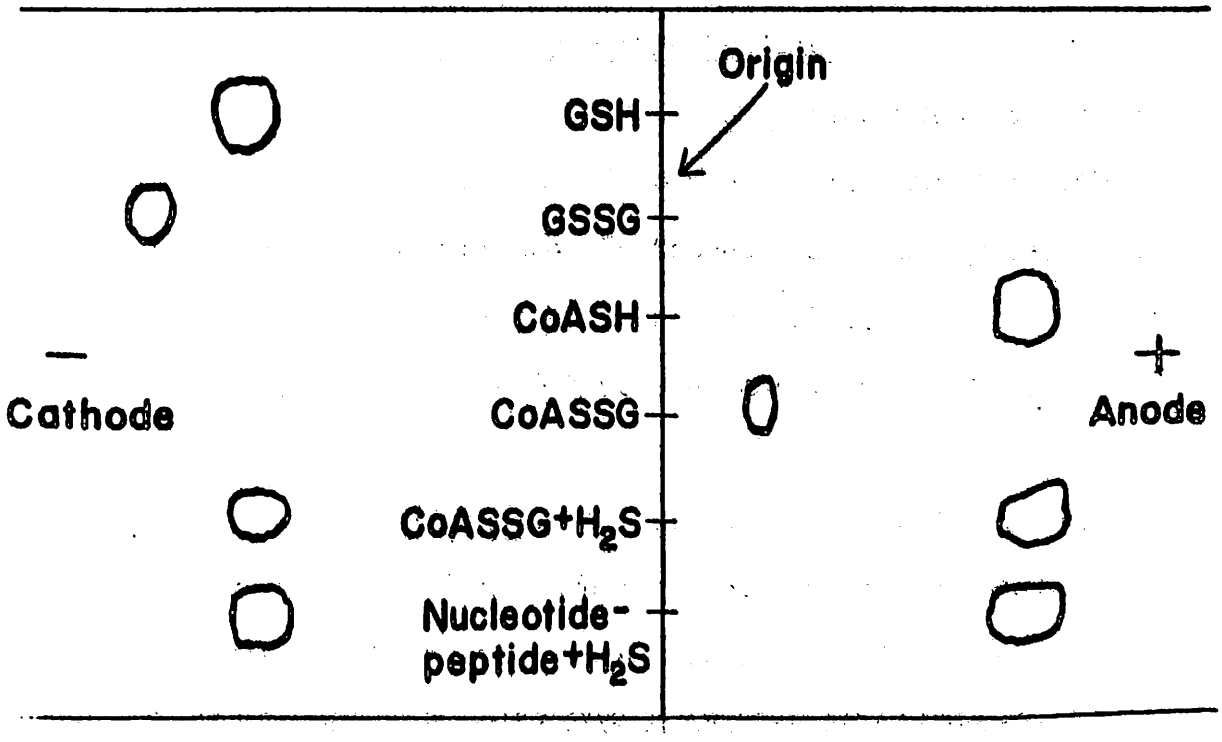
Comparative Properties of the Nucleotide-Peptide and Synthetic CoASSG

The results described above coupled with those previously reported by Wilken and Hansen (64) suggested that the bovine liver nucleotide-peptide might be a mixed disulfide of coenzyme A and glutathione, that is, CoASSG. Therefore, this compound was chemically synthesized in order to compare its properties with those of the nucleotide-peptide. The synthesis was conducted under conditions similar to those previously reported by Eldjarn and Pihl (214) for the preparation of a mixture of symmetrical and unsymmetrical disulfides.

Figure 9

Presence of a Glutathione Residue in Nucleotide-Peptide and in Synthetic CoASSG

This figure represents a reproduction of an electrophoretogram of the nucleotide-peptide and synthetic CoASSG after reduction with hydrogen sulfide. GSH is shown to be produced from each of these preparations. Standard markers of GSH, GSSG, CoA and CoASSG are shown for reference purposes. Electrophoresis was conducted in System 1. For details see text.


Origin
GSH
GSSG
CoASH
CoASSG
CoASSG+H_2S
Nucleotide-peptide+H_2S
—
Cathode
+
Anode

Oxidized glutathione and CoA were mixed in a 4:1 molar ratio in 0.05 M potassium phosphate pH 7.5. The solution was placed in a water bath at 35-38° C and oxygen was slowly bubbled through it for three hours. The CoASSG formed in such mixtures was purified by preparative paper chromatography in System 2, and electrophoresis in System 1. The CoASSG was detected by its ultraviolet absorption and by its reactivity with ninhydrin.

Electrophoretic and Chromatographic Mobilities.

(a) Electrophoresis in System 1 (207). The nucleotide-peptide was spotted on acid washed Whatman No. 3 MM paper (see Methods) with a spot of synthetic CoASSG as marker and electrophoresised in System 1 (see Methods). The results showed that both the isolated nucleotide-peptide and the synthetic CoASSG have the same mobility in this system (Table II).

(b) Chromatographic mobilities. The isolated nucleotide-peptide and synthetic CoASSG were spotted on Whatman No. 1 paper and chromatographed in Systems 2, 3, 4 and 5 (see Methods) for 15 hours with AMP, ADP and ATP as markers. Results showed that these two preparations have the same R_{AMP} value in four different solvents (Table II).

(c) Chromatographic mobility on a Dowex-1-formate resin column. Five micromoles of synthetic CoASSG were chromatographed on a Dowex-1-formate resin column (1.8 x 47 cm). The column was eluted with 100 ml water, 1400 ml 4 M formic acid and 1,000 ml 0.2 M ammonium formate in 4 M formic acid starting with 500 ml of water in a mixing flask. Ten ml fractions were collected. Absorbance of the fractions was measured at 260 mμ. The peak tube was found at fraction number 200 compared to

TABLE II

COMPARATIVE PROPERTIES OF ISOLATED NUCLEOTIDE-PEPTIDE AND SYNTHETIC CoASSG

Experiment	Isolated nucleotide-peptide	Synthetic CoASSG
	R_{AMP}	
Mobility studies[a]		
$M_{synthetic\ CoASSG\ in\ System\ 1}$	1.00	1.00
R_{AMP} in System 2	0.61	0.61
R_{AMP} in System 3	0.73	0.72
R_{AMP} in System 4	0.74	0.74
R_{AMP} in System 5	0.77	0.79
	μmoles/μmole adenosine	
Chemical analyses		
Organic phosphorus	3.32	2.76
Acid-labile phosphorus	0.37	0.32
Ninhydrin amino-equivalents		
Before hydrolysis	0.93	1.05
After hydrolysis[b]	4.47	4.60
Enzymatic assays[c]		
A. Initial rate of formation of GSSG on addition of 0.1 μmole of the compound tested (mμmoles per min)	18	19
B. GSSG formed per 0.05 μmole of compound tested (μmoles)	0.048-0.057	0.049-0.056

[a]See "Experimental Procedure" for a description of the electrophoretic and chromatographic systems used. $M_{\text{synthetic CoASSG}}$ is the electrophoretic mobility of the compound tested compared to that of synthetic CoASSG. R_{AMP} is the chromatographic mobility of the compound tested compared to that of AMP.

[b]The ninhydrin amino-equivalents obtained by assaying hydrolysates of adenosine have been subtracted from the values shown, in an attempt to correct for acid breakdown products of the adenosine portion of CoASSG. The value of the correction for the experiment shown was 0.17 μmole per μmole of adenosine.

[c]The enzymic assays were performed spectrophotometrically as described in the second part of the "Results" under spectrophotometric assay.

fraction number 183 (peak) when the nucleotide-peptide was chromatographed in the same system except that the compound was part of a crude acid-soluble nucleotide mixture from bovine liver. This result showed that the synthetic CoASSG has approximately the same mobility as the isolated nucleotide-peptide in the described system. The small discrepancy in mobility may have been due to a decrease in the effective capacity of the resin in the case in which the crude acid-soluble nucleotide mixture was applied to the resin or to a small difference in the amount of resin used in the two experiments.

Chemical Analysis

(a) Ninhydrin amino equivalents. The number of ninhydrin amino equivalents of the isolated nucleotide-peptide and the synthetic CoASSG were determined before and after acid hydrolysis using a quantitative ninhydrin colorometric analysis (213) (Table II). Glutamic acid was used as the standard (213). Before acid hydrolysis approximately one mole of ninhydrin amino equivalent per mole of adenosine was observed in both the isolated nucleotide-peptide and the synthetic CoASSG. After acid hydrolysis (6 N HCl, 110°, 17 hours) this value increased to approximately 4.5 in both the isolated nucleotide-peptide and the synthetic CoASSG. The latter value has been corrected for a small amount of ninhydrin positive acid breakdown products arising from the adenosine portion of CoASSG and the nucleotide-peptide.

(b) Organic phosphorus, acid-labile phosphorus, and inorganic phosphorus. Organic phosphorus, acid labile phosphorus, and inorganic phosphorus in both the nucleotide-peptide and synthetic CoASSG were determined by the Fisk and SubbaRow procedure (212). The results (Table II) showed that

the nucleotide-peptide and synthetic CoASSG contained three moles of organic phosphorus per mole of adenosine. Only 0.3 mole of ten minute acid-labile phosphorus per mole of base was present. No inorganic phosphorus was observed in either preparation.

(c) Amino acid composition. The amino acid composition of the nucleotide-peptide and CoASSG were compared by paper chromatography of acid hydrolysates. Samples of each compound, after chromatography in System 2 as the final purification step, were treated with performic acid for four hours at 0° (215) followed by removal of the excess reagent in a vacuum. The dried samples were hydrolyzed in triple distilled constant boiling hydrochloric acid in a vacuum at 110° for 17 to 20 hours and chromatographed in two dimensions as described previously (64). The separated amino acid components were detected with ninhydrin reagent B. The results are shown in Figure 10. Both the nucleotide-peptide and the synthetic CoASSG gave the same amino acid components, namely, cysteic acid, taurine, glycine, glutamic acid and β-alanine. The unidentified ninhydrin reactive component was observed in both the synthetic CoASSG and the isolated nucleotide-peptide. As mentioned in the "Introduction" chapter, Okuhara and Hansen (65) indicated that this unidentified component was not observed in their results. In repeated experiments, a sample of synthetic CoASSG or isolated nucleotide-peptide, after electrophoresis in System 1 as the final purification step, was hydrolyzed and chromatographed as described above. No unidentified component was found after spraying the paper with ninhydrin reagent B. In another experiment a preparation of synthetic CoASSG purified by electrophoresis in System 1 which did not contain the unidentified component yielded the unidentified component when it was subsequently chromatographed in

Figure 10

Chromatography of the Amino Acids Obtained from Hydrolysates of the Isolated Nucleotide-Peptide and Synthetic CoASSG

Isolated nucleotide-peptide or synthetic CoASSG was purified by System 2 chromatography (Methods) and hydrolyzed in triply distilled constant boiling hydrochloric acid in vacuum at 110° for 17 to 20 hours and chromatographed in two dimensions. For details see text.

Synthetic CoASSG
dye marker
alanine
glutamic acid
glycine
taurine
cysteic acid
origin
Isolated Nucleotide-Peptide
dye marker
alanine
glutamic acid
glycine
taurine
cysteic acid
origin
BUTANOL: PROPIONIC ACID: WATER
WATER: PHENOL

System 2 prior to hydrolysis. The unidentified component previously reported (64) is, therefore, an artifact which appears in preparations of CoASSG purified by chromatography in System 2.

(d) Enzymic assay. The enzymatic assays reported in Table II were performed with a DEAE column purified preparation of glutathione-CoASSG transhydrogenase from bovine kidney which will be described in the second section of this chapter. The assay was performed spectrophotometrically (see Assay of GSH-CoASSG Transhydrogenase). The enzymatic assay is based on the formation of GSSG which is subsequently reduced to GSH by TPNH and glutathione reductase. The initial rate of formation of GSSG on addition of 0.1 micromole of isolated nucleotide-peptide or synthetic CoASSG was the same (Table II). The number of micromoles of GSSG formed from 0.05 micromole of nucleotide-peptide or CoASSG varied within a limited range, but was approximately 0.05 micromoles. The variation was probably due to experimental error caused by the oxidation of GSH by atmospheric oxygen during the enzymatic assay. The results indicate that for every mole of CoASSG utilized one mole of GSSG is formed.

The results reported above led to the conclusion that the bovine liver nucleotide-peptide previously reported by Wilken and Hansen is the mixed disulfide of coenzyme A and glutathione which has the structural formula shown in Figure 11.

Assay, Purification and Properties of Glutathione-CoASSG Transhydrogenase

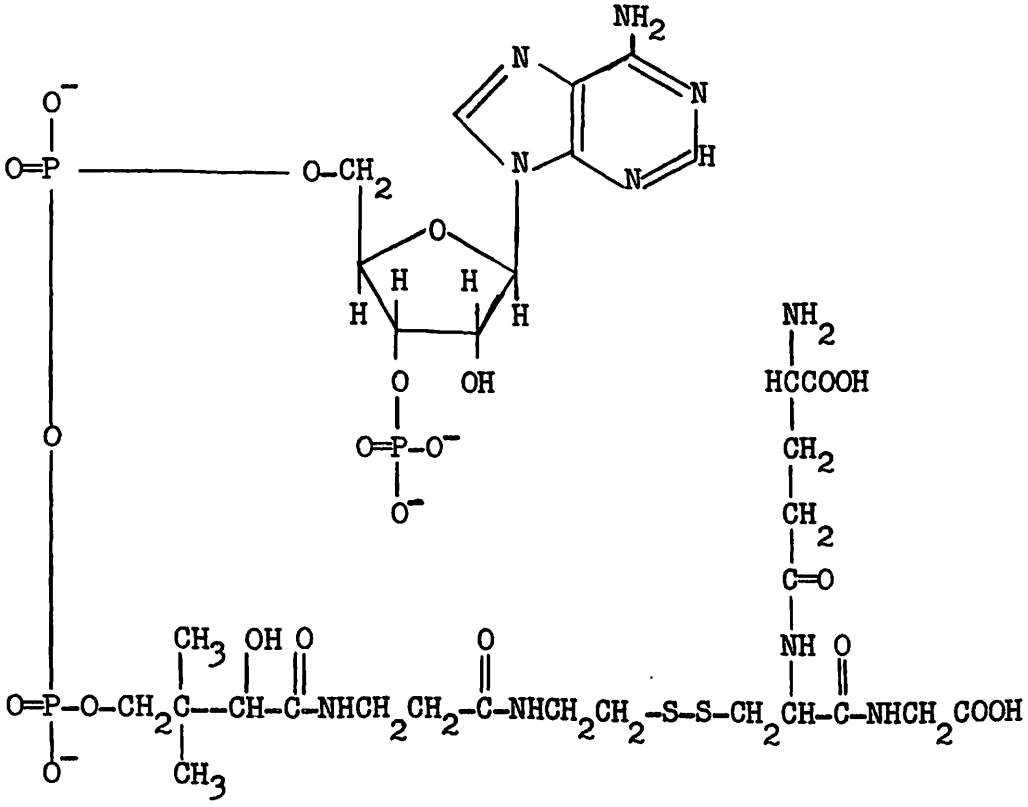
Assay of GSH-CoASSG Transhydrogenase

Two methods for measuring the reaction rate catalyzed by GSH-

Figure 11

Structure of the Unsymmetrical Disulfide of

Glutathione and Coenzyme A



CoASSG transhydrogenase were used depending upon the particular technical requirements of different experiments.

Spectrophotometric Assay. Glutathione-CoASSG transhydrogenase catalyzes the sulfhydryl-disulfide interchange shown in equation 49.

GSH:CoASSG Transhydrogenase

$$\text{CoASSG} + \text{GSH} \rightleftharpoons \text{CoASH} + \text{GSSG} \qquad (49)$$

Glutathione Reductase

$$\text{GSSG} + \text{TPNH} + \text{H}^{+} \longrightarrow 2\text{GSH} + \text{TPN}^{+} \qquad (50)$$

In the standard spectrophotometric assay, reaction 49 is coupled to the glutathione reductase assay for GSSG. The latter reaction is shown as reaction 50. Thus the rate of formation of GSSG in reaction 49 may be followed by measuring the decrease in absorption of TPNH at 340 mμ as the reaction proceeds. The decrease in absorbance at 340 mμ was followed in a Beckman DB spectrophotometer. Table III gives in detail the contents of the standard assay reaction mixture.

In the spectrophotometric assay the rate of change of absorbance is a function of five variables including the GSH-CoASSG transhydrogenase catalyzed reaction. Therefore, appropriate controls to correct for the other sources of absorbance change were necessary. The other variables which influenced the reaction rate measurements were: (a) the presence of approximately one percent GSSG in the commercial GSH used (except where GSH purified as described in "Methods" was used), (b) the direct air oxidation of GSH to GSSG during reaction velocity measurements, (c) the nonenzymatic sulfhydryl-disulfide exchange reaction between

TABLE III

STANDARD SPECTROPHOTOMETRIC GSH-CoASSG TRANSHYDROGENASE ASSAY COMPONENTS

Reagent	Volume	Final concentration
	ml	
1.0 M potassium phosphate pH 7.6	0.2	210 mM[d]
1 percent bovine serum albumin in 0.1 M potassium phosphate pH 7.6	0.1	0.105 percent[d]
1 mM TPNH in 10^{-4} M KOH	0.1	0.1 mM
Glutathione reductase[a]	0.1	0.194 units
GSH-CoASSG transhydrogenase	Varies	Varies
10 mM CoASSG[b]	0.01	0.1 mM
50 mM GSH[b]	0.01	0.5 mM
Water[c]	to 1 ml	

[a]The amount of glutathione reductase used should cause a change in 340 mμ absorbance of 1.2 ± 10 percent per minute (about 0.2 units) when assayed in the following assay mixture: 60 μM potassium phosphate pH 7.6, 0.1 percent bovine serum albumin, 0.1 mM TPNH, 0.1 mM GSSG and glutathione reductase dissolved in 0.05 percent bovine serum albumin in a final volume of one ml. GSSG is added last to initiate the reaction.

[b]Either CoASSG or GSH was added last to initiate the reaction. The effect of addition sequence of CoASSG and GSH is discussed in the text.

[c]Usually water was the first reagent added into the cuvette.

[d]The apparent discrepancy in the final concentration of phosphate and BSA is due to the fact that small amounts of these two compounds were added with other reagents.

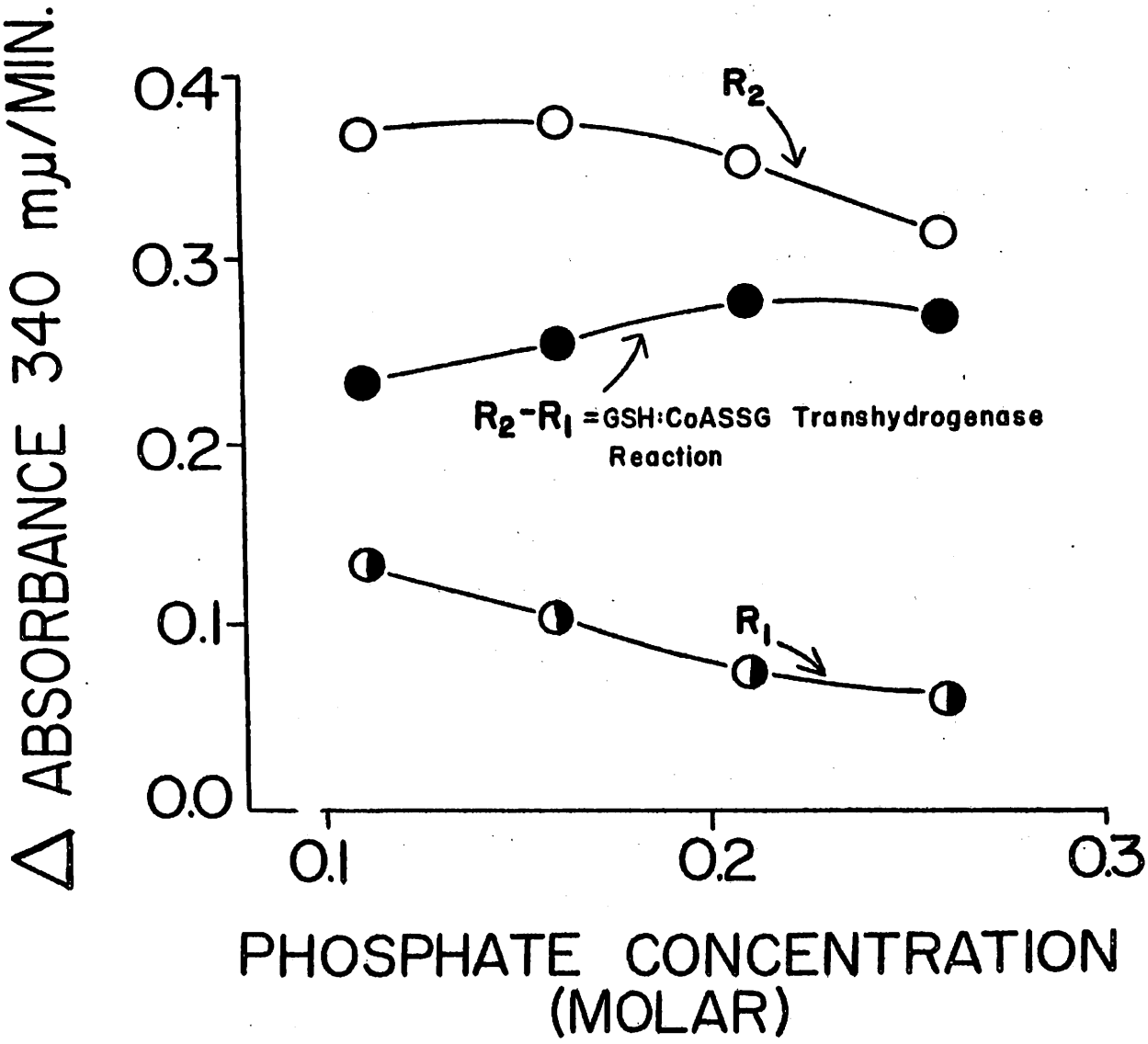
the substrates shown in equation 49 above and (d) the direct reduction of CoASSG by the commercial preparation of glutathione reductase. The relative contribution of two of these factors (b and c) was markedly influenced by pH. At the pH used in the standard assay (7.6), or below this pH, the contribution to the change in absorbance due to these two factors was negligible and could be ignored. At pH values higher than pH 7.6 or a concentration of GSH considerably higher than that used in the standard spectrophotometric assay these two factors could not be ignored. Figure 12 shows that the contribution of variable (d) to the rate of change of absorbance (R_1) could be decreased without appreciably affecting the GSH-CoASSG transhydrogenase contribution (R_2-R_1) by increasing the phosphate concentration of the assay mixture to 0.2 M. At lower concentrations of phosphate than the lowest shown in Figure 12 the contribution by variable (d) was considerably greater than that shown in Figure 12.

Different control assays were required depending on the order of addition of GSH and CoASSG to the reaction mixture. In the assay most commonly used, particularly during the enzyme fractionation experiment, CoASSG and GSH were added last and in that order. Prior to adding GSH the change in absorption was recorded for approximately 1.5 minutes to correct for the contribution of variable (d) listed above. The difference between this rate and the rate observed after GSH was added was taken as the GSH-CoASSG transhydrogenase contribution to the overall reaction rate (see Fig. 13A). Small contributions due to the variables (a), (b) and (c) could be corrected for by conducting an identical assay except in the absence of GSH-CoASSG transhydrogenase. During the fractionation of the enzyme these small contributions to the

Figure 12

Effect of Inorganic Phosphate Concentration on the Reaction Rates in the Spectrophotometric Assay of GSH-CoASSG Transhydrogenase and Glutathione Reductase

The reaction rates of the glutathione reductase catalyzed reduction of CoASSG is shown as R_1. The total reaction rate in the coupled system of GSH-CoASSG transhydrogenase and glutathione reductase is shown as R_2. The net rate of the GSH-CoASSG transhydrogenase catalyzed reaction is shown as R_2-R_1. For additional details see text.


Δ ABSORBANCE 340 mμ/MIN.
0.4
0.3
0.2
0.1
0.0
R2
R2-R1 =GSH:CoASSG Transhydrogenase Reaction
R1
0.1
0.2
0.3
PHOSPHATE CONCENTRATION (MOLAR)

over-all rate were ignored. The addition of GSH prior to the addition of CoASSG had the advantage that small amounts of GSSG in the GSH were converted to GSH prior to the measurement of the GSH-CoASSG transhydrogenase catalyzed reaction. This effectively removed any contribution due to variable (a) above. Contributions from variables (b), (c) and (d) were corrected for by conducting a single identical assay, except that no GSH-CoASSG transhydrogenase was added. The value in this nonenzymatic control assay was subtracted from the enzymatic assay described to obtain the final change in absorbance due to the GSH-CoASSG transhydrogenase reaction (Fig. 13B and C).

Electrophoretic Assay. Since the spectrophotometric assay could not be readily adapted to some of the experiments conducted, an assay system depending on the separation of the reaction products by electrophoresis was devised. The reactants shown in Table IV were pipetted into a depression of a spot plate in a constant temperature water bath, and kept in three individual drops. A mixture of CoASSG, buffer and water was in one drop while the GSH and enzyme were in separate drops. CoASSG, water, buffer, and GSH were mixed by a small stirring rod first and after five seconds the enzyme solution was also mixed in. The actual timing of the experiment was started when the enzyme was mixed with other reagents to make a complete reaction mixture. The reaction was stopped after a suitable time by adding a measured amount (usually 20 µl) of acid electrophoresis buffer (see Methods). An aliquot of known size (usually 30 µl) was spotted on a piece of Whatman 3 MM paper (acid washed) and electrophorised in System 1 at 80 volts per cm for 30 minutes. CoASSG spots (or occasionally CoA) were cut from the paper

Figure 13

Example of Spectrophotometric Assay

A) The trace of the reaction shown was obtained by doing a standard spectrophotometric assay (Table III) in which 0.1 μmole of CoASSG was added at point 1. The reaction was allowed to proceed for one minute and then 0.5 μmole of GSH was added at point 2. The difference in reaction rates R_2 and R_1 is the net rate of CoASSG cleavage catalyzed by GSH-CoASSG transhydrogenase.

B) The reaction was conducted in the same manner as in (A) except that the order of addition of GSH and CoASSG was reversed, that is, GSH was added at point 1 and CoASSG was added at point 2.

C) The reaction was conducted as in (B) except that no GSH-CoASSG transhydrogenase was added to the reaction mixture.

Reaction rate R_4 in (C) subtracted from reaction rate R_3 in (B) yields the net rate of CoASSG cleavage catalyzed by GSH-CoASSG transhydrogenase. The dashed lines (- - - -) represent extrapolations of the linear portions of the curves from which the reaction rates were obtained. For details see text.

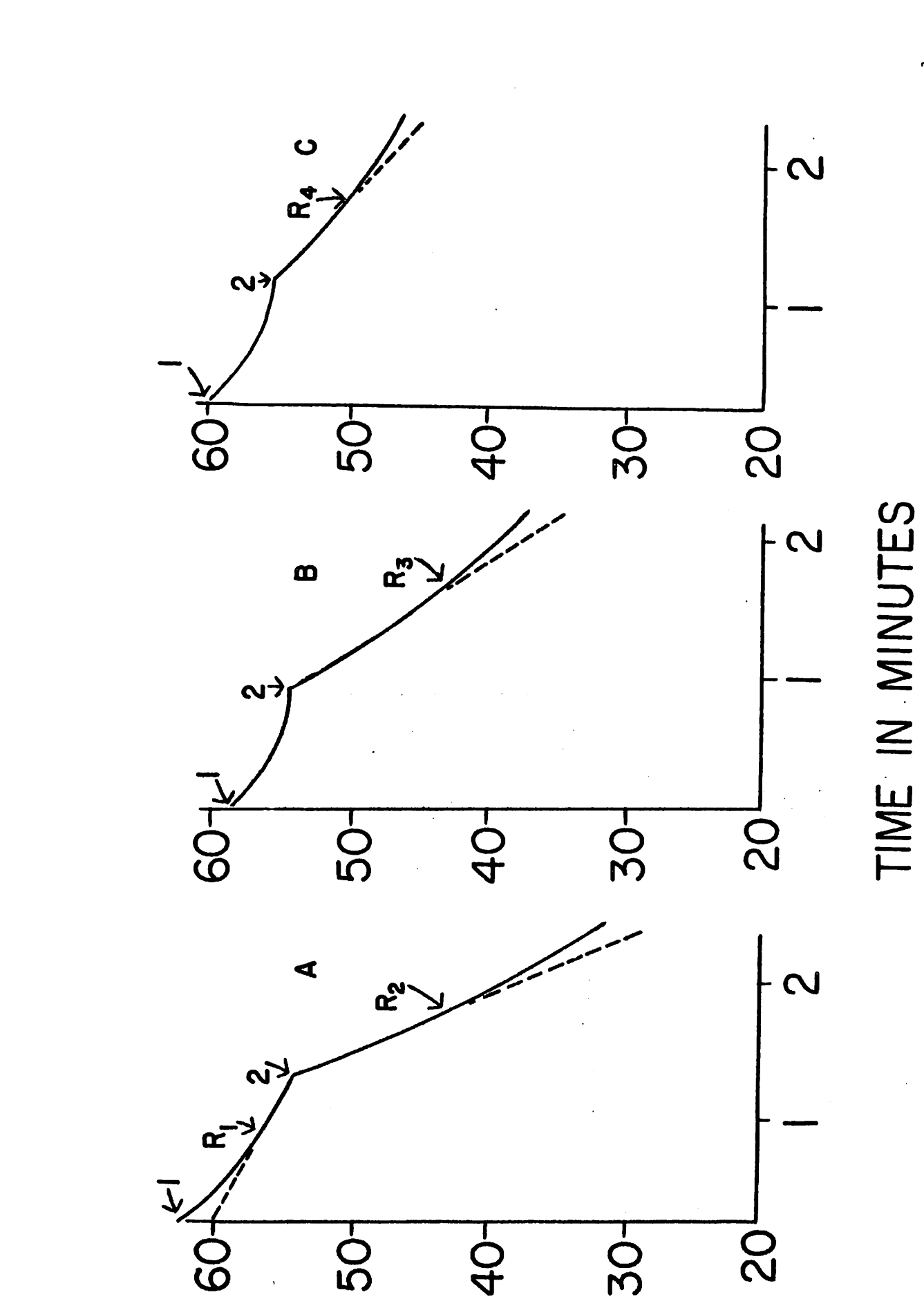

ABSORBANCE 340 mμ X 10²
60
50
40
30
20
1
2
A
R1
R2
B
R3
C
R4
TIME IN MINUTES

TABLE IV

ELECTROPHORETIC ASSAY FOR ACTIVITY OF GSH-CoASSH TRANSHYDROGENASE

Reagent	Volume	Final concentration
	µl	mM
Premixture containing CoASSG[a]	12	3
50 mM GSH	6	15
Transhydrogenase	2	Varies

[a]The premixture contained 0.6 µmole CoASSG, 10 µmole potassium phosphate and varing amount of KOH depending on final pH desired. Final volume 120 µl. In later experiments it was found better to use 1 M tris buffer of the appropriate pH in place of potassium phosphate and KOH. The technical details of the procedure are described in the text.

and eluted with water and the final volume was adjusted with water to one ml. The absorbance at 260 mμ was measured. The initial reaction rate was calculated in terms of the change in A_{260}/min. An assay without the transhydrogenase was required to correct for the nonenzymatic rate. Figure 14 shows an example of the time course of the electrophoretic assay.

Tissue Distribution of GSH-CoASSG Transhydrogenase Activity

Different tissues from a freshly sacrificed rat were minced by passing them through a stainless steel mincer and then homogenized in a micro-Waring Blendor with two volumes of water or 0.1 M potassium phosphate buffer pH 7.6, and finally centrifuged at 20,000 g for 30 minutes. The supernatant solutions were assayed for GSH-CoASSG transhydrogenase activity by the spectrophotometric assay. Table V shows the distribution of enzyme activity in different rat tissues. The enzyme is widely distributed in different tissues, the highest activity being found in the pancreas and brain while only low activity was observed in muscle and heart. Liver, lung and kidney contained intermediate amounts of the enzyme.

Enzyme Fractionation

Bovine kidney from freshly slaughtered animals was obtained from a local abattoir. The whole kidney was kept in ice for transportation to the laboratory. The outter membrane and the inner white connective and fatty tissues of the kidney were removed. The cortex and part of the medulla (400 g) were homogenized approximately two minutes in a Waring Blendor with two volumes of water and centrifuged at 14,000 x g

Figure 14

Example of an Electrophoretic Assay of GSH-CoASSG Transhydrogenase

The time course of the reaction was obtained by plotting the 260 mμ absorbance due to CoASSG disappearance (○) or CoASH formation (●) after different times of incubation of a complete reaction mixture. The nonenzymatic disappearance of CoASSG (◒) is also shown. For details see text and Table IV.

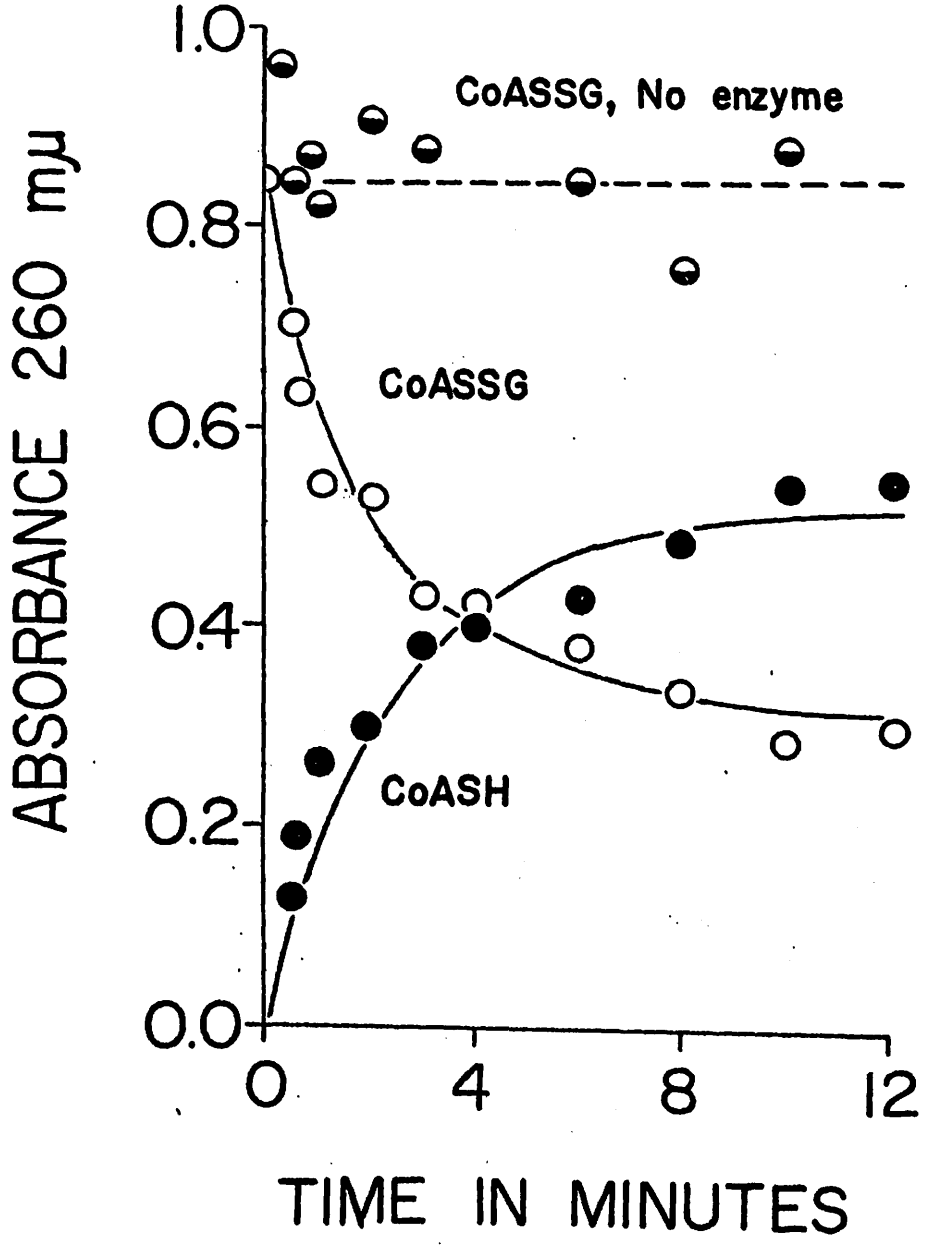

ABSORBANCE 260 mμ
1.0
0.8
0.6
0.4
0.2
0.0
CoASSG, No enzyme
CoASSG
CoASH
0
4
8
12
TIME IN MINUTES

TABLE V

DISTRIBUTION OF GLUTATHIONE-CoASSG TRANSHYDROGENASE ACTIVITY IN RAT TISSUES

Tissue	Specific Activity	
	Experiment[a] 1	Experiment[b] 2
	μmole CoASSG cleaved[c]/min/mg protein	
Pancreas	28.0	8.7
Brain	14.6	14.8
Liver	9.1	6.2
Kidney	5.4	4.9
Lung	5.2	3.3
Muscle	4.5	1.3
Heart	2.9	2.0

[a]In this experiment two volumes of 0.2 M potassium phosphate buffer pH 7.6 were used to extract the tissue.

[b]In this experiment two volumes of water were used to extract the tissue.

[c]The spectrophotometric assay described in the text was used in these experiments.

for 30 minutes. The supernatant solution was heated to 58° in an 80° water bath in 200 ml portions contained in a two liter stainless steel beaker. During the heating period the beaker was subjected to a swirling motion to avoid local heating. The mixture was kept at 58° for one minute in a 58° water bath and then was rapidly chilled in an ice bath to 5°. The combined fractions from the heat step were centrifuged at 14,000 x g for 30 minutes. The supernatant solution was fractionated by adding solid ammonium sulfate with mechanical stirring to 40 percent saturation (226 g/L, 216) and equilibrated for 10 minutes. The mixture was centrifuged at 14,000 x g for 30 minutes. The supernatant solution was slowly (approximately 20 minutes) taken to saturation with ammonium sulfate (418 g/L) and stirred for 10 minutes after the last ammonium sulfate addition. The mixture was centrifuged as above. The supernatant solution was removed from the precipitate as completely as possible by decantation and by allowing centrifuge bottles to drain while in an inverted position. The precipitate obtained was dissolved in water to a volume of 200 ml and then fractionated with acetone. The acetone step was done in a stainless steel beaker mounted over a magnetic stirrer and sitting in an acetone bath whose temperature could be adjusted by the addition of dry ice. Acetone at -15° was delivered into the beaker containing the enzyme solution as a stream directed at the side of the beaker. The solution inside the beaker was stirred by a magnetic stirring bar to avoid a high local concentration of acetone. The cooling bath temperature was decreased during the addition of acetone from 0° to approximately 10° to 15° below zero but not so rapidly as to cause the enzyme solution to freeze in the beaker. Upon reaching 58 percent acetone, the solution

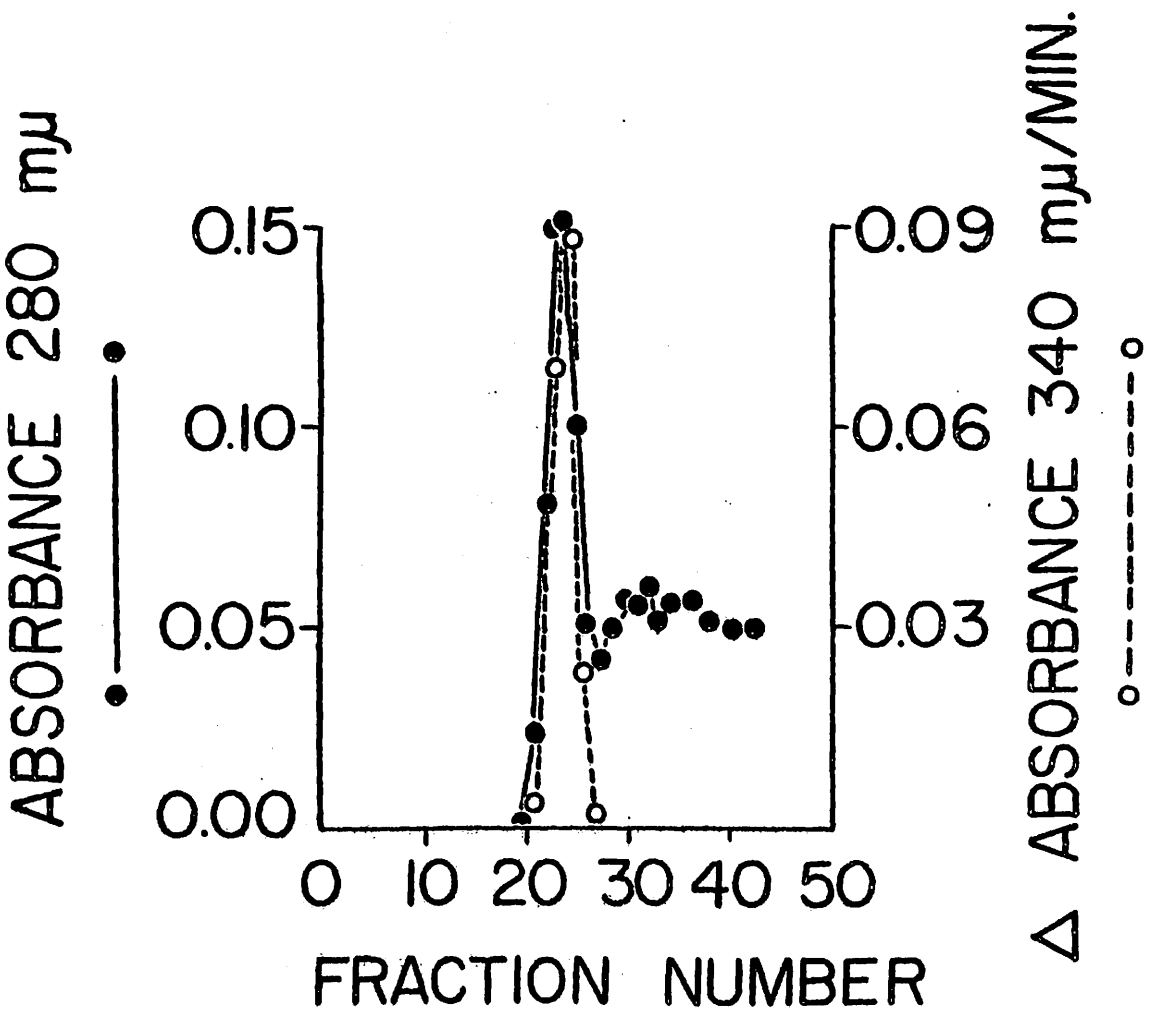
was equilibrated for 10 minutes and then centrifuged at 7,000 x g for one minute at -10°. The clear yellow supernatant solution was adjusted to 90 percent acetone at 15° to 20° below zero. After the addition of the acetone, the solution was equilibrated for 10 minutes and then centrifuged at 7,000 x g for one minute at -10°. The precipitate obtained was dissolved in a small volume (about 40 ml) of water and was dialyzed against two to three one-liter changes distilled water for five to twelve hours. The dialysate was then concentrated by lyophilization to about 10 ml. The concentrated enzyme solution was centrifuged to remove any insoluble material and then chromatographed on a DEAE cellulose (50 x 1.8 cm) column previously equilibrated with phosphate buffer (Methods). The column was eluted by a linear gradient (224) in which one liter of 0.005 M potassium phosphate buffer pH 7.6 was placed in the mixing chamber and one liter of 0.2 M potassium phosphate buffer pH 7.6 was placed in the reservoir. Five ml fractions were collected. The fractions containing protein were located by measuring the 280 mμ absorbance of each fraction (225). The GSH-CoASSG transhydrogenase activity was located by assaying aliquots of the fractions by using the spectrophotometric assay method. The enzymatic activity was found under the first protein peak (Fig. 15). The fractions containing enzyme were pooled and then divided into several tubes for storage at -10° until used.

Starting from the centrifuged crude kidney homogenate, aliquots from each step of the fractionation procedure were assayed for enzymatic activity and protein content (218). Table VI shows the results of a typical purification of the enzyme starting with 400 grams of tissue. In several early enzyme preparations, the protein content of each

Figure 15

Purification of GSH-CoASSG Transhydrogenase on DEAE Cellulose

Only the early portion of the protein elution pattern which contained the GSH-CoASSG transhydrogenase is shown. Most of the protein applied to the column was retained on the column. The protein content of each fraction was determined by its 280 mμ absorbance (●—●). The enzyme activity in the fractions were assayed spectrophotometrically (O--O).


ABSORBANCE 280 mμ
0.15
0.10
0.05
0.00
0.09
0.06
0.03
Δ ABSORBANCE 340 mμ/MIN.
0 10 20 30 40 50
FRACTION NUMBER

fraction was measured by a biuret method (226) through the acetone step. Then the 280/260 mμ absorbance method (225) was used for quantitation of protein eluted from the DEAE column because of its greater sensitivity. Later it was observed that the biuret and 280/260 methods agreed poorly using DEAE purified enzyme. Therefore, Lowry's method was used throughout the purification in subsequent preparations of the enzyme. Only the results obtained with this method are presented.

Further purification steps which were tried including starch gel electrophoresis, chromatography on CM-cellulose and phospho-cellulose columns were unsuccessful.

Validity of Assay with DEAE-Purified Enzyme

Figure 16 shows that the formation of GSSG as a function of time was linear for approximately one minute at several enzyme concentrations tested. Figure 17 shows that the rate of the reaction was linear with protein concentration over the range tested.

Products of the Reaction

The electrophoretic assay was used to identify the products of the enzymatic cleavage of CoASSG in the presence of GSH. Fifty mμmoles of CoASSG was allowed to react for three minutes with 250 mμmoles of GSH in the presence of 3.2×10^{-3} units of GSH-CoASSG transhydrogenase and subjected to electrophoresis in System 1 (Methods). Figure 18 shows the products of the enzymatic reaction. It also shows that no product was formed in a nonenzymatic control experiment. Appropriate standard markers are also shown. In the complete enzymatic reaction mixture, only unreacted GSH and CoASSG as well as the products

TABLE VI

FRACTIONATION OF GSH-CoASSG TRANSHYDROGENASE[a]

Treatment	Total activity	Total protein	Specific activity	Purification	Recovery
	units[b]	mg	units/mg		%
Crude extract	670	24,000	0.028	1.0	100
Heat	360	14,300	0.025	0.9	53.6
Ammonium sulfate 40-100%	365	8,850	0.041	1.48	54.3
Acetone 58-90%	68.7	47.9	1.435	51.5	10.2
DEAE	29.5	5.7	5.13	184.0	4.4

[a]The data shown represent a typical purification starting from 400 gm of bovine kidney.

[b]The number of units was determined using a spectrophotometric assay described in the text and expressed in micromoles of substrate reacted/min.

Figure 16

Time Course of the GSH-CoASSG Transhydrogenase Catalyzed GSSG Formation

The spectrophotometric assay was used employing enzyme purified through DEAE column chromatography (see text). The assay was conducted in the presence of 10, (○); 30, (●); and 50, (◑) ug of transhydrogenase as described in the legend of Figure 13A.

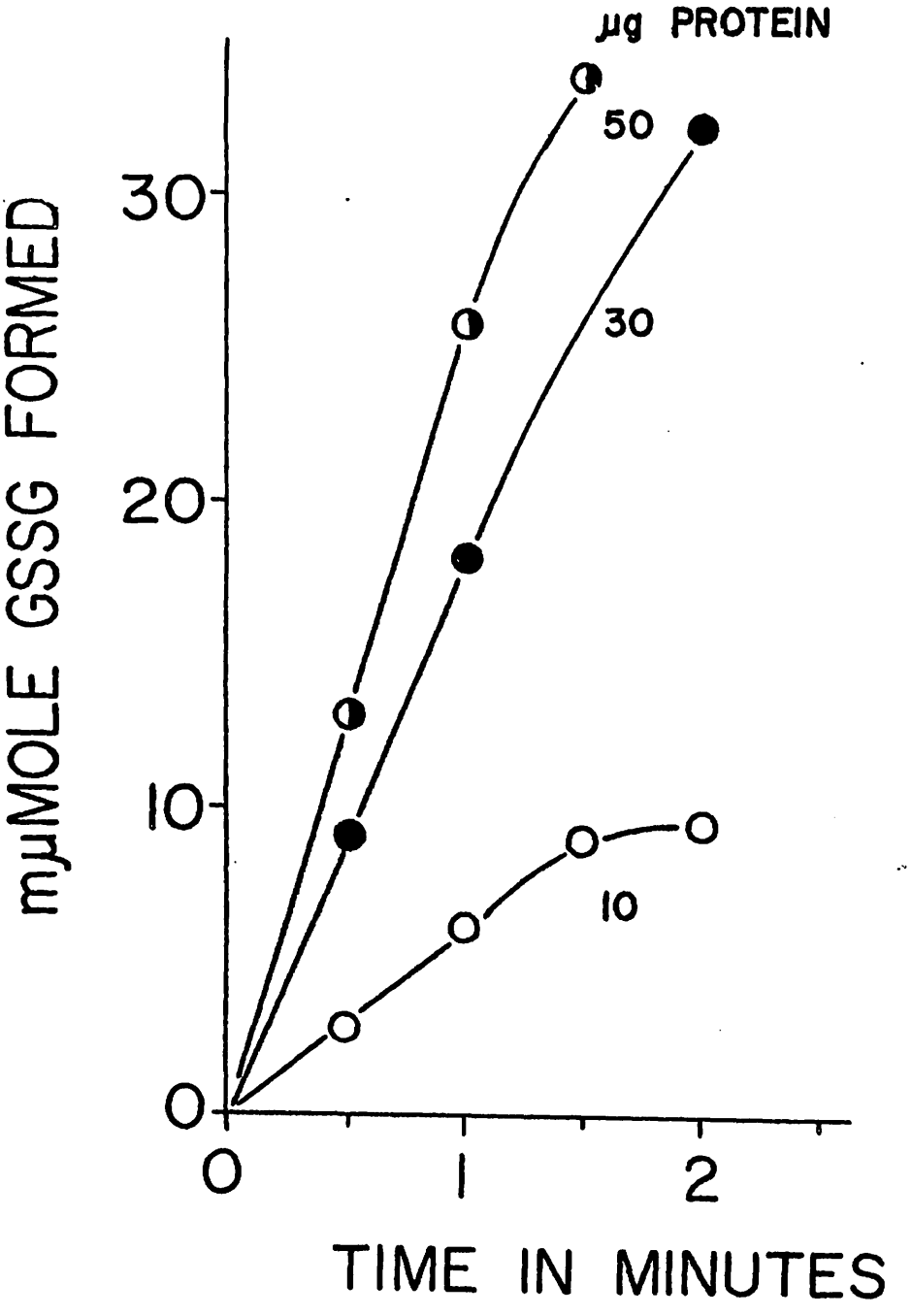

µg PROTEIN
50
30
10
mµMOLE GSSG FORMED
0
10
20
30
0
1
2
TIME IN MINUTES

Figure 17

Linearity of the Initial Reaction Velocity with the Concentration of GSH-CoASSG Transhydrogenase

The spectrophotometric assay was used with enzyme purified by DEAE column chromatography (see text). The assay was conducted as described in Figure 13A.

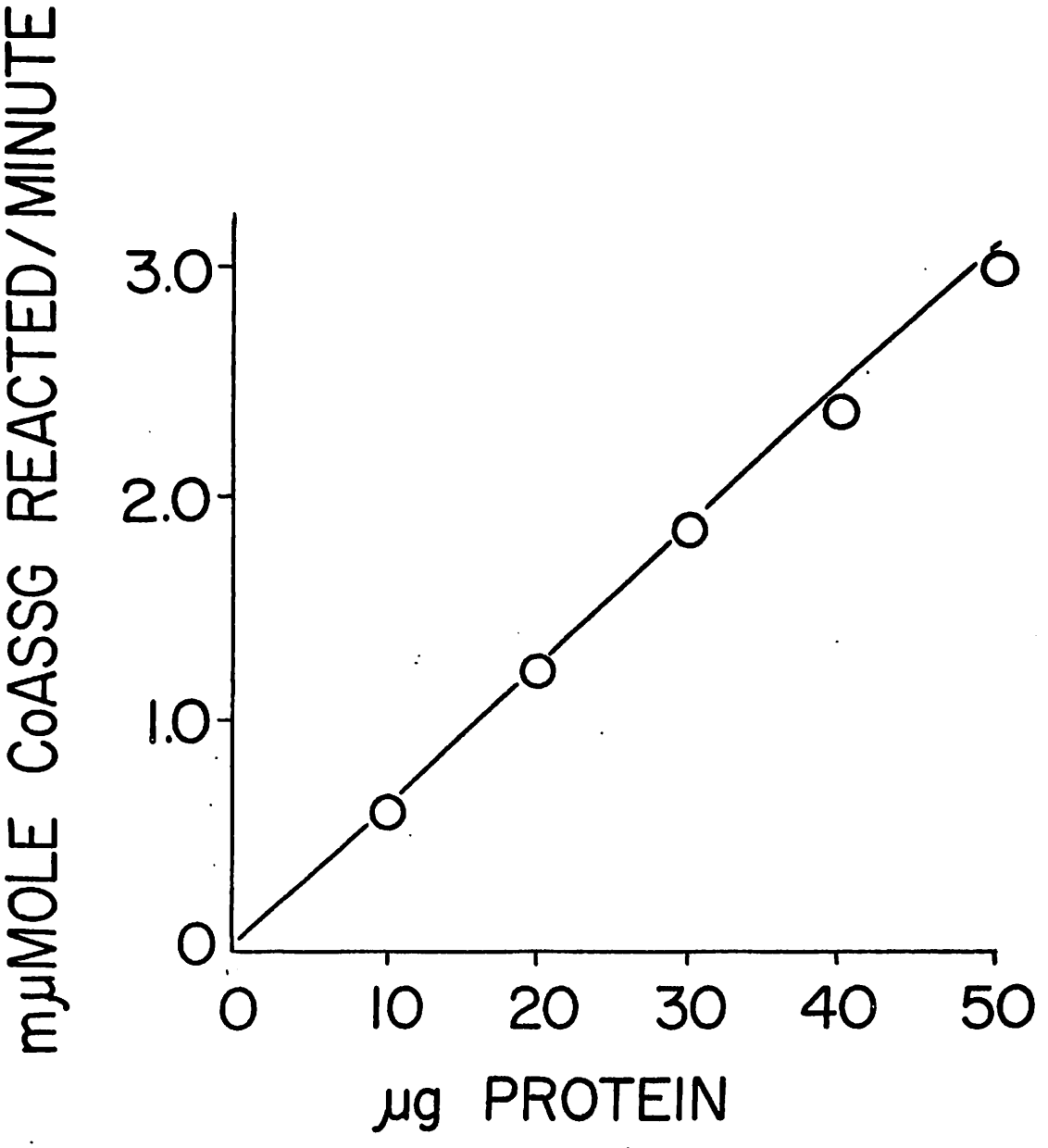
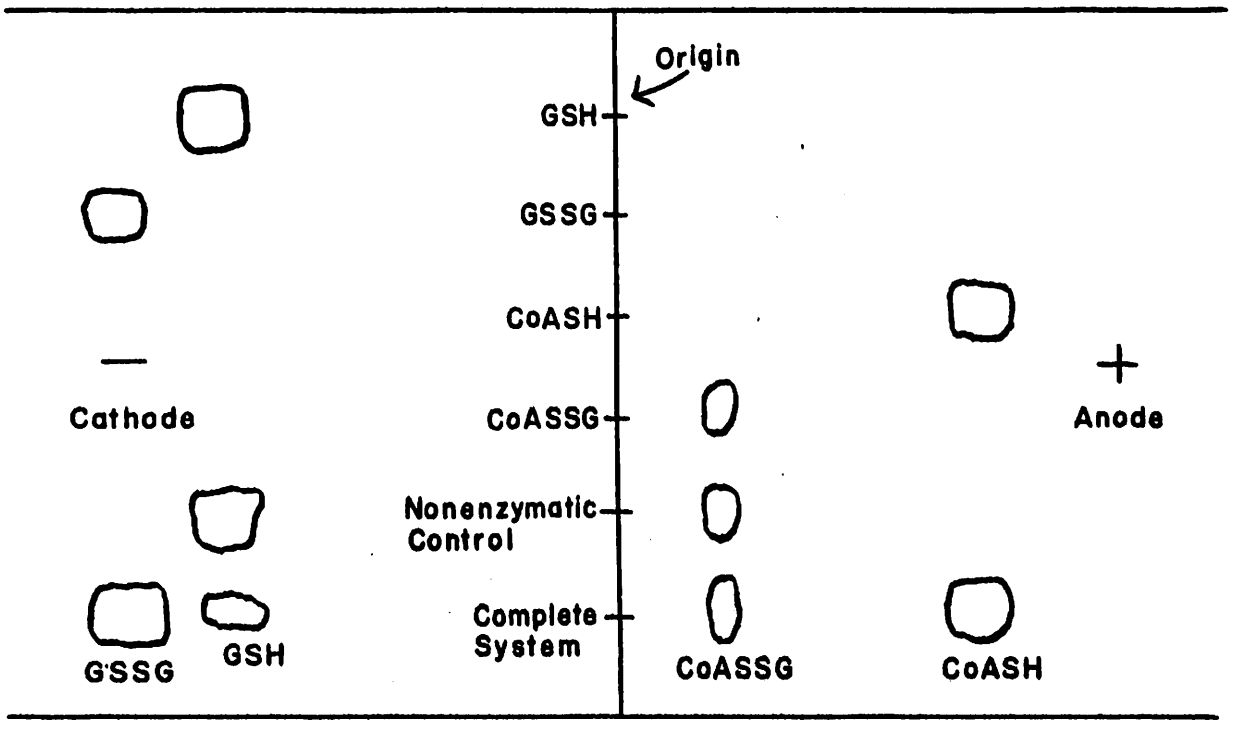

mμMOLE CoASSG REACTED/MINUTE
3.0
2.0
1.0
0
0
10
20
30
40
50
μg PROTEIN

Figure 18

Electrophoretic Separation of the Products of the GSH-CoASSG Transhydrogenase Reaction

The complete reaction mixture contained 50 mμmoles of CoASSG, 250 mμmoles of GSH and 3.2×10^{-3} units of GSH-CoASSG transhydrogenase (determined by a spectrophotometric assay) in 0.05 M inorganic phosphate pH 7.0. The final volume was 15 μl. The reaction mixture was incubated at 25° for three minutes and then 20 μl of acid electrophoresis buffer (Methods) was added to stop the reaction. The mixture was separated by electrophoresis in System 1 (Methods). The nonenzymatic control contained the same components as the complete system except that no enzyme was added. The mobility of the reactants (nonenzymatic control) and products (complete reaction mixture) are shown as well as the mobilities of standard CoA, CoASSG, GSH and GSSG.


Origin
GSH
GSSG
CoASH
CoASSG
Nonenzymatic Control
Complete System
Cathode
Anode
GSSG
GSH
CoASSG
CoASH

coenzyme A and oxidized glutathione were detected.

Stoichiometry

Figure 14 shows the disappearance of CoASSG and the appearance of an equivalent amount of coenzyme A as a function of time in an electrophoretic assay. In addition it verifies the earlier conclusion that the nonenzymic reaction between GSH and CoASSG is slow compared to the enzymatic reaction. Since one mole of GSSG (Table II) and one mole of CoA is formed per mole of CoASSG cleaved, the data suggest that equal amounts of reactants react to form equal amounts of products.

Equilibrium Constant

The final concentrations of the reactants and products of an incubation mixture at equilibrium have not been determined. The equilibrium constant for the reaction can be estimated, however, from the data obtained from the reaction mixtures as shown in Figure 14 in conjunction with the assumption that one to one stoichiometry occurs in the reaction. After correcting the data for background absorbance eluted from the electrophoresis paper, it was calculated that nearly 80 percent of the CoASSG was consumed in the reaction mixture described in Figure 14. The estimated equilibrium constant at pH 6.9 and 25°, therefore, is approximately unity.

Reversibility

Although the reversibility of the enzyme catalyzed reaction has not been studied in detail reversibility has been demonstrated.

Figure 19 shows the disappearance of coenzyme A and appearance of CoASSG during the incubation of CoA and GSSG in the presence of enzyme. Little or no nonenzymatic reaction occurred in the same time period. The experiment was conducted in a manner similar to the usual electrophoretic assay except that CoA (0.76 μmoles) and GSSG (1 μmole) were substituted for CoASSG and GSH, respectively. The incubation was conducted at pH 6.7 and 25° in the presence of 35×10^{-3} units of enzyme as determined by the spectrophotometric assay.

Specificity

The DEAE purified bovine kidney GSH-CoASSG transhydrogenase preparation was found to form GSSG when several symmetrical or unsymmetrical disulfides were substituted for CoASSG. In these experiments, 0.1 micromole of several disulfides were tested in the spectrophotometric assay system, and the results were compared to those obtained with 0.1 micromole of CoASSG. The results showed that CoASSG and most of the disulfides having a glutathione moiety are the best substrates (Table VII). Although it was shown in experiment 2 that TSSG and PSSG gave a slightly higher initial rate than CoASSG, the actual assay showed that when these two latter disulfides were tested curvature toward a slower reaction rate was observed sooner than when CoASSG was used as substrate.

In order to find out whether the activity observed with other disulfides was due to a single enzyme or due to several enzymes with similar catalytic activities a heat denaturation experiment was performed. A DEAE purified enzyme preparation was heated at 80° for different lengths of time and the heated enzyme was assayed spectrophoto-

Figure 19

Reversibility of the GSH-CoASSG Transhydrogenase Catalyzed Reaction

The reaction was followed by using a modified electrophoretic assay as described in the text. The appearance of CoASSG (○) or disappearance of CoASH (◒) in the presence of enzyme are shown as a function of time. A nonenzymatic CoASH disappearance (●) is also shown.

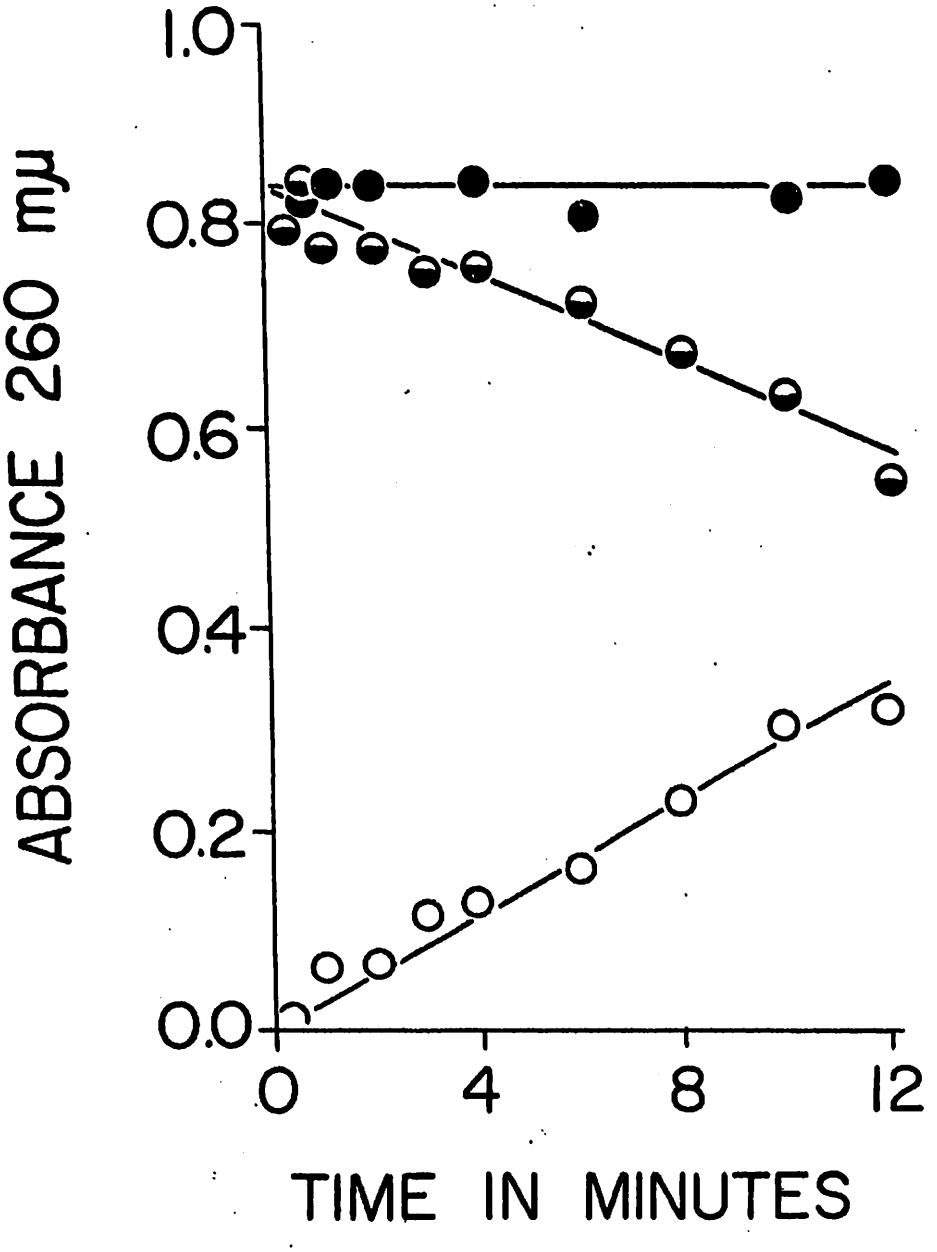

ABSORBANCE 260 mμ
1.0
0.8
0.6
0.4
0.2
0.0
0
4
8
12
TIME IN MINUTES

TABLE VII

GSH-CoASSG TRANSHYDROGENASE DISULFIDE SPECIFICITY

Disulfides[a] tested	Enzyme sources	
	Purified enzyme	Crude kidney extract
	percent activity	
Experiment 1[b]		
CoASSG	100	100
Cystine	54	14
Homocystine	16	15
Lipoic acid	0	5
Pantothine	18	16
Lysozyme	---	8
Insulin	1	1
RNase	3	---
Experiment 2[b]		
CoASSG	100	---
PSSG	115	---
TSSG	105	---
Cystine	64	---
Homocystine	11	---
CoASSC	27	---
Pantothine	13	---
CoASSCoA	2.5	---
Experiment 3[b]		
CoASSG	100	---

TABLE VII (CONTINUED)

Disulfides[a] tested	Enzyme sources	
	Purified enzyme	Crude kidney extract
	percent activity	
CSSG	98	---
HSSG	9	---
CSST	19	---

[a]The change in absorbance per minute at 340 mμ observed with CoASSG was 0.087 in experiment 1, 0.157 in experiment 2, and 0.143 in experiment 3. In the remaining assays, the indicated disulfide was substituted for CoASSG in an otherwise standard spectrophotometric assay as described in the text. Each disulfide was tested at a final concentration of 10^{-4} M. A dash (---) indicates not assayed. See text for a further discussion of the activities found with TSSG and PSSG. See page 1 for a list of abbreviations of the compounds found in this table.

[b]Three different preparations of DEAE-purified GSH-CoASSG transhydrogenase were used in experiments 1, 2, and 3, respectively.

metrically using different disulfides as substrate. Figure 20 shows the results expressed as the percent of the initial activity remaining after heating the enzyme for different lengths of time. The results show that of those disulfides tested the same rate of deactivation of the enzyme was obtained when the residual activity obtained with those unsymmetrical disulfides containing a glutathione moiety (and also PSSP) were compared to the deactivation rate with CoASSG. The deactivation rates obtained with CSSC and HSSH were different from that obtained with CoASSG as substrate. These results indicate that in this enzyme preparation the activity obtained with PSSP and those disulfides which contain a glutathione moiety may be due to a single enzyme. The activity obtained with cystine and homocystine may be due to contaminating enzymes in the GSH-CoASSG transhydrogenase preparation.

^{35}S GSH-GSSG Exchange Reaction Catalyzed by GSH-CoASSG Transhydrogenase

Glutathione labeled with ^{35}S (0.25 µmole) was mixed with 0.25 µmole of unlabeled GSSG in the presence of 0.01 units of GSH:CoASSG transhydrogenase and 10 µmoles of phosphate buffer. The final volume was 0.1 ml and the pH was 7.0. The mixture was incubated at 25° C for 30 minutes. Twenty µl of the acid electrophoresis buffer (System 1, see Methods) was added. The acidified reaction mixture was subjected to electrophoresis in System 1 on Whatman 3 MM paper. The distribution of isotope between GSH and GSSG was detected by a strip counter and compared to the distribution of isotope observed in similar experiments conducted at pH 7.0 or pH 2.0 except that no GSH-CoASSG transhydrogenase was added. Figure 21 depicts the qualitative results. No isotope redistribution was observed when ^{35}S GSH and GSSG were incubated at

Figure 20

Percent Enzyme Activity Remaining with Different Substrates for GSH-CoASSG Transhydrogenase after Partial Heat Deactivation

The enzyme was heated at 80° for the time period indicated. Twenty ug of the heated enzyme were assayed spectrophotometrically using CoASSG, (○); TSSG, (◐); PSSG, (◑); pantothine, (●); cystine, (◒) or homocystine (◓) as substrate. Abbreviations of the unsymmetrical disulfides are given on page 1.

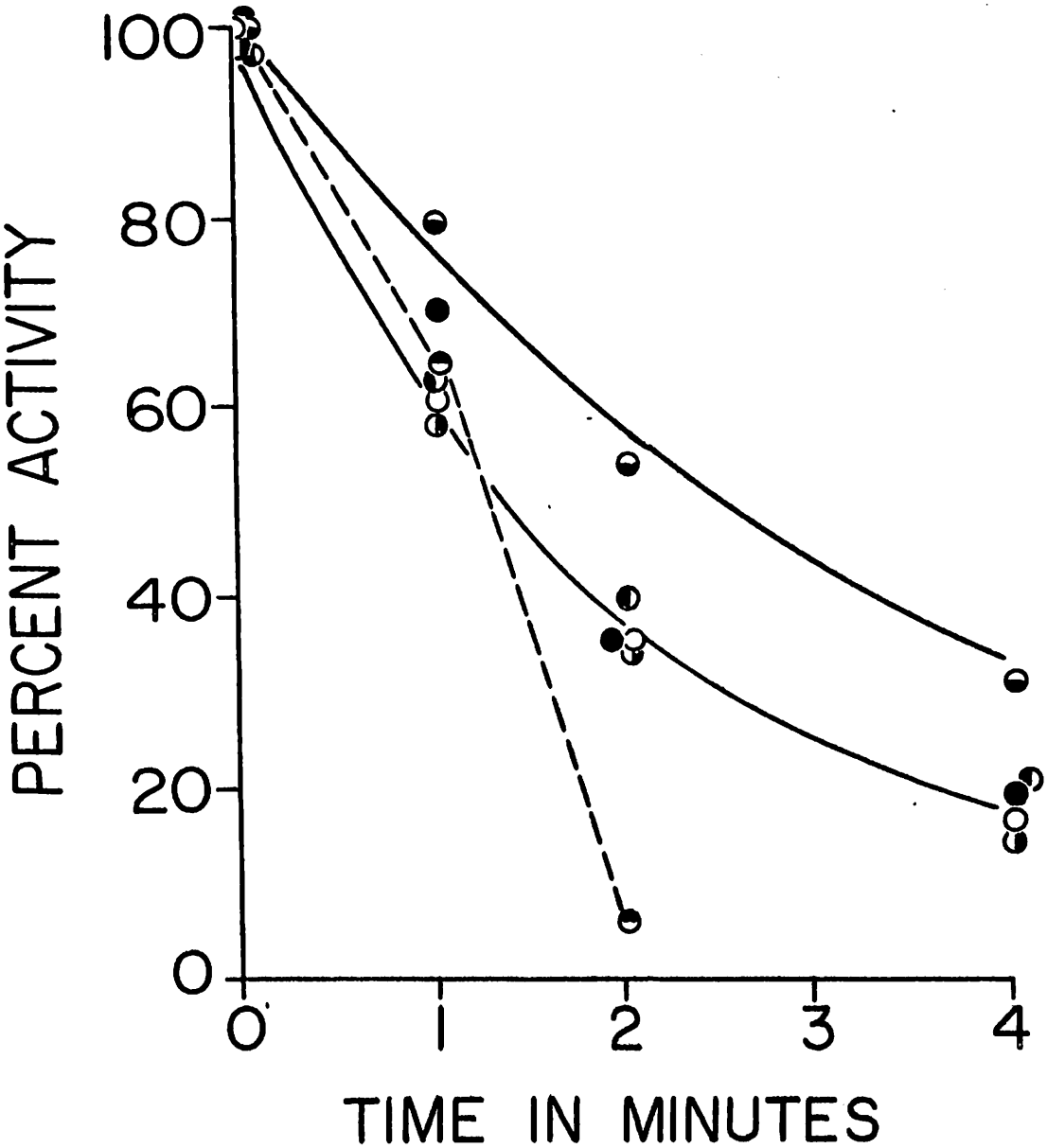
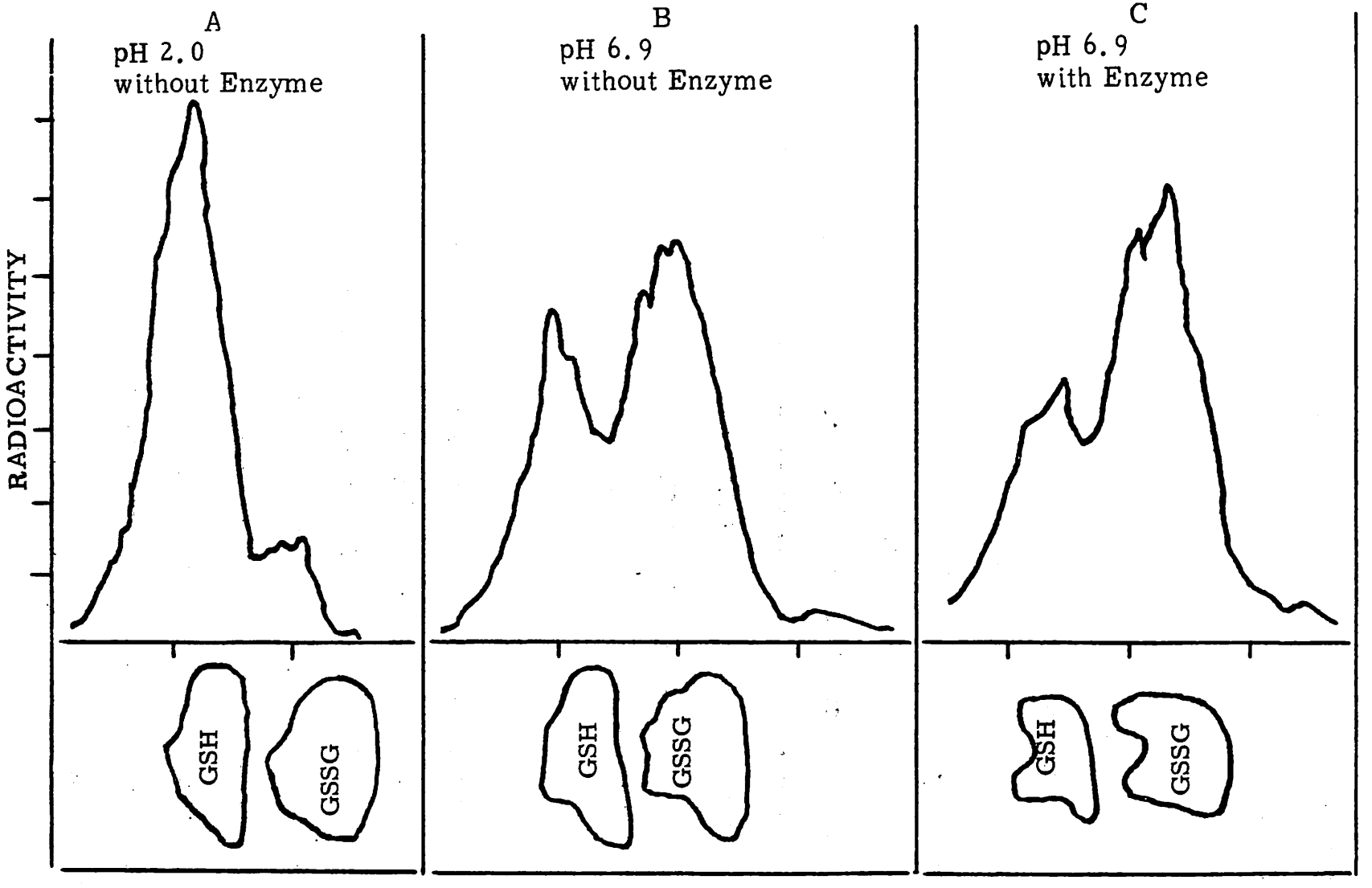

100
80
60
40
20
0
PERCENT ACTIVITY
0
1
2
3
4
TIME IN MINUTES

Figure 21

The Distribution of ^{35}S Between GSH and GSSG in the GSH-GSSG Exchange Reaction Catalyzed by GSH-CoASSG Transhydrogenase

The reaction was started with equal amounts of ^{35}S GSH and unlabeled GSSG. A and B show results of nonenzymatic experiments. Results shown in C were obtained from an enzymatic experiment. The reactions were conducted at the indicated pH value. Other experimental details are presented in the text.


A
pH 2.0
without Enzyme
B
pH 6.9
without Enzyme
C
pH 6.9
with Enzyme
RADIOACTIVITY
GSH
GSSG
GSH
GSSG
GSH
GSSG

pH 2.0 and about equal radioactivity was distributed in both forms of the glutathione in a nonenzymatic experiment at neutral pH. In contrast, a larger portion of radioactivity was found in GSSG when ^{35}S GSH and GSSG were incubated in the presence of the enzyme at neutral pH.

The experiment described above was followed by a more quantitative experiment. Equal amounts of ^{35}S GSH and unlabeled GSSG were incubated as described in the legend of Figure 22. Glutathione and GSSG were separated after varying times of reaction by electrophoresis in System 1. The separated compounds were detected by ninhydrin reagent A. The paper containing the compounds was cut out and placed in counting vials and the purple color was decolorized as described in "Methods." Ten ml of scintillation fluid (Methods) was added to each vial and counted in a liquid scintillation counter (Packard 314 A). Figure 22 shows that in the first minute the GSH-CoASSG transhydrogenase accelerated the initial rate of isotope exchange between GSH and GSSG approximately 3.6 times compared to a nonenzymatic control experiment.

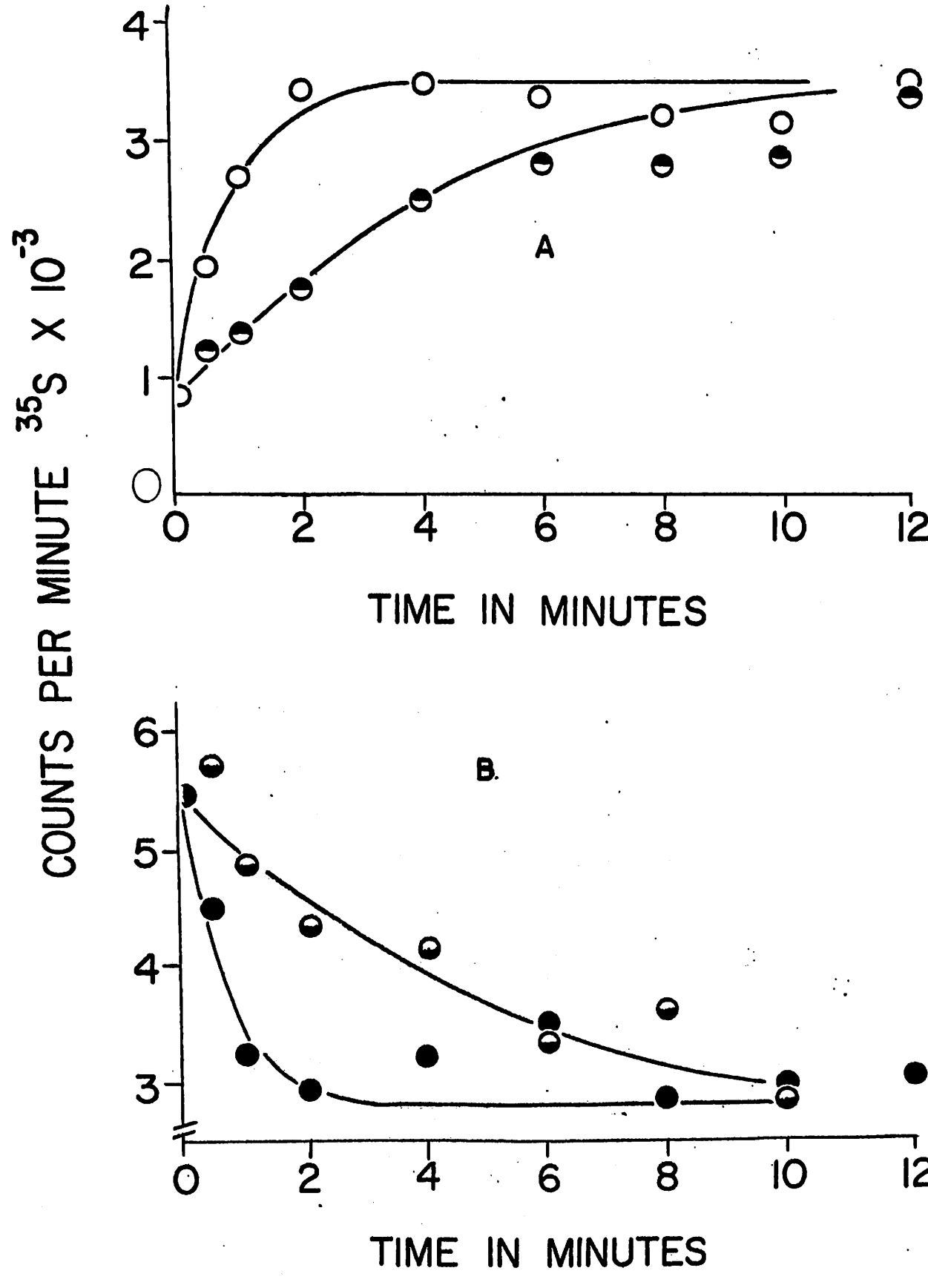
pH Optimum

The standard electrophoretic assay was used in the studies of the pH optimum of GSH-CoASSG transhydrogenase because a wider range of pH could be more readily studied than if the spectrophotometric assay system were used. The pH value of the reaction mixture was adjusted with 1 M KOH and measured with a Coleman micro electrode (Coleman 3-611, 216). The reaction was stopped after 10, 20 and 30 seconds. Aliquots of each of the acidified reaction mixtures were separated by electrophoresis in System 1 (Methods). The CoASSG spots were cut from the paper and eluted in one ml of H_2O. The 260 mμ absorbance from each

Figure 22

Time Course of the Isotope Exchange Between ^{35}S GSH and GSSG Catalyzed by GSH-CoASSG Transhydrogenase

The reaction mixture contained 10 micromoles of potassium phosphate buffer pH 7.6, 1.5 micromoles of unlabeled GSSG, 1.5 micromoles of ^{35}S GSH (containing 0.021 microcuries per micromole) and 7.4×10^{-3} units of DEAE-column purified GSH-CoASSG transhydrogenase in a final volume of 0.1 ml. At various time intervals 10 microliter aliquots of enzymatic (○ or ●) or nonenzymatic (◒ or ◒) incubation mixtures were added to 20 microliters of System 1 electrophoresis buffer (Methods) to stop further sulfhydryl-disulfide interchange from occurring. The GSH and GSSG were separated by electrophoresis and counted. ^{35}S GSSG formation is indicated in Figure 22A and ^{35}S GSH disappearance is indicated in Figure 22B. Additional details are presented in the text.


COUNTS PER MINUTE ^{35}S X 10^{-3}
A
TIME IN MINUTES
B.
TIME IN MINUTES

spot was measured.

Figure 23A shows the enzymatic and the nonenzymatic plus enzymatic rate of disappearance of CoASSG at various pH values. Figure 23B shows the activity as a function of pH constructed from the data in Figure 23A. In Figure 23B the pH curve for the enzymatic reaction has been corrected for any nonenzymatic contribution. The nonenzymatic reaction does not proceed rapidly at a pH value of 7.6 or below; however, it is rapid at pH 8.2 and above. Although the pH optimum of the enzyme catalyzed reaction is about 8.2 a significant amount of activity remains at lower pH values where little or no nonenzymatic interchange occurs.

Molecular Weight

Whitaker (227) reported that an excellent linear correlation between the logarithm of molecular weight of a protein and the ratio of its elution volume, V, to the void volume V_o of the column was found for Sephadex G-100. This technique was adopted for the determination of the molecular weight of glutathione-CoASSG transhydrogenase.

Sephadex G-100 beads were allowed to thoroughly swell in 0.1 M potassium phosphate pH 7.6 containing 0.001 M EDTA for several days in the cold room. The fine particles of the beads were removed by decantation three times. The beads were packed in a column (1.35 x 100 cm) with 0.1 M potassium phosphate pH 7.6. The column was washed by the same buffer for three more days in the cold under a constant hydrostatic pressure on top of the column so that the whole column could be uniformly packed without further shrinking or swelling.

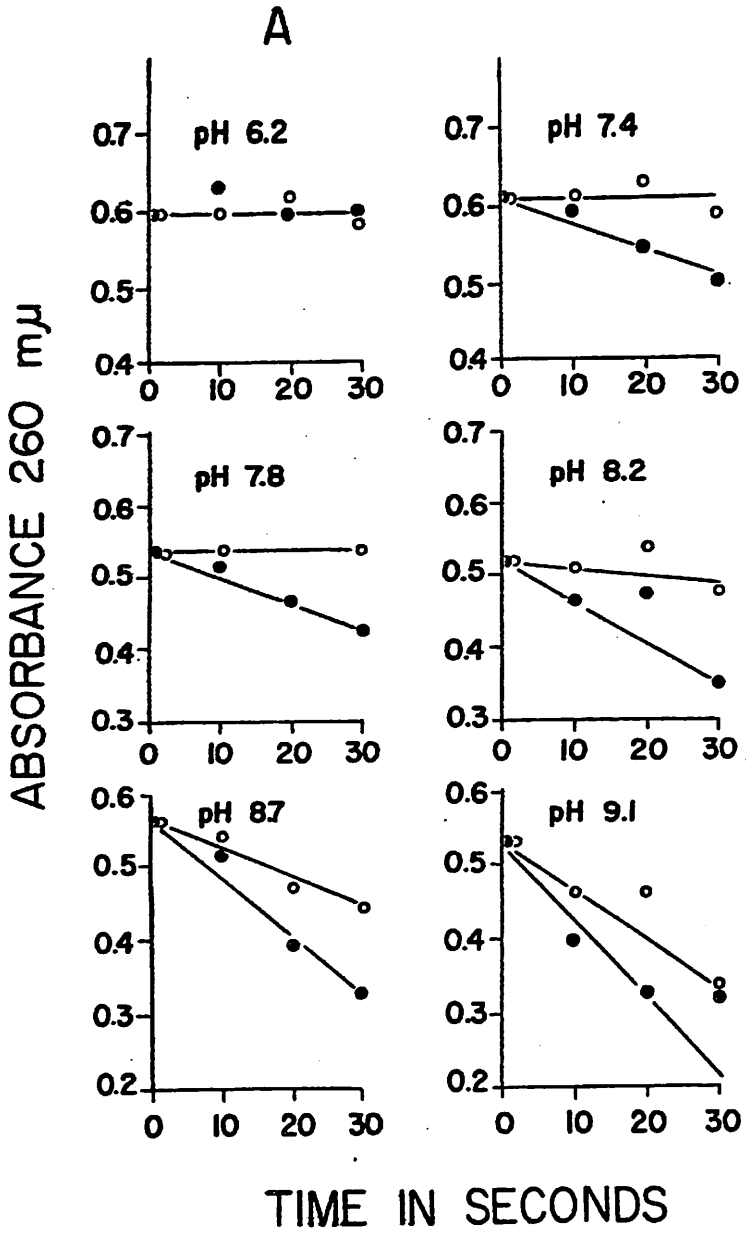
Five mg each of cytochrome c, egg albumin and bovine serum albumin were dissolved in 0.5 ml of 0.2 M potassium phosphate buffer

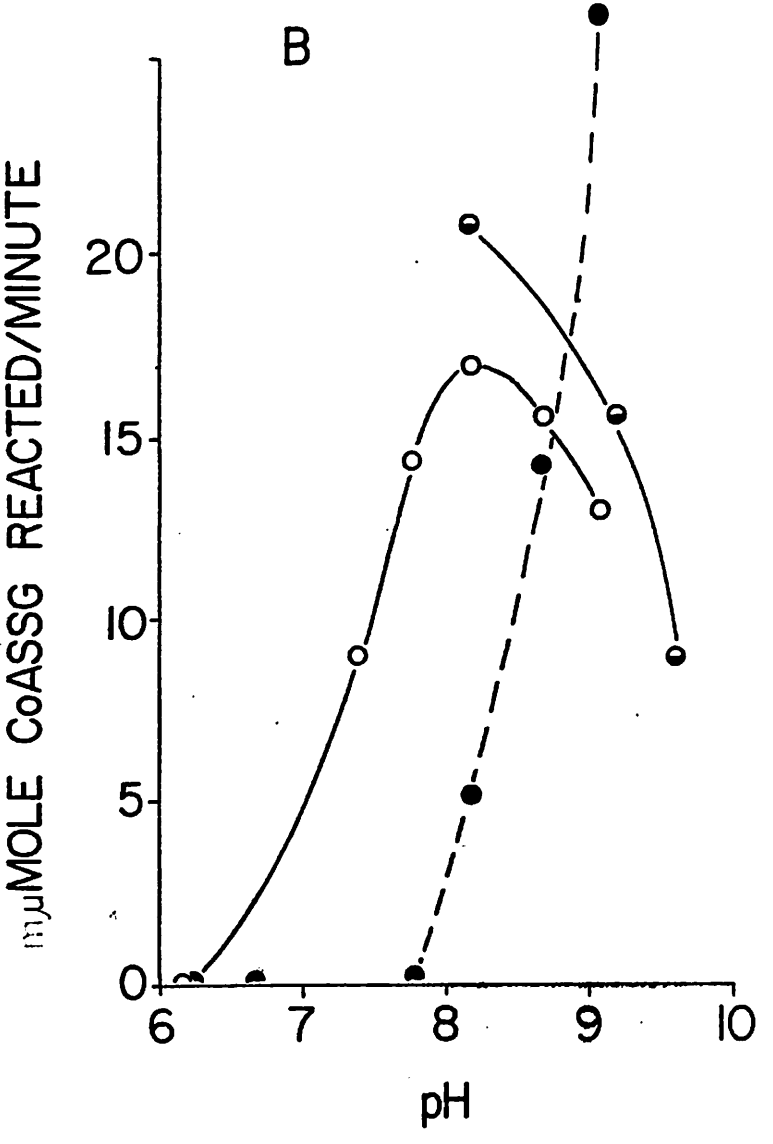
Figure 23

Effect of pH on the Rate of the Nonenzymatic and the GSH-CoASSG Transhydrogenase Catalyzed Cleavage of CoASSG

A) The nonenzymatic reaction (○) and the reaction in the presence of enzyme (●) were assayed using the electrophoretic assay described in Table II.

B) The initial reaction rates obtained in part A have been converted to mμmoles of CoASSG disappearing per minute and plotted versus pH. The rate obtained in the absence of enzyme was subtracted from the rate obtained in the presence of enzyme to obtain the net rate due to the presence of enzyme. The nonenzymatic pH curve is shown as closed circles (●) and the enzymatic pH curve is shown as open circles (○). The enzymatic pH curve shown as a mixed character (◒) was obtained in a separate experiment not shown in part A.


A
pH 6.2
pH 7.4
pH 7.8
pH 8.2
pH 8.7
pH 9.1
ABSORBANCE 260 mμ
TIME IN SECONDS


B
mμMOLE CoASSG REACTED/MINUTE
pH

pH 7.6 which contained 0.0178 units of DEAE purified GSH-CoASSG transhydrogenase. Then, 0.5 ml of concentrated catalase solution (10 ml of one mg/ml catalase was concentrated to one ml using Biodriex, then centrifuged) was added to the mixture. The mixture was applied to the column and was eluted with 0.1 M potassium phosphate buffer pH 7.6. One ml fractions were collected. The position of elution of each of the proteins was detected by measuring the absorbance of each fraction at 280 mμ. Figure 24A shows the elution pattern obtained. The GSH-CoASSG transhydrogenase activity was detected by the spectrophotometric assay and was found mainly together with cytochrome c. Figure 24B shows the linear correlation between the ratio V/V_o and the logarithm of the molecular weight of the proteins used as standards. Using these data as a standard curve and the measured V/V_o for GSH-CoASSG transhydrogenase, the molecular weight of this enzyme was estimated to be 12,300.

It is known that metal ions particularly those of iron and copper catalyze the oxidation of sulfhydryl groups (72). The close association of the GSH-CoASSG transhydrogenase activity with cytochrome c in the Sephadex G-100 experiment suggested the possibility that the porphyrin iron in cytochrome c might catalyze the sulfhydryl-disulfide interchange between GSH and CoASSG. This possibility was tested by using the standard spectrophotometric assay except that the transhydrogenase was replaced by cytochrome c or reduced cytochrome c. A cytochrome c solution was adjusted to the same concentration as the GSH-CoASSG transhydrogenase according to 280 mμ absorption measurements and assayed spectrophotometrically. No transhydrogenase activity was found. When the same amount of cytochrome c was reduced by ascorbic

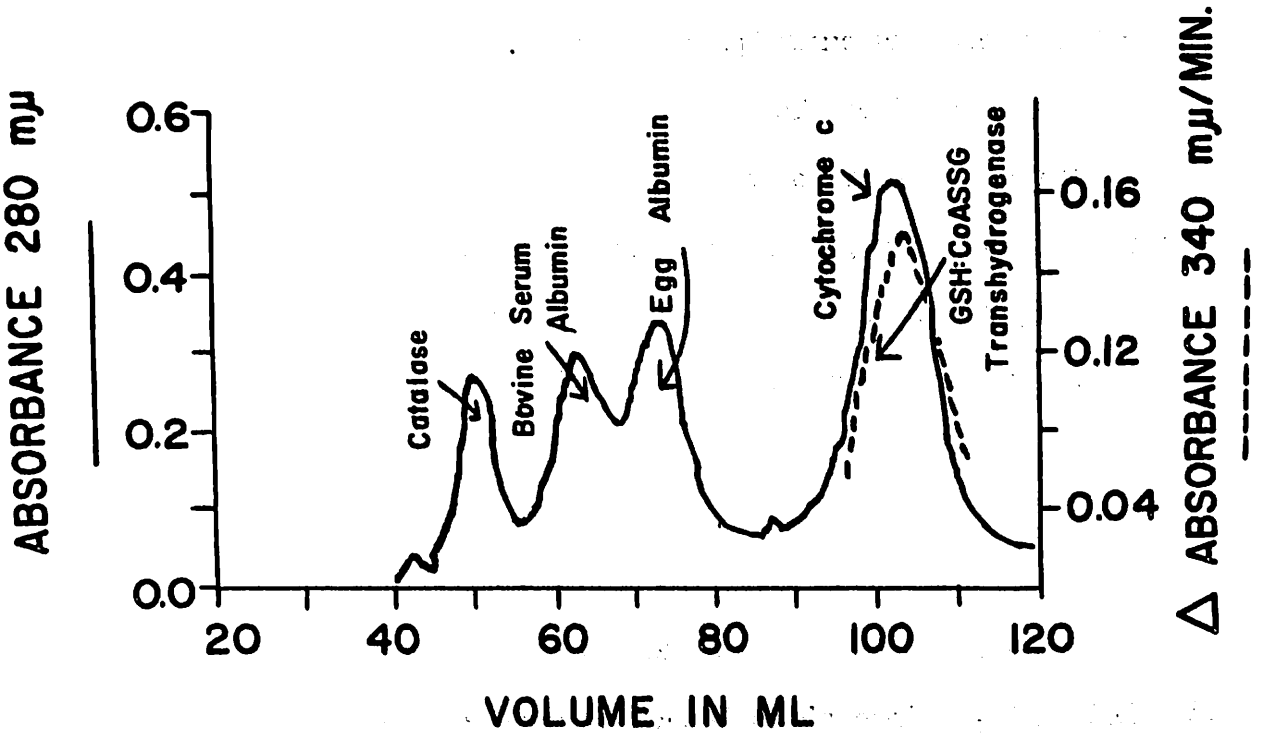
Figure 24

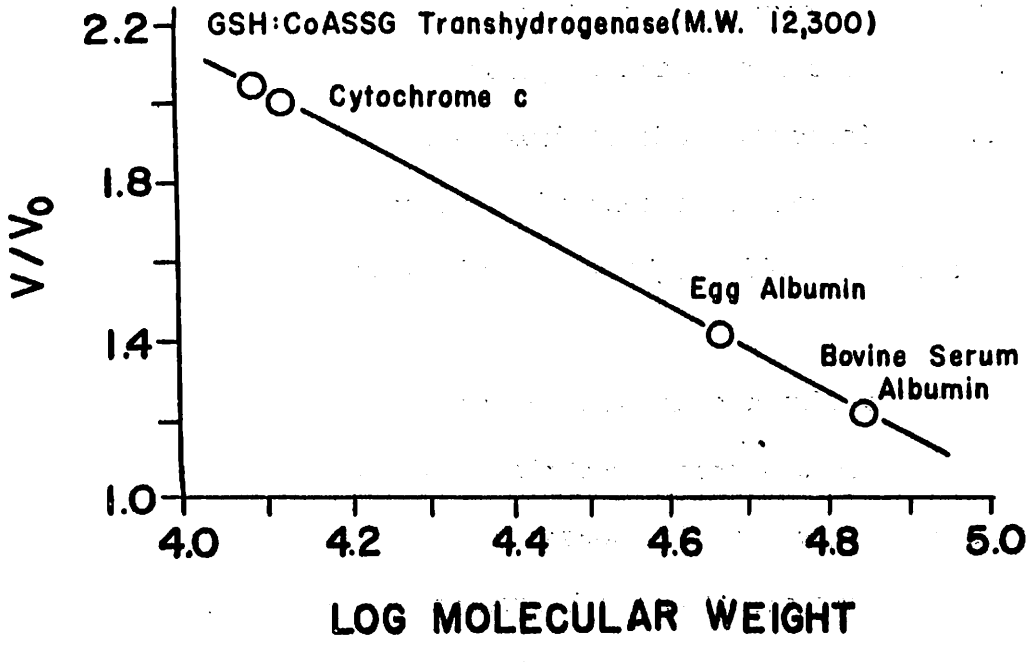
A) Chromatography of GSH-CoASSG Transhydrogenase and Other Proteins of Known Molecular Weight on Sephadex G-100

The GSH-CoASSG transhydrogenase sample was a lyophilized, DEAE-purified preparation (see text). Fractions containing protein shown by the solid curve were detected by their 280 mμ absorbance. GSH-CoASSG transhydrogenase activity shown by the broken line curve was detected using the standard spectrophotometric assay.

B) Determination of Molecular Weight of GSH-CoASSG Transhydrogenase

The ratio of the protein elution volume to the column void volume (V/V_o) obtained from the data in Figure 24A is plotted against the logarithm of the molecular weight of known proteins. The void volume, V_o, was assumed to be equal to the elution volume of the peak tube in the elution of catalase.


ABSORBANCE 280 mμ
Δ ABSORBANCE 340 mμ/MIN.
0.6
0.4
0.2
0.0
0.16
0.12
0.04
20
40
60
80
100
120
VOLUME IN ML
Catalase
Bovine Serum Albumin
Egg Albumin
Cytochrome c
GSH:CoASSG Transhydrogenase


GSH:CoASSG Transhydrogenase(M.W. 12,300)
Cytochrome c
Egg Albumin
Bovine Serum Albumin
V/V0
2.2
1.8
1.4
1.0
4.0
4.2
4.4
4.6
4.8
5.0
LOG MOLECULAR WEIGHT

acid and assayed no transhydrogenase activity was observed. Ascorbic acid itself was tested in a standard spectrophotometric assay and was found not to effect the catalytic activity of the transhydrogenase. Therefore, it may be concluded that neither cytochrome c nor reduced cytochrome c has any any transhydrogenase activity, and the association of the transhydrogenase activity with cytochrome c in the Sephadex G-100 chromatogram is simply due to the similarity of the molecular weight of these two proteins.

Michaelis Constants

Since the reaction catalyzed by GSH-CoASSG transhydrogenase requires two substrates, the graphic method of determining the Michaelis constants in two substrate systems reported by Florini and Vestling (228) was used. Theoretically, a reciprocal plot of 1/V against 1/[GSH] at a constant CoASSG concentration gives a straight line intersecting the vertical axis at $1/V_m$ for that CoASSG concentration. A series of values of $1/V_m$ is obtained by using different concentration of CoASSG. These $1/V_m$ values are replotted against the reciprocal of the CoASSG concentration at which they were obtained resulting in a straight line which intersects the abcissa at $-1/K_m$CoASSG. A corresponding procedure gives $-1/K_m$GSH.

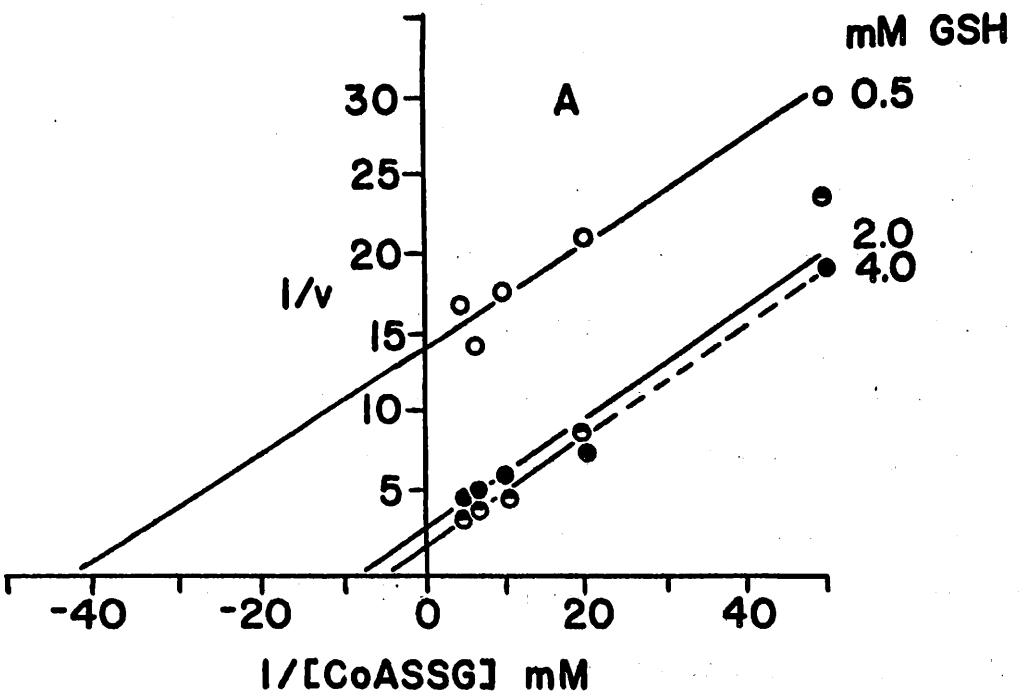
The change in initial reaction rate as a result of varing the GSH concentration was determined at different constant concentrations of CoASSG and vice versa. The spectrophotometric assay was used in these experiments. The first experiment following this procedure gave the results shown in Figure 25A and 25B. Figure 25A appeared to indicate that increasing the concentration of GSH increased the maximum velocity

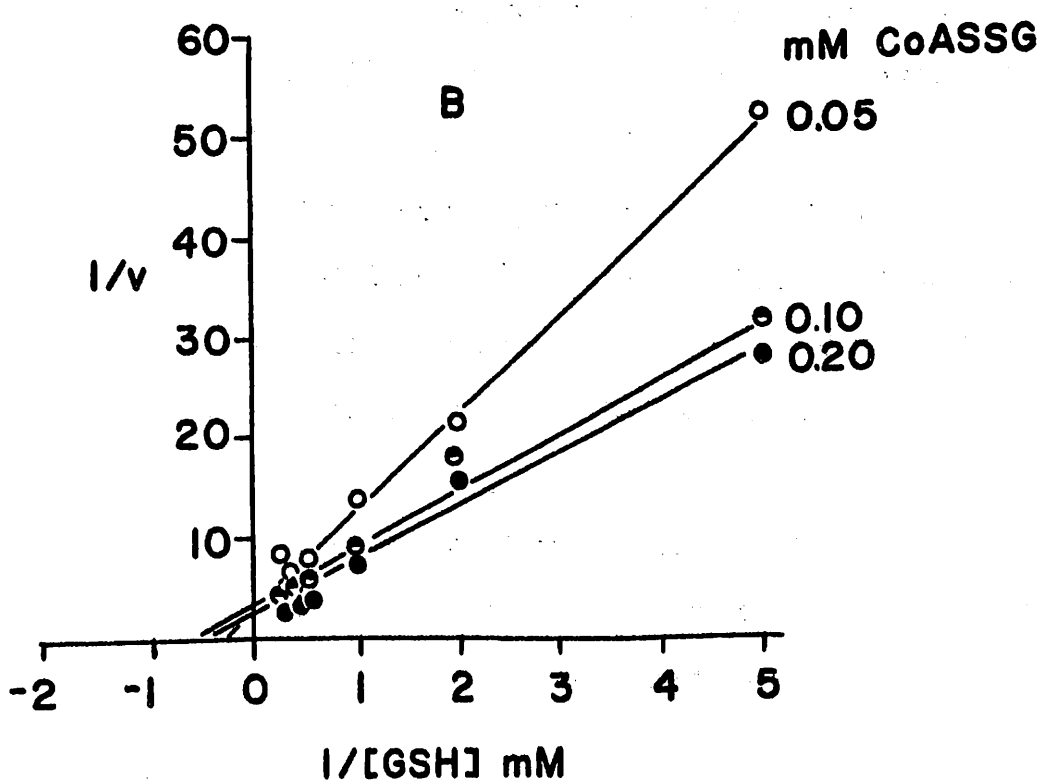
Figure 25

Lineweaver-Burk Plots for the Two Substrates GSH and CoASSG of the GSH-CoASSG Transhydrogenase Catalyzed Reaction

A) A plot of the reciprocal velocity versus the reciprocal CoASSG concentration at different constant GSH concentrations is shown. The GSH concentrations used were 0.5 mM, (O); 2.0 mM, (●); and 4.0 mM, (◓). The standard spectrophotometric assay was used (Figure 13B and 13C) except for varing the concentration of CoASSG and GSH.

B) A plot of the reciprocal velocity versus the reciprocal GSH concentration at different constant CoASSG concentrations is shown. The CoASSG concentrations used were 0.05 mM, (O); 0.1 mM, (◓); and 0.2 mM, (●). The assay was as described for part A.


mM GSH
A
0.5
2.0
4.0
30
25
20
15
10
5
1/v
-40
-20
0
20
40
1/[CoASSG] mM


mM CoASSG
B
0.05
0.10
0.20
60
50
40
30
20
10
1/v
-2
-1
0
1
2
3
4
5
1/[GSH] mM

and increased the K_m for CoASSG. Figure 25B appeared to indicate that increasing the CoASSG concentration had no appreciable effect on the maximum velocity but decreased the K_m GSH. The effects observed in these experiments appear to have been due to activation of the enzyme by one of its substrates, GSH, as described below.

The results shown in Figure 25A suggested the possibility that GSH activates the enzyme. This possibility was tested by pretreating the enzyme with 3.7 mM GSH in 0.2 M phosphate pH 7.6 at 25° for five minutes before conducting the spectrophotometric assay. Activation by GSH was observed. The time course of the activation is shown in Figure 26. From these data, a time period of activation of five minutes was chosen for all further experiments. Table VIII shows that pretreating the enzyme with 3.7 mM GSH as in experiment 2 caused approximately a 3.6 fold activation compared to the nontreated enzyme in experiment 1. When the enzyme was pretreated without GSH no activation was observed (experiment 3). Experiment 4 shows that enzyme pretreated in 0.74 mM CoASSG was not activated. In experiment 5, GSH was pretreated in the absence of the enzyme prior to assay. No activation was observed under these conditions which indicates that the increased rate of reaction observed after activation by GSH in experiment 2 was not due to the nonenzymatic oxidation of GSH to GSSG during the pretreatment period.

The activated enzyme also gave a linear relationship between the amount of enzyme protein and the initial reaction rate. This is shown in Figure 27. The same enzyme preparation was used in experiments shown in Figure 27 and Figure 17.

The observation that the enzyme was activated by its substrate,

Figure 26

Time Course of Activation of GSH-CoASSG Transhydrogenase by GSH

Twenty micrograms of enzyme were pretreated in 4.17 mM GSH under the conditions described in the text for different time periods as indicated. The activity of the enzyme was then assayed by the spectrophotometric method (Figure 13B and 13C).

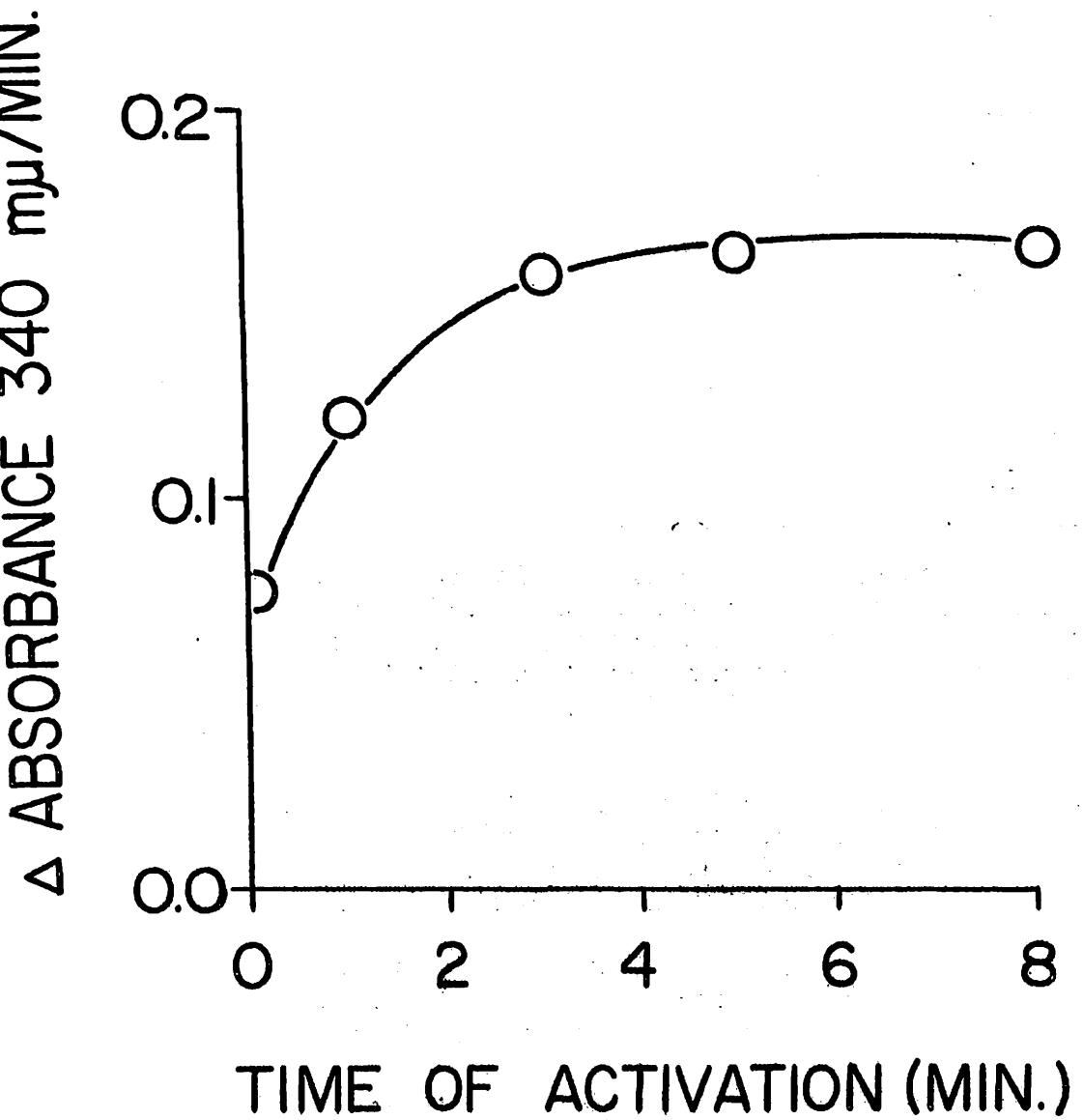

Δ ABSORBANCE 340 mμ/MIN.
0.2
0.1
0.0
0
2
4
6
8
TIME OF ACTIVATION (MIN.)

TABLE VIII

ACTIVATION OF GSH-CoASSG TRANSHYDROGENASE BY GSH

Experiment	Initial rate
	ΔA 340 mμ/min
1. Standard assay[a]	0.048
2. Enzyme pretreated with GSH[b]	0.177
Duplicate	0.180
3. Enzyme pretreated with water[c]	0.035
Duplicate	0.045
4. Enzyme pretreated with CoASSG[d]	0.040
5. GSH pretreated with water[e]	0.055

[a]The standard assay contained a final concentration of 0.1 micromole CoASSG, 0.5 micromole GSH, 20 micrograms transhydrogenase and the rest of the spectrophotometric assay reagents. For details see text and Figure 13B and 13C. In all the experiments to be described the final concentrations of reagents at the time of assay were identical to the standard assay.

[b]Forty micrograms of enzyme were mixed with 0.02 ml GSH solution (50 mM) in 0.2 M phosphate buffer pH 7.6, final volume 0.27 ml. The mixture was incubated at 25° for five minutes. An aliquot (0.135 ml) of this mixture was assayed. No additional GSH was added.

[c]Like experiment 2 except that the GSH solution was replaced by 0.02 ml of water in the premixture. An aliquot (0.135 ml) of the premixture was assayed with the addition of 0.5 micromole of GSH.

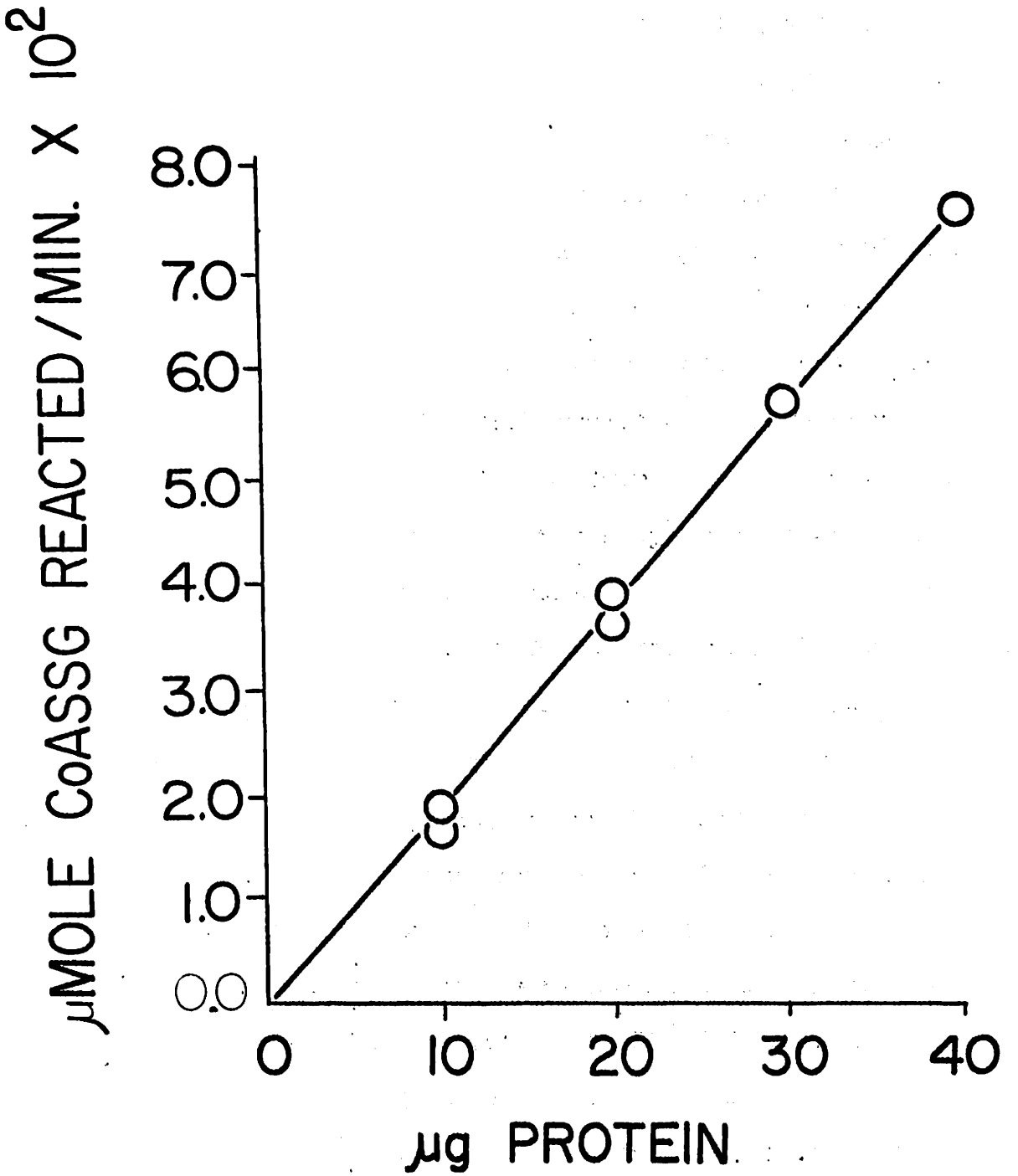
[d]Like experiment 2 except that the GSH solution was replaced by 0.02 ml of CoASSG solution (10 mM) in the premixture. An aliquot (0.135 ml) of this mixture was assayed with the addition of the required amount of GSH. No additional CoASSG was added.

[e]Like experiment 2 except that the transhydrogenase solution was replaced by 0.2 ml of water in the premixture. An aliquot (0.135 ml) of this mixture was assayed with the addition of 20 micrograms of enzyme solution which had not been pretreated.

Figure 27

Linearity of the Initial Velocity with the Concentration of GSH-CoASSG Transhydrogenase after Activation by GSH

The spectrophotometric assay (Figure 13B and 13C) was used to assay an enzyme preparation purified through the DEAE-column chromatography step (text). The amount of enzyme as indicated was pretreated with 4.17 mM of GSH at 25° for five minutes prior to assay. For details of the activation procedure see the text.


μMOLE CoASSG REACTED / MIN. X 10^2
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
0
10
20
30
40
μg PROTEIN

GSH, led to the reexamination of the K_m values of both substrates as described above after the enzyme had been activated.

The activation was performed in a manner similar to that described above except that 0.1 ml of enzyme solution (20 µg protein) was first pretreated with 4.16 mM GSH in the presence of 0.1 M potassium phosphate pH 7.6 at 25° for five minutes directly in the assay cuvette prior to assay. The remaining spectrophotometric assay reagents were added as described in Table III except that the final amount of CoASSG and GSH was varied. Additional GSH solution was added to the cuvette from a concentrated stock solution (50 mM) to make up the required final GSH concentration. CoASSG was the last reagent added to the cuvette. The Lineweaver-Burk plots of the data are shown in Figure 28A and 28B. The best fitting lines have been obtained by least squares. The series of values of $1/V_m$ at different constant GSH concentrations obtained from Figure 28A and of $1/V_m$ at different constant CoASSG concentrations obtained from Figure 28B were replotted against $1/[GSH]$ and $1/[CoASSG]$, respectively (Figure 28C). Straight lines were obtained which cut the base line at $-1/K_m$ GSH corresponding to a K_m for GSH equal to 2.29×10^{-4} M and $-1/K_m$ CoASSG corresponding to a K_m for CoASSG equal to 6.97×10^{-5} M. The two lines obtained have essentially the same ordinate intercept which corresponds to the reciprocal of the maximum velocity obtainable at infinite concentration of GSH and CoASSG. The maximum velocity obtained from Figure 28C is equal to a change in absorbance at 340 mµ/min/20 µg protein of 0.413 at 25° or 0.0665 µmoles/min/20 µg of protein. A previous section showed that the molecular weight of the enzyme is 12,300. Therefore, 20 µg protein corresponds to a minimum of 1.625×10^{-3} micromoles of protein. The minimum molecular activity of

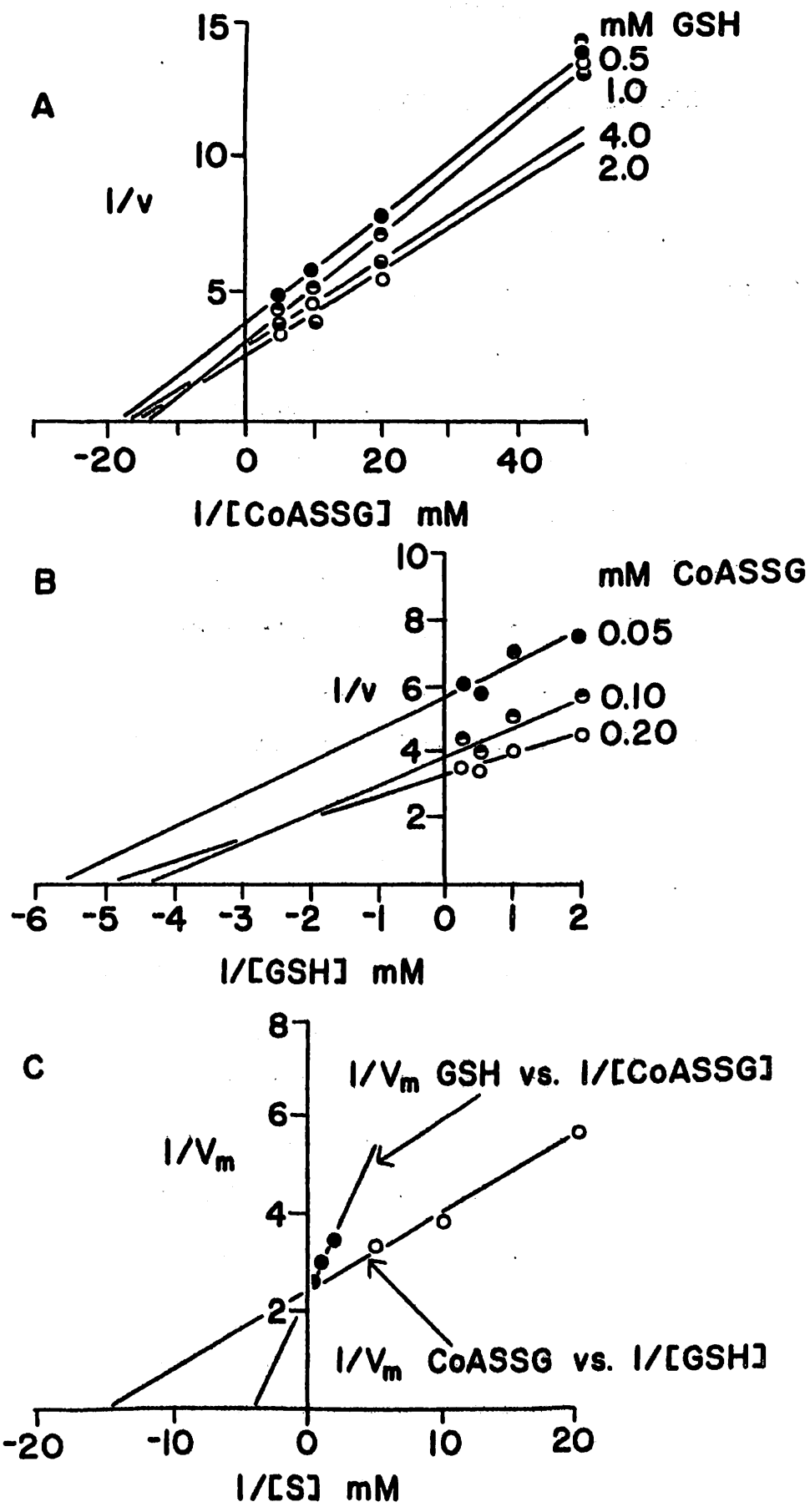
Figure 28

Determination of the K_m for GSH and CoASSG

A) A plot of the reciprocal velocity versus the reciprocal CoASSG concentration at different constant GSH concentrations is shown. The GSH concentrations used were 0.5 mM, ● ; 1.0 mM, ◒ ; 2.0 mM, ○ ; and 4.0 mM, ◔ . The assay conditions were identical to those described in Figure 26A except that the enzyme was first activated with GSH as described in the text.

B) A plot of the reciprocal velocity versus the reciprocal GSH concentration at different constant CoASSG concentrations is shown. The CoASSG concentrations used were 0.05 mM, ● ; 0.1 mM, ◒ ; and 0.2 mM, ○ . The assay was as described above in part A.

C) A plot of the reciprocal maximum velocities obtained in parts A and B above versus the reciprocal of the appropriate constant concentration of GSH or CoASSG used is shown. From these data the K_m for GSH was 2.29×10^{-4} M and the K_m for CoASSG was 6.97×10^{-5} M. The maximum velocity with saturating concentrations of both substrates was calculated to be equal to the formation of 3.3 μmoles/min/mg protein or a minimum molecular activity of 41.


A
15
10
5
1/v
mM GSH
0.5
1.0
4.0
2.0
-20
0
20
40
1/[CoASSG] mM
B
10
8
6
4
2
1/v
mM CoASSG
0.05
0.10
0.20
-6
-5
-4
-3
-2
-1
0
1
2
1/[GSH] mM
C
8
6
4
2
1/Vm
1/Vm GSH vs. 1/[CoASSG]
1/Vm CoASSG vs. 1/[GSH]
-20
-10
0
10
20
1/[S] mM

GSH-CoASSG transhydrogenase calculated from these data is 40.9.

Stability of the Purified GSH-CoASSG Transhydrogenase and Activation by GSH

The stability of the DEAE-purified enzyme to storage at -10° has been variable. One preparation was completely stable for approximately six weeks but lost 70 percent of its activity on storage for about seven months. Another preparation lost about 70 percent of its activity during six weeks storage. The enzyme is unstable to freeze-thawing and loses essentially all of its activity within five successive cycles of freezing and thawing using a dry ice-acetone bath to freeze the enzyme and a 25° water bath to thaw it. The loss of activity on freeze thawing is not regained by preincubation with GSH.

Two experiments have been conducted to find out at which step in the isolation procedure the enzyme acquired the property of being activated by GSH. The results showed that activation by GSH was not acquired during the purification procedure. This finding suggested that GSH activation occurred only in an enzyme preparation after it had been stored. Thus the enzyme preparation described above which had lost 70 percent of its activity in seven months was tested for activation by GSH. The results indicated that the enzyme could be activated to 89 percent of its original activity. With other enzyme preparation described above which lost 70 percent of its activity in six weeks only 50 percent of its original activity could be regained by activation with GSH. These results indicate that at least two types of inactivation may occur on storage of the enzyme, one which is reversible by GSH and one which is not.

CHAPTER V

DISCUSSION

The results reported in this thesis have led to the conclusion that the bovine liver nucleotide-peptide previously isolated (64) is the unsymmetrical disulfide of coenzyme A and glutathione. The present results are in agreement with those previously reported (64, 65) except for two. The first concerns the difference in results about the presence (64) or absence (65) of the unidentified ninhydrin reactive component in the acid hydrolysate of the isolated CoASSG. It was found in this research that the presence or absence of this unidentified ninhydrin reactive component in an acid hydrolysate of the isolated or synthetic CoASSG depends on the final purification procedure employed. Hydrolysates of CoASSG purified by electrophoresis in System 1 did not contain the unidentified component; however, the same preparation of CoASSG yielded the unidentified component when it was subsequently chromatographed in System 2 prior to acid hydrolysis. Thus the unidentified component previously reported (64) appears to be an artifact due to one method of purification and is not a component of CoASSG. The second difference concerns the value for the ninhydrin amino equivalents after acid hydrolysis. The previously reported value (64) appears to have been slightly overestimated. The unidentified component in the previous preparations of CoASSG presumably contributed to the number of amino equivalents determined. In addition, in contrast to these studies, in

the earlier studies no corrections were made for small amounts of ninhydrin-reactive materials liberated from the adenine moiety of the compound during acid hydrolysis.

CoASSG and other disulfide derivatives of coenzyme A previously have been observed in coenzyme A preparations (98, 103, 107, 108) isolated from biological materials. These compounds presumably are formed as a result of direct oxidation of the appropriate sulfhydryl compounds or by a sulfhydryl-disulfide exchange reaction probably between coenzyme A and another disulfide. Whether the bovine liver CoASSG was originally present in the tissue or was formed during some phase of its isolation is, therefore, open to question. CoASSG might arise in the cell, however, by a direct oxidation between free CoA and GSH. Although most of the glutathione found in most cells is primarily in the reduced form, small amounts of GSSG have been reported to occur (cited in 229). Thus, it is also possible that CoASSG might be formed by a sulfhydryl-disulfide interchange between CoA and GSSG.

In the study of the metabolism of CoASSG, a bovine kidney enzyme, GSH-CoASSG transhydrogenase, which catalyzes the sulfhydryl-disulfide exchange reaction between this disulfide and GSH with the formation CoASH and GSSG, was observed. This enzyme was found to be distributed in several rat tissues and has been partially purified from bovine kidney. Although the enzyme has been purified 184 fold from bovine kidney the best preparations still catalyze the sulfhydryl-disulfide exchange reaction between GSH and several other disulfides. Black (179) has reviewed the subject of GSH transhydrogenases and has pointed out that it is not known whether there are several enzymes which catalyze a sulfhydryl-disulfide exchange between GSH and a particular disulfide or

whether a single enzyme lacking a high degree of specificity catalyzes the observed reactions. For instance, Racker found that at a suitable concentration, cystine also serves as a substrate of partially purified GSH-homocystine transhydrogenase (179). More recently, Tietze and Katzen (197) found that an electrophoretically homogeneous preparation of GSH-insulin transhydrogenase also accelerates the rate of reappearance of RNase activity following reduction of the latter enzyme in 8 M urea. In neither case has it been established whether the activities observed with two substrates were due to one or more than one enzyme. In order to find out whether the activity of GSH-CoASSG transhydrogenase on other disulfides was due to a single enzyme or due to several enzymes with similar activities, a heat denaturation experiment was performed. The results showed that the enzyme has essentially the same rate of deactivation when CoASSG or several other unsymmetrical disulfides containing a glutathione moiety were tested as substrate. The deactivation rates obtained with cystine and homocystine were different from that obtained with CoASSG as substrate. These results indicate that the activity of the best enzyme preparation on those disulfides which contain a glutathione moiety may be due to a single enzyme. The activity obtained with cystine and homocystine is probably due to contaminating enzymes in the GSH-CoASSG transhydrogenase preparation. Therefore, GSH-CoASSG transhydrogenase appears to be a different enzyme than GSH-homocystine transhydrogenase.

The DEAE purified GSH-CoASSG transhydrogenase preparation is not active on insulin and therefore appears to be a different enzyme than GSH-insulin transhydrogenase. It is interesting to note, however, that the K_m value for CoASSG using the former enzyme is 7.0×10^{-5} M is very

close to the K_m value for insulin using the beef liver GSH-insulin transhydrogenase which is 5.0×10^{-5} M.

DEAE purified GSH-CoASSG transhydrogenase has been tested at several concentrations of enzyme for its ability to substitute for or enhance the activity of the microsomal enzyme which renatures reduced RNase. The solubilized microsomal enzyme was prepared according to Goldberger et al. (205). In neither type of experiment did the GSH-CoASSG transhydrogenase cause a significant increase in RNase reactivation over that obtained in appropriate control experiments. Therefore, tentatively it may be concluded that the microsomal enzyme which renatures reduced RNase and GSH-CoASSG transhydrogenase are different enzymes.

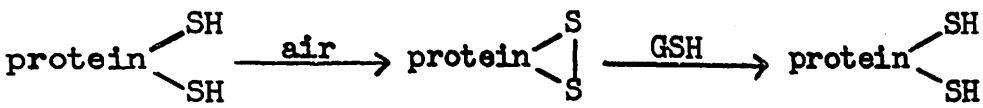
Although several enzymes which catalyze sulfhydryl-disulfide exchange reactions have been reported, their physiological function has not been established. One such enzyme which reactivates reduced RNase may function as a terminal reaction in the synthesis of RNase and perhaps other proteins by aiding in the formation of the correct tertiary structure. The physiological role of GSH-homocystine transhydrogenase and GSH-insulin transhydrogenase is uncertain. Similarly, the physiological role of GSH-CoASSG transhydrogenase remains to be established. Since the reaction is reversible, the possibility exists that it may function in the control of the level of the functional or sulfhydryl form of coenzyme A in the cell. However, most tissues contain sufficient glutathione reductase such that if the TPNH and glutathione reductase concentrations were adequate any CoASSG formed in the cell would be reconverted to coenzyme A. This raises the possibility that the function of the enzyme is to recover coenzyme A which might be converted to CoASSG either by a direct oxidation of coenzyme A and glutathione or

by a nonenzymatic sulfhydryl-disulfide exchange reaction between CoA and GSSG.

The basic problem of the necessity of the transhydrogenase type of enzyme in tissues was questioned by Pihl et al. (188). They reinvestigated the role of GSH in the mechanism of reduction of various disulfide compounds. Their results showed that a rat liver enzyme preparation containing glutathione reductase and TPNH source did not directly reduce disulfides other than GSSG. The other disulfides tested were reduced if GSH was added to the reaction mixture. They suggested that the reduction of these disulfides occurred as a result of a two-step nonenzymatic sulfhydryl-disulfide interchange reaction, which produced GSSG, coupled to glutathione reductase. Although they did not exclude the possibility of the occurrence of enzymes which might catalyze sulfhydryl-disulfide reactions, they felt that the "nonenzymatic" reaction rates between GSH and disulfides were fast enough "to make the postulation of such enzymes unnecessary." This suggestion has been criticized (179) because the experiments of Pihl et al. (188) were conducted under conditions which would not have detected an enzymatically catalyzed sulfhydryl-disulfide reaction. Thus, their conclusion that such a reaction was nonenzymatic could have been erroneous.

The results reported in this thesis with GSH and CoASSG show that the nonenzymatic exchange reaction takes place at a rapid rate only when the pH becomes relatively high. On the contrary the enzymatically catalyzed reaction occurs at a lower and more physiological pH. It even occurs at a pH value lower than that at which no nonenzymatic reaction occurs. Thus, it appears that the enzymatic reaction may have significance at physiological pH values.

GSH was found to be an activator of GSH-CoASSG transhydrogenase in some preparations of the enzyme. Recent results suggested that the DEAE purified enzyme may be subject to deactivation by aging and that this deactivated enzyme is reactivated by GSH. This may be due to the oxidation of one or more sulfhydryl groups in the protein to form an inactive disulfide form of the enzyme. During the activation by GSH the disulfide form could be reduced to an active sulfhydryl form of the enzyme. These speculations are presented in one possible schematic form in equation 51.

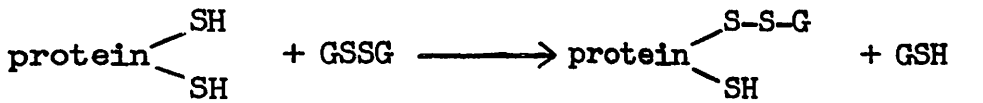


(51)

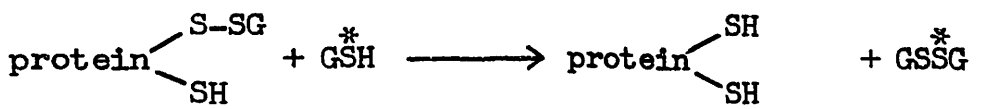
active inactive active

The protein might alternatively form an inactive disulfide dimer which can be reactivated by GSH.

A finding which bears on the enzyme mechanism was that the enzyme catalyzes an isotope exchange between ^{35}S-GSH and GSSG. Assuming a sulfhydryl form of the enzyme is the active form, the following reactions would account for the observed exchange.



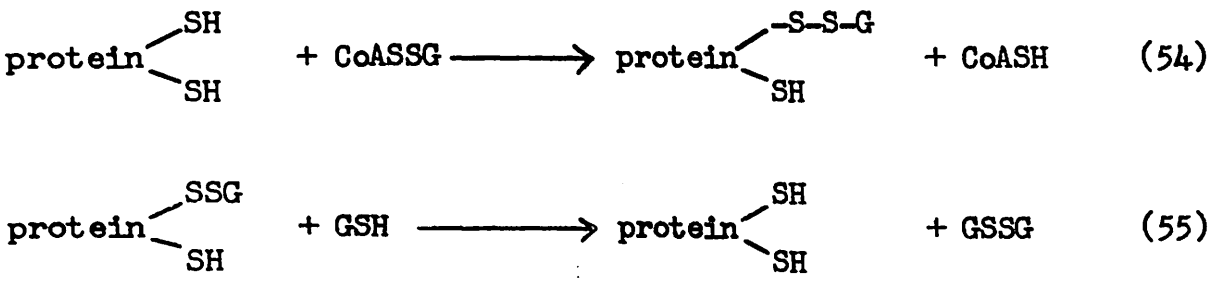
(52)



(53)

Coupling these two proposals, the mechanism of the GSH-CoASSG transhydrogenase catalyzed sulfhydryl-disulfide exchange between GSH and CoASSG

may be speculated to occur as follows:



Rall and Lehninger (187) found that liver glutathione reductase had no activity on cystine or homocystine. Pihl and Eldjarn (188) indirectly showed that several mixed disulfides containing a GSH residue were not reduced by TPNH and glutathione reductase. In the present experiments some of these observations have been confirmed and extended. Of the several symmetrical and unsymmetrical disulfides tested in Table VII only one, namely CoASSG, was found to oxidize TPNH at an appreciable rate in the presence of the glutathione reductase preparation employed. Whether this reaction is due to nonspecificity of glutathione reductase or to a small amount of a second enzyme specific for CoASSG remains to be established.

SUMMARY

The bovine liver nucleotide-peptide previously isolated and partially identified by Wilken and Hansen (70) was completely identified and was found to be an unsymmetrical disulfide of coenzyme A and glutathione (CoASSG). In the study of the metabolism of CoASSG, an enzyme which catalyzes the reaction

$$\text{CoASSG} + \text{GSH} \rightleftharpoons \text{CoASH} + \text{GSSG}$$

was detected in several tissues tested. The enzyme was purified from bovine kidney approximately 184 fold in five percent yield. Its equilibrium constant is near unity at pH 6.9 and 25°. The pH optimum of the enzyme is pH 8.2. The molecular weight of the enzyme determined by gel filtration is 12,300. The Michaelis constants of GSH and CoASSG are 2.3×10^{-4} M and 7.0×10^{-5} M, respectively. The activity of the enzyme with several disulfides other than CoASSG is discussed.

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