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STUDIES ON THE BEHAVIOR OF ALPHA-SUBSTITUTED CYSTINES IN AN ENZYMATIC REACTION AND IN AN ENZYME MODEL SYSTEM.

BY

JOHN F. G. DIEDERICH

A Dissertation

Submitted to the Faculty of Graduate Studies through the
Department of Chemistry in Partial Fulfillment
of the Requirements for the Degree of
Doctor of Philosophy at the
University of Windsor

Windsor, Ontario

1966

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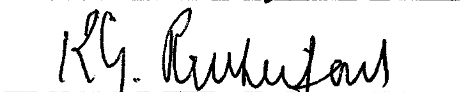

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ABSTRACT

Several new alpha-substitued cystines have been synthesized. The behavior of these compounds, α-methyl-, α-ethyl-, α-n- propyl-, α-n -butyl-, α-isopropyl-, and α-phenyl-DL-cystine, in an enzymatic system and a non-enzymatic model reaction has been studied. This problem was undertaken as a part of a general programme concerned with the effects of alpha-substitution in cystines.

The substituted cystines are cleaved neither by cystathionase (also called homoserine deaminase), nor are they degraded in an enzyme model system consisting of pyridoxal phosphate, with or without cupric ions. The alpha-hydrogen is required for reactivity. L-cystine reacts even in the absence of added metal ions, but its rate of degradation is increased by copper.

The enzyme homoserine deaminase, which has been isolated from rat liver and purified to some extent, is inhibited competitively by L-cystine and the substituted sulfur amino acids. Competition by the natural substrate, L-cystine, is greatest. The inhibitors apparently act by combining with the internal Schiff base of the enzyme, formed by the coenzyme, pyridoxal phosphate, and the apoenzyme.

In the non-enzymatic system the substituted amino acids form unreactive Schiff bases, whose absorption spectra have been determined. The rate of formation of the Schiff bases has been followed spectrophotometrically and can be used to predict the relative magnitude of the inhibition of the enzyme.

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ABBREVIATIONS

ArSSAr	5,5' - dithiobis - (2-nitrobenzoic acid)
ArSH	ArSSAr in the reduced form (aryl mercaptan)
PLP	Pyridoxal phosphate
EDTA	Ethylenediamine tetraacetic acid
DPNH	Reduced diphosphopyridine nucleotide

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CHAPTER I

INTRODUCTION

Preliminary Remarks

This study was undertaken as part of a general programme concerned with the effect of the substitution of the alpha-hydrogen of cystine by alkyl or aryl groups. For instance, the effect of this type of substitution on the polarographic reduction of cystine is being studied in this laboratory at the present time.

In general, a lack of a hydrogen atom on the carbon next to the carbonyl group tends to make an amino acid inert in many enzymatic reactions. For example, α-methyl-DL-glutamic acid did not react in dehydrogenation or transamination reactions and inhibited decarboxylation of glutamic acid (1). After α-methylalanine and α-methyl-α-amino-n-butyric acid had been administered to dogs, the amino acids were largely lost in the urine (2,3). In another study α-methyl-DL-serine did not undergo oxidative deamination in the presence of L- or D-amino oxidase (4). However, there are examples in the literature of enzymatic and non-enzymatic reactions undergone by alpha-substituted amino acids. Snell and co-workers (5,6) observed that α-methylserine, when heated in aqueous solution with pyridoxal and Cu^{++}, was degraded to alanine and other products. The amino acid was also attacked by cell-free extracts of a bacterium of the genus *Pseudomonas*; D-alanine was produced in the reaction; α-hydroxymethylserine and α-ethylserine also acted as

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substrates (6,7). Snell and his group studied as well the non-enzymatic decarboxylation of some α-methyl-substituted amino acids (8,6). They observed that α-aminoisobutyrate, in the presence of pyridoxal, lost carbon dioxide and yielded isopropylamine; this reaction was accompanied by the formation of acetone in a decarboxylation-dependent transamination reaction. Production of carbon dioxide was inhibited rather than catalysed by metal ions; α-methylserine underwent a similar set of reactions.

It has been observed that α-methyl-DL-cystine is degraded very slowly in hot aqueous alkali, compared to the unsubstituted amino acid (9). Bergel and co-workers noticed during their work on an enzyme model system for cysteine desulfhydrase that α-methyl-DL-cysteine is not acted upon by pyridoxal phosphate and vanadium salts (10). Schwimmer and Kjaer (11) reported that the C-S-lyase of Albizzia lophanta, which acts optimally on S-ethyl-L-cysteine to yield free thiol, pyruvate, and ammonia, does not use α-methyl-DL-cysteine or S-benzyl-α-methyl-DL-cysteine as substrates, though L-cysteine is degraded by the enzyme.

In this laboratory interest was developed in the effect of various substituted groups, methyl, ethyl, n-propyl, n-butyl, isopropyl, and phenyl, in enzymatic and non-enzymatic (enzyme model system) reactions. The enzyme cystathionase was chosen, because it can be partially purified, and because it requires pyridoxal phosphate as a coenzyme. The latter takes part in some interesting enzymatic reactions and has been used in many studies of enzyme model systems. The purpose of this work was to see, if substituted cystines are substrates of cystathionase; and whether they act as metabolic antagonists toward some of the natural substrates of the enzyme. It was hoped that experiments with model systems, consisting of cystines, pyridoxal phosphate and metal ions, might

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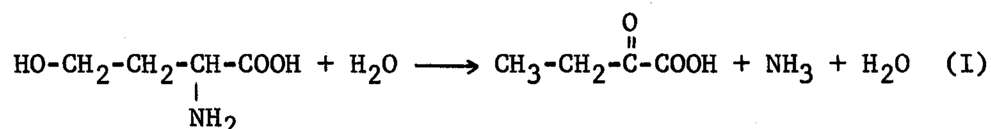
result in a better understanding of the mechanisms of enzymatic and non-enzymatic desulfhydration reactions. Results of inhibition studies with several substrates of the enzyme could help to clarify the function of the enzymatic reaction studied in sulfur amino acid metabolism, particularly with respect to its control.

Historical Development - Properties of Cystathionase

The enzyme cystathionase catalyzes in general a variety of reactions (α, β - β-, or γ-eliminations) in which ROH or RSH are removed from a considerable number of amino acids and closely related compounds containing hydroxyl, thiol, or thioether groups. The products of the reaction are usually ammonia, a keto acid and a thiol compound (or water in the case of homoserine). When cystathionine is the substrate γ-elimination takes place. The enzyme catalyzes the removal of water from the hydroxyamino acid, homoserine, in a similar type of reaction. Thioethers and disulfide amino acids such as cystine or djenkolic acid are cleaved by β- or α,β -elimination.

There is some confusion in the literature, because the enzyme is named according to the reaction which it catalyzes. When L-homoserine is the substrate, homoserine deaminase (12) or homoserine dehydratase (L-homoserine hydrolyase (deaminating) (EC 4.2.1.15)) catalyzes the reaction (13):



$$HO\text{-}CH_2\text{-}CH_2\text{-}\underset{\displaystyle NH_2}{\underset{|}{CH}}\text{-}COOH + H_2O \longrightarrow CH_3\text{-}CH_2\text{-}\overset{\displaystyle O}{\overset{\|}{C}}\text{-}COOH + NH_3 + H_2O \quad (I)$$

In this reaction removal of water from L-homoserine is followed by

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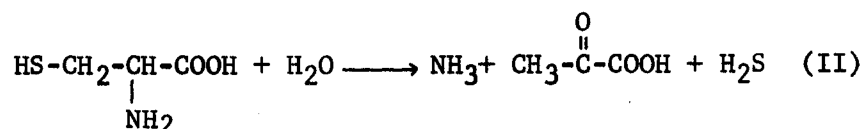
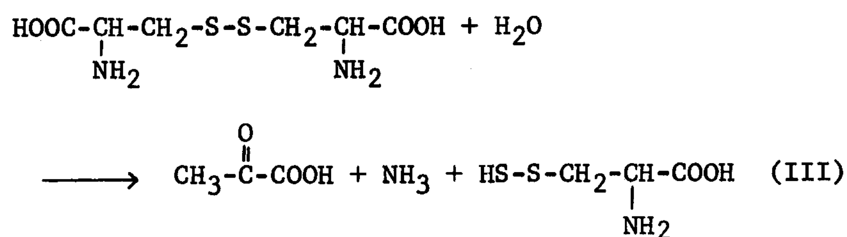
rearrangement of the double bond in the intermediate, vinylglycine, and subsequent hydrolysis liberating ammonia (13). Equivalent amounts of α-ketobutyrate and ammonia are liberated (14).

With L-cysteine as the substrate, the enzyme is called cysteine desulfhydrase or by its systematic name L-cysteine hydrogensulfide-lyase (deaminating (EC 4.4.1.1)). The main reaction products in this case are pyruvic acid, ammonia and hydrogen sulfide as shown in equation II (15).



It has been shown (16,17,18), however, that cystine, which is always present in small amounts and is spontaneously regenerated, is the actual substrate in a cyclic reaction. The unstable intermediate, thiocysteine, (alanine hydrogen disulfide) is formed from cystine as shown in equation III.



The lability of the sulfur of the intermediate leads to the production of sulfur and hydrogen sulfide; the reaction will be discussed in more detail in a subsequent section. Several side reactions account for a lack of stoichiometry observed among the reaction products (17). With cystine as substrate the enzyme has also been called cystine desulfurase (19).

Cystathionase has the same systematic name as homoserine deaminase.

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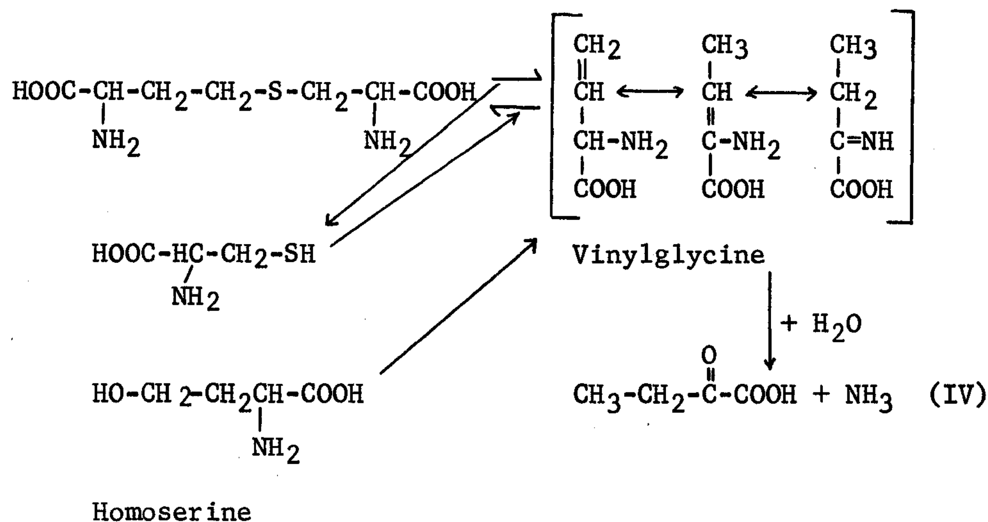
In its presence cystathionine is converted to cysteine, ammonia, and α-ketobutyric acid. This is shown in equation IV (20).



In this reaction Greenberg (14) observed that the amounts of ammonia and alpha-ketoacid exceeded the quantity equivalent to the substrate added; some H_2S was also formed. He concluded, therefore, that cysteine was decomposed by a desulfhydrase activity; this yielded pyruvate plus additional ammonia.

Matsuo and Greenberg (14) observed that there is a much higher activity of the enzyme in the liver than in heart or kidney. Rat liver has the highest concentration. Mudd et al. (21) reported that the activity of the enzyme in the brain is low, although one of its substrates, cystathionine, is found in high concentrations in this organ in man and monkey. Chatagner and Trautmann (22) obtained "soluble desulfurase" from the non-particulate fraction of rat liver. Fernandez observed the highest activity in the supernatant fractions of dog liver homogenates, compared to nuclear or mitochondrial fractions (23). Jolles - Bergeret also reported highest activity in the non-particulate fraction of rat liver (24).

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Fromageot was the first to describe formation of H_2S from L-cysteine in an enzymatic system (25). Smythe (26) obtained an enzyme preparation from rat liver that catalyzed the desulfhydration of cysteine. Carrol and coworkers (27) observed that an extract from rat liver catalyzed the cleavage of cystathionine; cysteine and α-ketobutyrate were identified as reaction products; the same keto acid was also formed when homoserine was the substrate. In 1950 Binkley (28) purified an enzyme from rat liver to a considerable extent. The preparation catalyzed the deamination of homoserine; a residual activity of cystathionase was always noted. The formation of hydrogen sulfide was reported when cystathionine or cysteine were incubated with the enzyme (29). In 1958 Greenberg (14) was able to prepare a highly purified, crystalline cystathionase sample from rat liver. Madlo in 1960 (30) purified the enzyme from rat liver 200-fold. Recently Kato et al.(19) and Fernandez (23) described the preparation of highly purified samples of cystathionase by procedures similar to Greenberg's (14) from liver of rat and dog, respectively.

The specific activity (expressed as μ moles/hr./mg. protein) of the enzyme toward several hydroxy amino and sulfur amino acids was observed to be as follows (20): L-homoserine, 340; L-serine, 6.8; O-acetyl -DL-homoserine, 6.3; L-cystathionine, 270; L-djenkolic acid, 103; L-cysteine, 18.4; S-methyl-L-cysteine, 1.8; S-ethyl-L-cysteine, 1.5; L-methionine, 1.1. Binkley and Okeson (31) reported that S-alkyl cysteines are substrates for cystathionase. They give the reactivity of a series of these derivatives of cysteine in terms of percent cleaved: L-cysteine, 35; S-methyl-L-cysteine, 21; S-ethyl-, 16; S-n-propyl-, 10; S-n-butyl-L-cysteine, 2. D-cystine and D-cysteine were inert (32).

Pyridoxal phosphate is required as a coenzyme. The colourless

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apoenzyme alone is inactive. The yellow holoenzyme has an absorption peak in neutral solution (pH 7.5) in the range 370 - 470 mμ with a maximum at 427 mμ, due to the apoenzyme-bound coenzyme (14). When the enzyme was reduced with sodium borohydride, no activity was observed with homoserine as substrate and no added coenzyme; there was little activity in the presence of added PLP. Possibly under these conditions the azomethine form of the bound coenzyme is reduced (33). Four moles of PLP are bound to one mole of enzyme (34). The coenzyme is probably bound to a lysine residue of the apoenzyme by a Schiff base type linkage as suggested by Snell for many pyridoxal-dependent enzymes (6).

The only activator of the enzyme reported is PLP. Inhibitors are the type of reagent that reacts with sulfhydryl or carbonyl groups (14). Among the former, metal ions such as Cu^{++}, Hg^{++}, and Cd^{++} were studied as well as p-chloromercuribenzoate and N-ethylmaleimide. Inhibition by certain of these inhibitors was reversed in part by 2,3-dimercaptopropanol. Inhibition was observed with the carbonyl reagents, cyanide, hydroxylamine, and semicarbazide. These react with the carbonyl group of PLP. Pyruvate is a competitive inhibitor (35). Cystathionase activity in rats was inhibited by thyroxine administration (23). An interesting observation is that substrates, such as homoserine, act as competitive inhibitors of other reactions. This will be discussed in detail in one of the following sections.

The optimum pH for activity toward homoserine or cystathionine is 8. The enzyme preparation is stable for several months in neutral phosphate buffer at -20°, if the concentration is about 1% (14). Cystathionase has a molecular weight of about 1.9×10^{5}. The isoelectric point of the enzyme is 6.05 (14).

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The activity of the enzyme, purified by Greenberg, toward different substrates decreases in the order: Homoserine > cystathionine > djenkolic acid > lanthionine > cysteine. With L-homoserine a K_m of 2×10^{-2} M was determined and a V_{max} of 2020 μ moles/hr./mg. protein; this allowed the calculation of a turnover number of 6400 moles/min./mole of enzyme. Similarly a K_m of 3×10^{-3} M, V_{max} of 738, and turnover number of 2340 were obtained for L-cystathionine (14). Kato _et al_.(19) determined the following Michaelis constants: L-homoserine, 3.0×10^{-2} M; L-cystathionine, 3.7×10^{-3} M; L-cysteine, 5.2×10^{-4} M; L-cystine, 9.3×10^{-5} M.

The enzyme does not contain firmly-bound metal ions (30,34). It is active with respect to homoserine and cystathionine without addition of metal ions (36); metal ions do not stimulate activity (37).

When cysteine and homoserine are incubated in the presence of the enzyme, cystathionine is formed (20). However, this indication of the reversibility of the cystathionase reaction is not considered to be of importance _in vivo_ (20). Brueggemann and Waldschmidt (35) were not able to observe formation of cysteine after incubation of pyruvate, ammonia, and hydrogen sulfide with cysteine desulfhydrase.

The mechanism of the cleavage of cystine and cysteine has been clarified in recent years. During a study of the reaction of the disulfide in a pyridoxal model system Cavallini _et al_. (38) proposed that thiocysteine (alanine hydrogen disulfide) is an intermediate in a cyclic reaction by which the amino acid is degraded. Indirect, but strong evidence, was also obtained for the presence of this intermediate in the corresponding enzymatic reaction (18). Flavin later gave convincing support to this theory, when he isolated the unstable alkyl hydrogen

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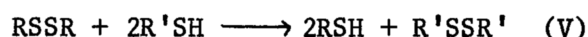
disulfide as a derivative (16). Recently Greenberg and coworkers produced further positive evidence. They showed that in the degradation of djenkolic acid, $HOOC-CH(NH_2)-CH_2-S-CH_2-S-CH_2-CH(NH_2)-COOH$, by cystathionase, cysteine, pyruvic acid, H_2S, ammonia, and formaldehyde are formed (39). In analogy to the reaction having cystine as substrate, S-thiomethylcysteine was formed as an unstable intermediate; this compound decomposed and yielded cysteine, ammonia, and formaldehyde.

The first report that cystine and not cysteine is the true substrate for cysteine desulfhydrase appeared in 1961. Mondovi gave evidence that pyruvate is produced from cysteine, only, if oxidation to cystine is permitted. The same group of workers observed that cysteine under nitrogen produces less pyruvate than in air (17). They also showed (40) rather convincingly that desulfhydration of cysteine is depressed by mercaptoethanol, because this thiol reduces traces of cystine present according to equation V.



$$RSSR + 2R'SH \longrightarrow 2RSH + R'SSR' \quad (V)$$

Hypotaurine, $R'SO_2H$, also inhibits the desulfhydration reaction. The sulfinate could react with the intermediate, thiocysteine to yield cysteine and a thiosulfonate (equation VI).

$$RSSH + R'SO_2H \longrightarrow R'SO_2SH + RSH \quad (VI)$$

Rather convincing evidence has accumulated that the different activities observed with preparations of cystathionase, homoserine deaminase, or cysteine desulfhydrase, are all possessed by the same protein. Binkley (29) and Kato _et al_. (19) noticed that throughout a purification of enzyme from rat liver the ratio of cystathionase to cysteine desulf-

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hydrase remained a constant. A similar phenomenon concerning the ratio of cystathionase to homoserine deaminase activity was described by Greenberg (14). Reconstitution after dissociation of the holoenzyme into apoenzyme and coenzyme restored this ratio of activities (20). Chatagner and Trautmann (22) suggested that cysteine desulfhydrase and cystathionase activity are on the same protein, since the two reactions involved varied in parallel with changes in the conditions of rats. Chromatographic studies by Cavallini _et al_. (17) indicate that cystathionine and cystine are degraded by the same enzyme protein. The authors observed two peaks which exhibited both enzyme activities. They concluded that the enzyme might be heterogeneous, that is, consist of distinct proteins or isozymes. However, more recently (41) they showed that one peak represents the holoenzyme and the other the apoenzyme. Mondovi (42) was not able to separate cystathionase and cysteine desulfhydrase activity by column electrophoresis.

Cystathionase and Sulfur Amino Acid Metabolism

The synthesis and metabolic fate of cysteine are summarized in FIG. 1 and FIG. 2.

Cysteine, a nonessential amino acid, is formed in mammals from methionine, an essential amino acid. The steps in the biosynthesis of cysteine may be summarized as follows (15).

1. Homocysteine is produced from methionine by demethylation:

$$CH_3\text{-}S\text{-}CH_2\text{-}CH_2CH(NH_2)\text{-}COOH \xrightleftharpoons{-CH_3} HS\text{-}CH_2\text{-}CH_2\text{-}CH(NH_2)\text{-}COOH \quad \text{(VIIa)}$$

2. Condensation of homocysteine with serine forms cystathionine:

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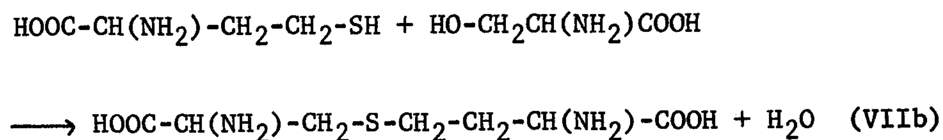
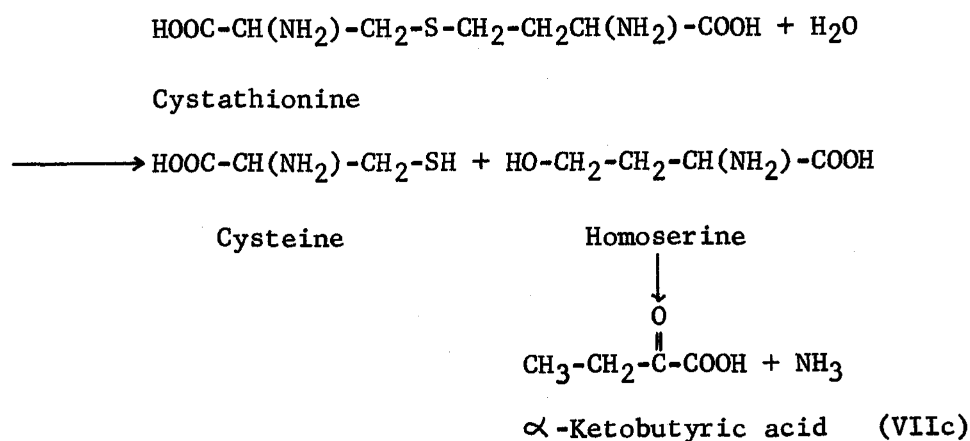


$$HOOC\text{-}CH(NH_2)\text{-}CH_2\text{-}CH_2\text{-}SH + HO\text{-}CH_2CH(NH_2)COOH$$

$$\longrightarrow HOOC\text{-}CH(NH_2)\text{-}CH_2\text{-}S\text{-}CH_2\text{-}CH_2\text{-}CH(NH_2)\text{-}COOH + H_2O \quad \text{(VIIb)}$$

3. Cleavage of cystathionine, a reaction catalyzed by cystathionase, to cysteine and homoserine; the latter is rapidly converted to α-ketobutyrate and ammonia by the same enzyme. Oxidative decarboxylation of the keto acid yields propionyl CoA, which is converted to succinyl CoA and enters the citric acid cycle as such:



$$HOOC\text{-}CH(NH_2)\text{-}CH_2\text{-}S\text{-}CH_2\text{-}CH_2CH(NH_2)\text{-}COOH + H_2O$$

Cystathionine

$$\longrightarrow HOOC\text{-}CH(NH_2)\text{-}CH_2\text{-}SH + HO\text{-}CH_2\text{-}CH_2\text{-}CH(NH_2)\text{-}COOH$$

Cysteine Homoserine

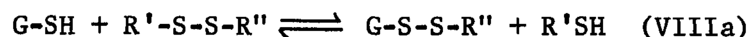
$$\downarrow$$

$$CH_3\text{-}CH_2\text{-}\overset{\overset{O}{\|}}{C}\text{-}COOH + NH_3$$

α-Ketobutyric acid (VIIc)

Some of the more important steps in the metabolism of cysteine may be summarized as shown in FIG. 1.

The dietary equivalence of cysteine and cystine has been established (15). Little free cystine is present in cells. The disulfide of proteins is formed apparently by oxidation of cysteine residues after their incorporation into polypeptide chains. In the presence of oxygen and cations such as Fe^{+++}, cystine may be formed from cysteine non-enzymically. Glutathione reductase can reverse this oxidation. Glutathione (γ-glutamylcysteinyl glycine) reacts non-enzymatically with any disulfide to form a mixed disulfide.



$$G\text{-}SH + R'\text{-}S\text{-}S\text{-}R'' \rightleftharpoons G\text{-}S\text{-}S\text{-}R'' + R'SH \quad \text{(VIIIa)}$$

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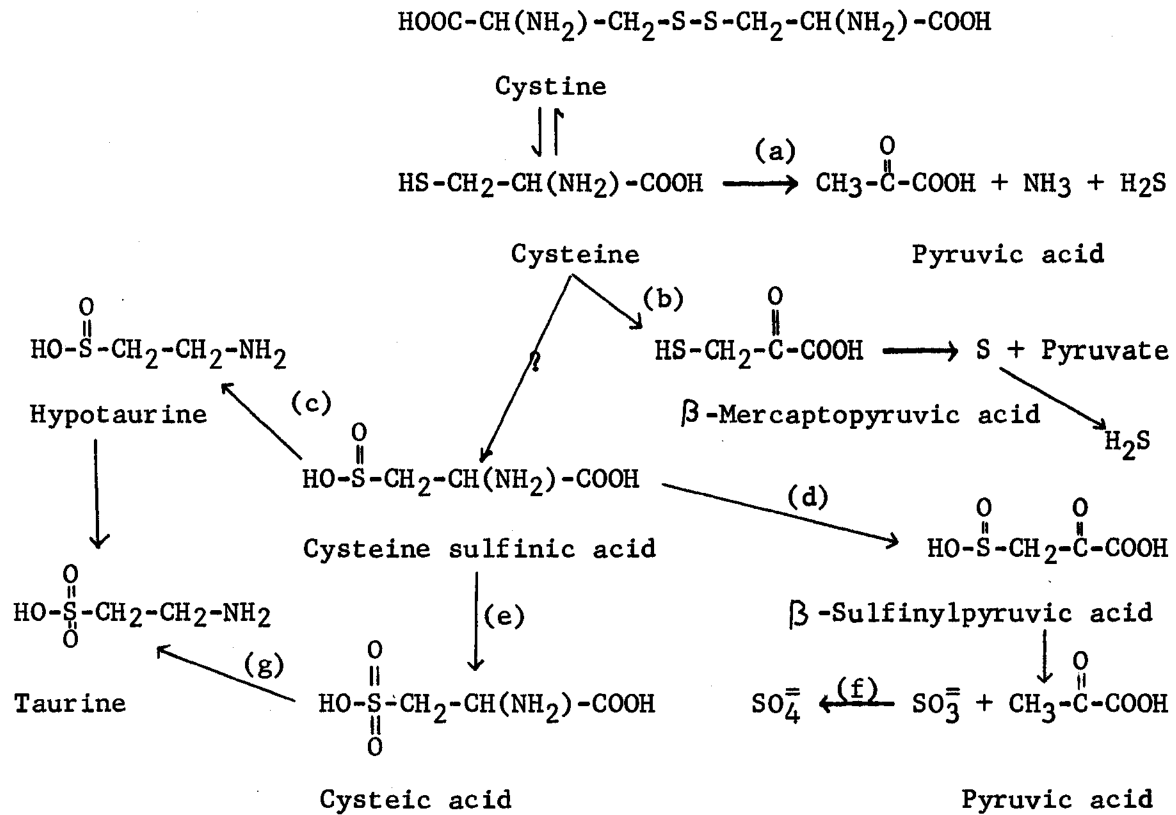
FIG. 1

METABOLISM OF CYSTEINE



(a): cysteine desulfhydrase

(b): cysteine aminotransferase

(c): cysteine sulfinate decarboxylase

(d): transaminase

(e): cysteine sulfinic acid dehydrogenase

(f): sulfite oxidase

(g): decarboxylase

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Oxidized glutathione is formed when a second molecule of glutathione reacts with the mixed disulfide.

$$G\text{-}SH + G\text{-}S\text{-}S\text{-}R'' \longrightarrow G\text{-}S\text{-}S\text{-}G + R''\text{-}SH \quad (VIIIb)$$

Glutathione reductase then catalyzes the reaction

$$TPNH + H^{+} + G\text{-}S\text{-}S\text{-}G \rightleftharpoons TPN^{+} + 2G\text{-}SH \quad (VIIIc)$$

In this manner cystine, R'-S-S-R", is reduced to cysteine by the cell (15). A specific reductase has not been discovered in mammalian systems.

Cysteine can be replaced by methionine in the diet of mammals. When rats were fed a protein-rich diet,increased cystathionase levels were observed (43). The feeding of methionine or ethionine had a similar effect; in addition an accumulation of homoserine was observed (44). High levels of the latter compound also induced an increase in the enzyme. Rat liver cystathionase decreased in pyridoxine deficiency (45) and in the metabolic disorder of cystathionuria (46). The sparing action of L-cystine for the dietary requirement of growing rats for methionine was observed by Womack and Rose (47).

Cystathionine synthetase catalyzes the formation of L-cystathionine from homocysteine and serine (48) and also the dehydration of L-serine, a reaction in which pyruvic acid and ammonia are formed. Recently Kato _et al_. published several papers on "Control Mechanism in the Rat Liver Enzyme System Converting L-Methionine to L-Cystine". They set out to account for the methionine-sparing action of L-cystine. In their first report (49), they suggested that the control mechanism works on the formation of cystathionine, a reaction catalyzed by cystathionine synthetase-serine dehydratase (SDH). This enzyme was repressed when rats

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fed on low methionine diet were supplemented with cystine. The activity of SDH was inhibited in crude extracts of rat liver by the reaction product of an enzyme fraction and L-cystine. A pre-incubation period was necessary for L-cystine to form this serine dehydratase inhibitor. Subsequently (19) the authors crystallized the enzyme, which formed an inhibitor of SDH from the sulfur amino acid, and showed it to be cysteine desulfhydrase. They made the observation that the activities, which caused the degradation of L-homoserine and L-cystine and the formation of the serine dehydratase inhibitor from the sulfur amino acid, reside on a single protein. In 1966 Kato _et al_. (50) showed that this non-competitive inhibitor is elemental sulfur. They also measured the competitive inhibition of cystathionase and homoserine deaminase by low concentrations of L-cystine and L-cysteine. However, they present no inhibition constants. It appeared that a complex of sulfur and cystine desulfurase was actually a more efficient inhibitor of serine dehydratase than an aqueous suspension of sulfur. L-cysteine and L-cystine were preferentially decomposed by cystathionase when cystathionine was present. The inhibition by L-cystine was stronger than that by L-cysteine. The report presents a summarizing diagram of the reactions from methionine to the degradation of cystine. Inhibitory effects were exerted by:

(1) cysteine on cystathionase,

(2) cystine on cystathionase,

(3) homocysteine on serine dehydratase,

(4) sulfur on serine dehydratase,

(5) sulfur on cystathionine synthetase.

However, the last inhibition above was considered to be somewhat uncertain, because only the SDH reaction had been studied with respect to

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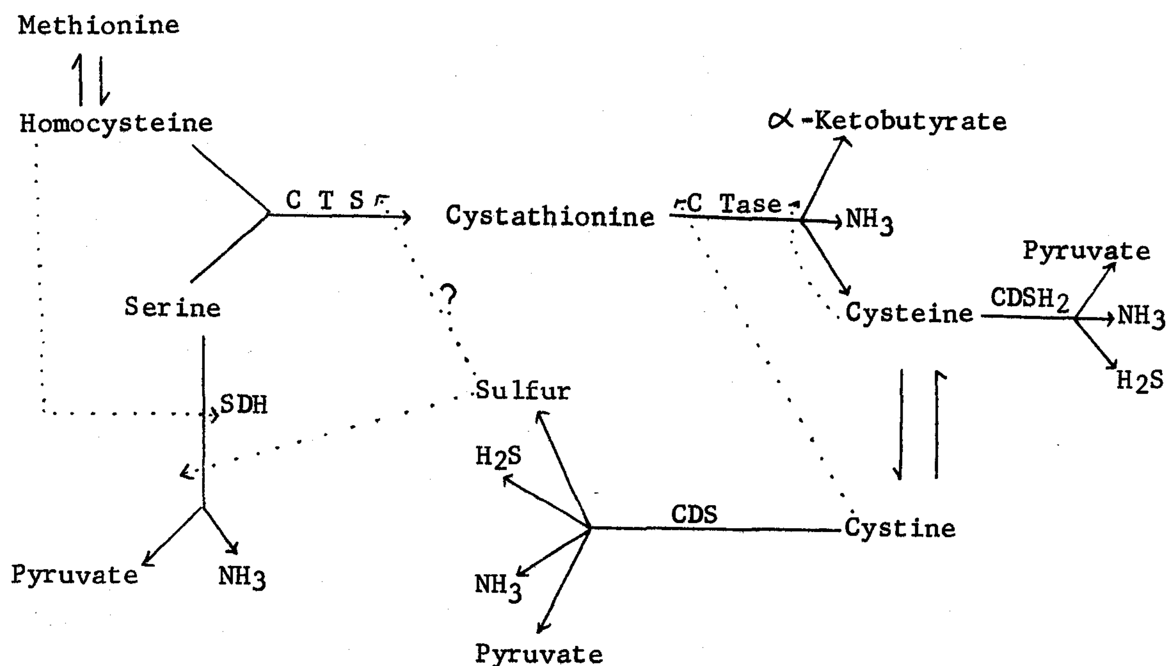
FIG. 2

INHIBITION IN THE RAT LIVER ENZYME SYSTEM
CONVERTING METHIONINE TO CYSTINE



CTS: cystathionine synthetase

$CDSH_2$: cysteine desulfhydrase

SDH: serine dehydratase

CDS: cystine desulfurase

C Tase: cystathionase

....>: inhibition is indicated by broken arrows

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this type of inhibition. These patterns of inhibition are summarized in FIG. 2 (50).

Homocysteine apparently inhibits serine dehydratase to prevent the breakdown of serine, which may be used for the synthesis of cysthathionine. Cystathionine synthetase-serine dehydratase is inhibited by sulfur, probably, because the first irreversible step in the breakdown of methionine is catalyzed by this enzyme. The degradation of cystathionine is also controlled (by inhibition by cysteine and cystine), as well as its formation, perhaps, to allow very careful control of the level of this thioether. However, it seems unclear why sulfur should exert control on the formation of cystathionine and the breakdown of serine.

The competitive inhibition of cystathionase by cystine has also been reported recently by Aronov (51).

In several microorganisms studied inorganic sulfur is fixed into organic compounds such as cysteine and then transferred to methionine (15). The metabolism of cysteine in fungi and bacteria has been summarized recently. (52,53,54,55,56).

In *Escherichia coli* and *Salmonella typhimurium* transsulfuration proceeds in the direction from cysteine to homocysteine (57), in *Neurospora crassa* it is reversible, while in mammals the pathway leads from homocysteine to cysteine. For instance, in *E.coli* cysteine is converted to methionine in the following sequence: cysteine + O-succinyl homoserine $\xrightarrow{a}$ cystathionine $\xrightarrow{b}$ homocysteine $\xrightarrow{c}$ methionine (IX), where enzyme a is cystathionine synthetase, b is cystathionase, and c is called homocysteine methylase. All of these enzymes are repressed by growth of *E.coli* on methionine (58).

Flavin and coworkers have studied two cystathionine cleavage enzymes in *Neurospora* (59,60,61). One of these splits the thioether to

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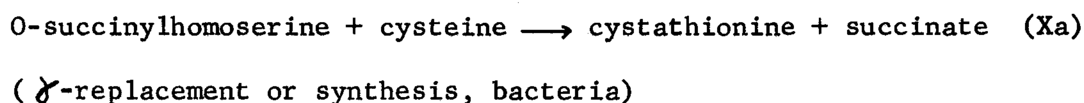
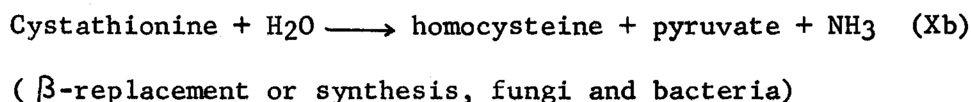
homocysteine, pyruvate, and ammonia (β-elimination or cleavage); it is also found in some bacteria. The other catalyzes predominantly γ-elimination to yield cysteine and α-ketobutyrate. Delavier-Klutchko and Flavin (62) have purified a β-cleavage enzyme from E.coli, which catalyzes the formation of homocysteine, pyruvate, and ammonia. The synthesis and degradation of cystathionine in bacteria, fungi and mammals may be summarized as follows (56):



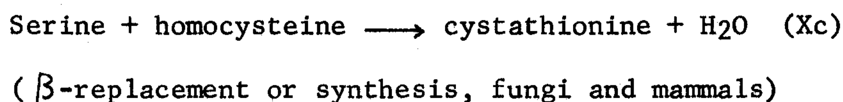
O-succinylhomoserine + cysteine ⟶ cystathionine + succinate (Xa)

(γ-replacement or synthesis, bacteria)



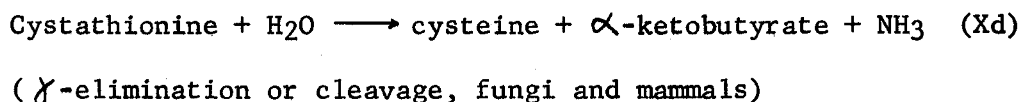
Cystathionine + H_2O ⟶ homocysteine + pyruvate + NH_3 (Xb)

(β-replacement or synthesis, fungi and bacteria)



Serine + homocysteine ⟶ cystathionine + H_2O (Xc)

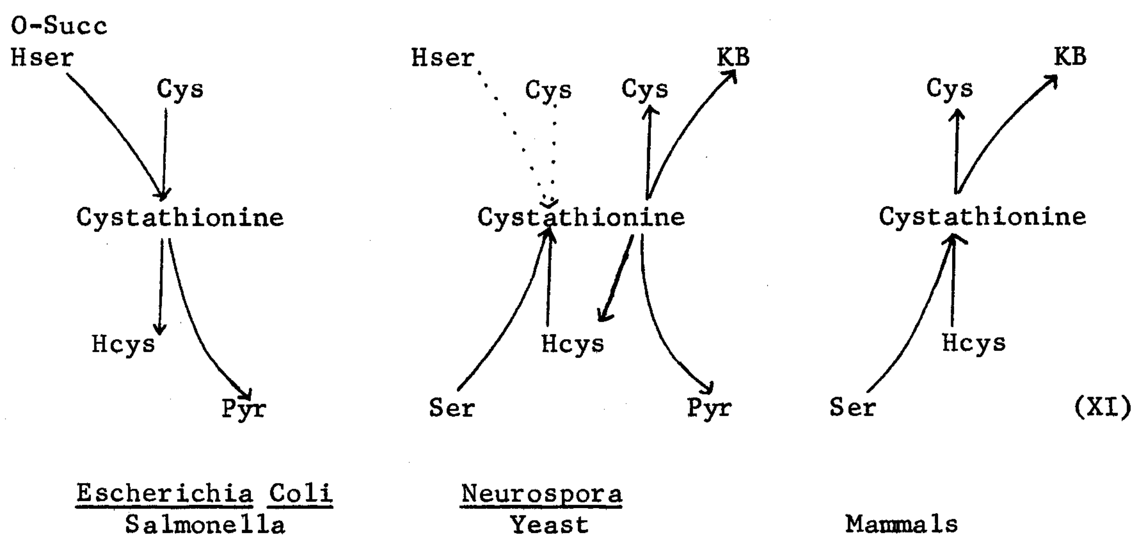
(β-replacement or synthesis, fungi and mammals)



Cystathionine + H_2O ⟶ cysteine + α-ketobutyrate + NH_3 (Xd)

(γ-elimination or cleavage, fungi and mammals)

Delavier-Klutchko and Flavin (62) describe the following patterns of transsulfuration:



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where O-Succ Hser = O-Succinylhomoserine,

Hser = homoserine, KB = α-ketobutyrate,

Hcys = homocysteine, and Pyr = pyruvate.

The liver cystathionase and Neurospora γ-cleavage enzyme have very similar properties. Both enzymes readily catalyze β-elimination from thioethers and disulfide amino acids. The effective substrates for both seem to be "double ended" compounds, with the exception of homoserine. However, liver cystathionase catalyzes only γ-elimination, that is, formation of cysteine, α-ketobutyrate, and ammonia, while the Neurospora enzyme also catalyzes the production of homocysteine and pyruvate. Furthermore, with L-homoserine as substrate, the reaction with liver enzyme is much faster (60).

The degradation of cystathionine by the γ-cleavage enzyme of Neurospora is inhibited 91 percent by L-cysteine (60). The inhibition by L-serine and L-homoserine (substrate) of the decomposition of cystathionine and cystine was determined also. Both were competitive inhibitors. With L-homoserine the K_i was 8×10^{-3} M for cystathionine and 6×10^{-3} M for cystine. With L-serine as inhibitor the same K_i value of 1.3×10^{-2} M was obtained with each substrate.

Rowbury and Woods (63) noted that the breakdown of cystathionine in E.coli was inhibited considerably by DL-homocysteine (a product). However, the amino acid had only a small effect on the formation of the enzyme. The ability to metabolize cystathionine and cysteine was decreased by growth of the organism in the presence of methionine. Reappearance of cysteine desulfhydrase activity accompanied that of cystathionase when the organism was transferred to a methionine-free medium.

Flavin observed competition between thioether and disulfide

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substrates of the Neurospora γ-cleavage enzyme (16). L-cystine inhibited the degradation of cystathionine, lanthionine that of cystathionine.

In yeast and Aspergillus niger an enzyme has been discovered which forms cysteine directly from serine and hydrogen sulfide (64,65). Serine sulfhydrase from chicken liver has been shown to catalyze the reverse reaction in which serine and hydrogen sulfide are formed from cysteine (35). Thus there are two enzymes at least, which liberate H_2S from cysteine directly.

Formation of Schiff Bases from Pyridoxal Phosphate (or Pyridoxal) and Amino Acids and their Reactions. Mechanism of the Non-Enzymatic and Enzymatic Degradation of Cystine

Several extensive discussions and reviews of the role of pyridoxal in model systems and enzymatic reactions have been presented in the literature (6,66,67,68,69,70).

Many reactions of amino acids are catalyzed by pyridoxal phosphate enzymes. Among these are transamination, raceminzation, decarboxylation, elimination of the α-hydrogen together with a β-substituent (cysteine desulfhydration) or a γ-substituent (cystathionine cleavage or deamination of homoserine) (70). Most of these reactions have been duplicated in model systems in which pyridoxal or pyridoxal phosphate may serve as catalyst, usually in the presence of metal ions. Snell (6,70) and Braunstein (67) have tried to explain the manifold catalytic role of vitamin B_6 in terms of chemical properties of pyridoxal and pyridoxal phosphate. They proposed that a Schiff base is formed between the amino acids and pyridoxal (phosphate) with a subsequent withdrawal of electrons from the

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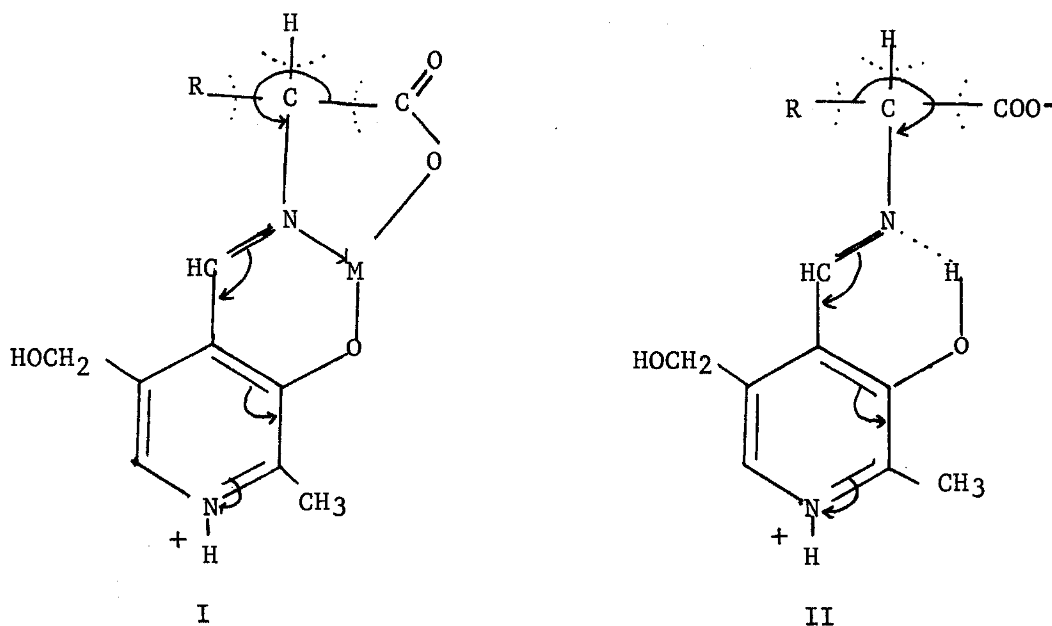
bonds connected to the α-carbon atom of the amino acid toward the electrophilic nitrogen of pyridoxal. This would lead to a general weakening of the bonds on the α-carbon. The consequent reactions would be determined in a non-enzymatic model system by the structure of the amino acid and in many cases the presence of a metal ion, as well as the reaction conditions. In enzymatic systems the nature of the apoenzyme plays a crucial role. It takes over the function of the metal ion, but with a much higher efficiency. However, not many details are known about the process of formation and hydrolysis of the Schiff base intermediate. FIG. 3 shows the structure of a metal-chelated and hydrogen-bonded aldimine of pyridoxal and an amino acid (6).

FIG. 3

ALDIMINES OF PYRIDOXAL AND AN AMINO ACID



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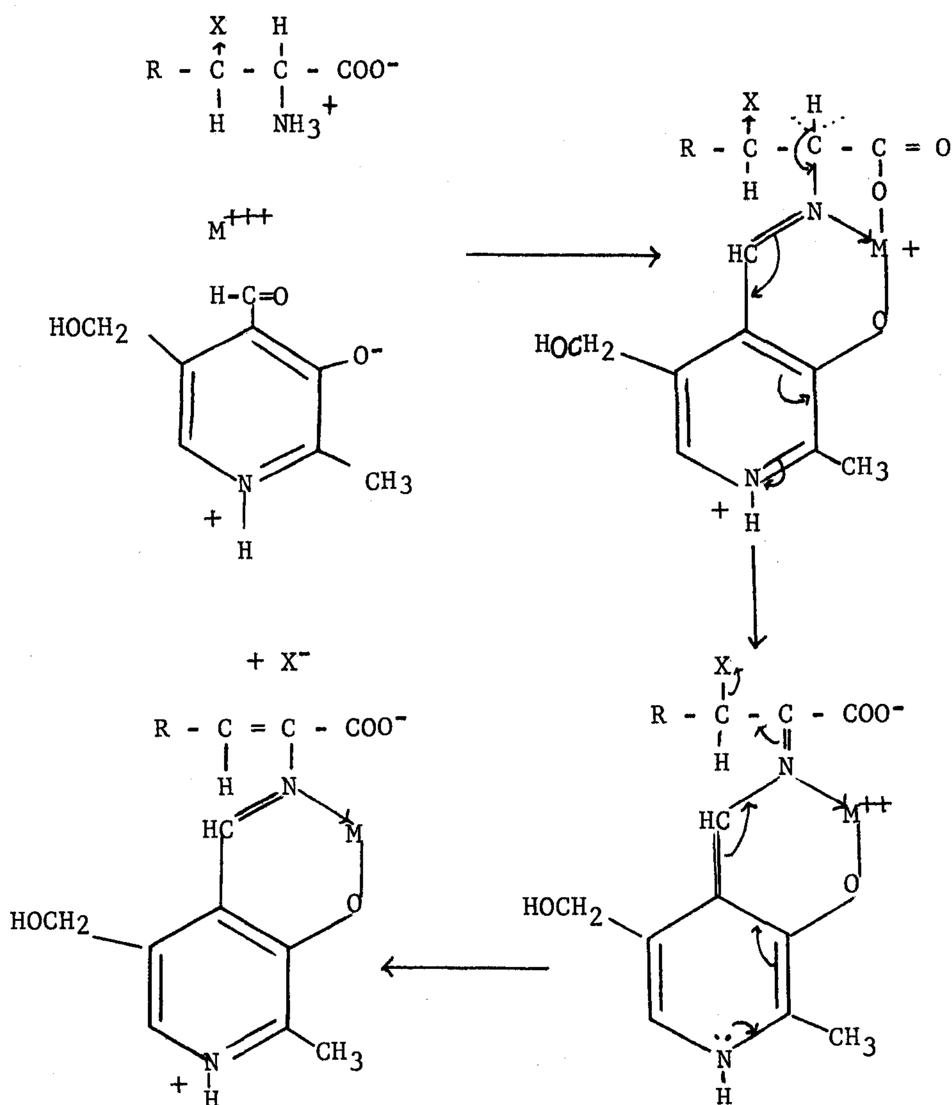
In a discussion of the general mechanisms involved in the Vitamin B_6 catalyzed reactions of amino acids, Snell and coworkers (70) present the reactions expected and observed, when there is an electron attracting group X at the β-carbon of the amino acid. In this case one would expect the following set of reactions:



(XII)

In this manner -SH of cysteine, and HSSR' of cystine are eliminated in non-enzymatic (38,71,72) and enzymatic reactions (16,17,73,74). The resulting Schiff base of aminoacrylic acid hydrolyzes to pyruvic acid

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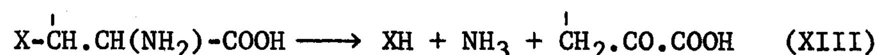
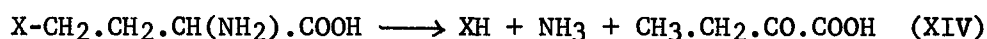
and ammonia and pyridoxal or pyridoxal phosphate. One can summarize this α-β-elimination as follows (67):



$$X\text{-}\overset{|}{C}H.CH(NH_2)\text{-}COOH \longrightarrow XH + NH_3 + \overset{|}{C}H_2.CO.COOH \qquad (XIII)$$

The more electronegative the β-substituent, the more rapid is the rate of the model reaction (75).

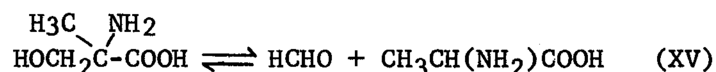
In a similar elimination reaction a β-hydrogen and a polar γ-substituent are removed according to the equation:



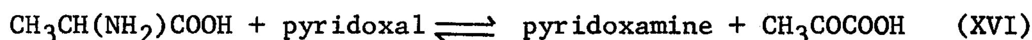
$$X\text{-}CH_2.CH_2.CH(NH_2).COOH \longrightarrow XH + NH_3 + CH_3.CH_2.CO.COOH \qquad (XIV)$$

For instance, in the case of homoserine, X = OH, for that of cystathionine X = $HOOC.CH(NH_2).CH_2S$. The nature and cause of these reactions is essentially the same as that of the α-β-elimination reactions of β-substituted amino acids (67).

It should be emphasized that according to the general mechanism proposed, transamination and β-elimination reactions such as that undergone by cysteine or cystine (70) depend on the presence of a labile hydrogen atom on the alpha-carbon. However, as pointed out by Snell (6) the cleavage of α-amino-β-hydroxyamino acids and the decarboxylation of amino acids do not depend on this α-hydrogen. When α-methylserine was heated in aqueous solution with pyridoxal and Cu^{++} or Al^{+++}, the amino acid was degraded according to the equations (5):



$$HOCH_2C(CH_3)(NH_2)\text{-}COOH \rightleftharpoons HCHO + CH_3CH(NH_2)COOH \qquad (XV)$$



$$CH_3CH(NH_2)COOH + \text{pyridoxal} \rightleftharpoons \text{pyridoxamine} + CH_3COCOOH \qquad (XVI)$$

The alpha-substituted serine was also used as a substrate by a cell-free extract of a bacterium of the genus Pseudomonas (6,7) according to the

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equation:

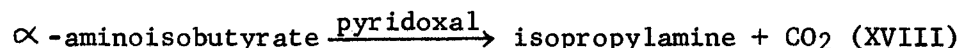
(+)-α-Methylserine + THF $\longrightarrow$ D-alanine + 5,10-methylene-THF (XVII)

In 1957 Kalyankar and Snell (76) reported that α-methylalanine and α-methylserine cause the formation of pyridoxamine from pyridoxal, although they cannot transaminate by the general mechanism proposed, because of the lack of the alpha-hydrogen. They suggested that decarboxylation accompanied by partial transamination occurred. Because the non-enzymatic, pyridoxal-catalyzed decarboxylation reaction is much slower than several competing reactions, transamination, for instance, this reaction is hard to demonstrate. It was thought in recent experiments (8) that alpha-substitution would minimize competing reactions. The non-enzymatic decarboxylation of α-aminoisobutyric acid, α-methylserine, and α-phenylglycine was studied. Some very interesting results were obtained:

(1) Formation of CO_2 was inhibited rather than catalyzed by metal ions.

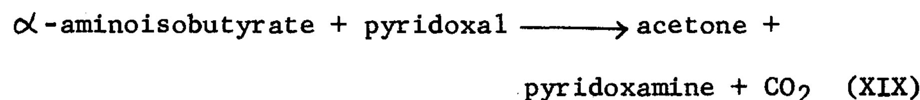
(2) α-Aminoisobutyrate underwent two types of reactions,

(a) a decarboxylation,



α-aminoisobutyrate $\xrightarrow{\text{pyridoxal}}$ isopropylamine + CO_2 (XVIII)

(b) and a decarboxylation-dependent transamination reaction.



α-aminoisobutyrate + pyridoxal $\longrightarrow$ acetone + pyridoxamine + CO_2 (XIX)

The reaction

isopropylamine + pyridoxal $\longrightarrow$ acetone + pyridoxamine (XX)

did not occur.

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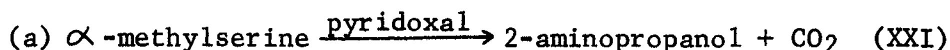
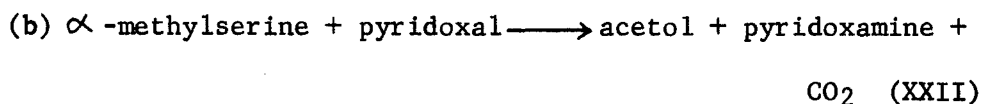
(3) In the absence of metal ions α-methylserine underwent the following reactions:



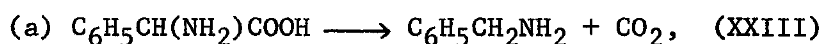
(a) α-methylserine $\xrightarrow{\text{pyridoxal}}$ 2-aminopropanol + CO_2 (XXI)



(b) α-methylserine + pyridoxal $\longrightarrow$ acetol + pyridoxamine + CO_2 (XXII)

These two reactions are similar to those undergone by α-aminoisobutyrate. But in the presence of metal ions (which form a chelate bond with the carboxyl group and thus make its removal more difficult) formaldehyde was formed from α-methylserine as shown before (equation XV).

The other amino acid, α-phenylglycine also underwent several reactions:

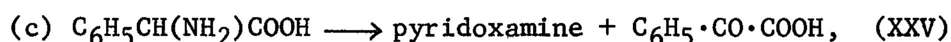


(a) $C_6H_5CH(NH_2)COOH \longrightarrow C_6H_5CH_2NH_2 + CO_2$, (XXIII)

a decarboxylation reaction slowed down in the presence of metal ions; the electron-withdrawing phenyl group apparently helped to facilitate removal of CO_2.

(b) $C_6H_5CH(NH_2)COOH \longrightarrow C_6H_5CHO$ + pyridoxamine + CO_2, (XXIV)

a decarboxylation-dependent transamination reaction.



(c) $C_6H_5CH(NH_2)COOH \longrightarrow$ pyridoxamine + $C_6H_5 \cdot CO \cdot COOH$, (XXV)

a normal transamination reaction, possible, since an alpha-hydrogen atom is available,

(d) $C_6H_5COCOOH \longrightarrow CO_2 + C_6H_5CHO$ (XXVI)

As a result of these studies and others (77), the pyridoxal catalyzed decarboxylation reactions were formulated as (6):

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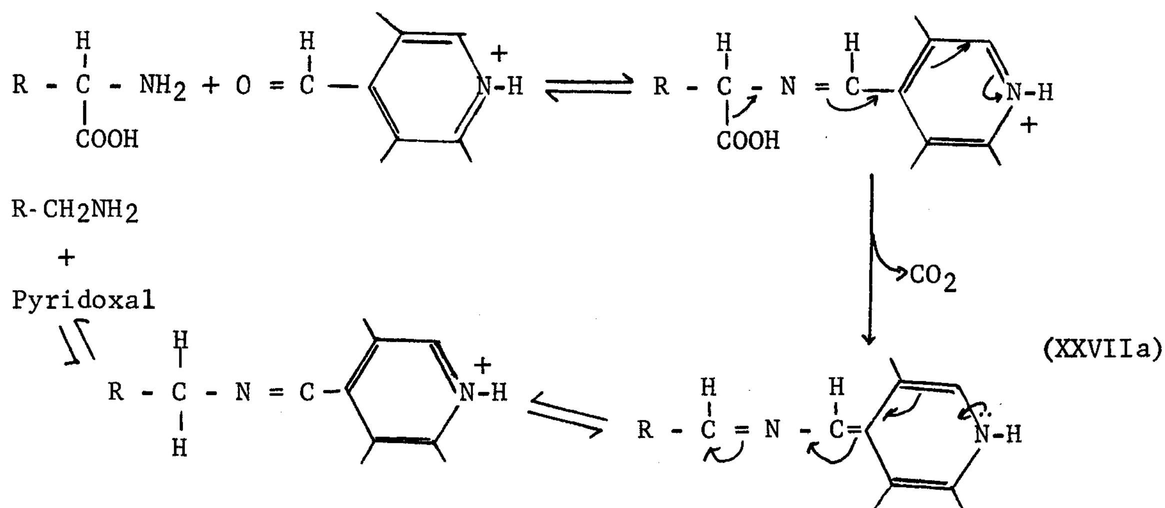

(XXVIIa)

The reactions undergone by α-substituted amino acids in the absence of metal ions can be visualized as follows (6):

Pyridoxal

+

R' | R - C - COOH | NH_2 ⇌ ($-H_2O$) R' | R - C - COOH | N ... H | HC | O | CH3 | + N | H

Pyridoxamine

+

R - C - R' ‖ O

$+H_2O$ ⇌

Pyridoxal

+

R' | R - C - NH_2 | H

⇌ $+ H_2O$

R' | R - C ‖ N | H | C | H^+ | H | O | CH3 | + N | H ⟷ R' | R - C ‖ N + CO_2 + H^+ | HC | H | O | N | H | CH3 ⟷ R' | R - C^- ← H^+ | N | HC | H | O | + N | H | CH3

(XXVIIb)

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Pyridoxal and pyridoxal phosphate and their azomethine complexes are yellow under neutral or alkaline conditions (34,67). Blakley (78) obtained spectrophotometric evidence for the formation of a Schiff base between glycine and pyridoxal phosphate. PLP alone showed an absorption maximum at 388 mμ ($\mathcal{E}_{max.}$ 5700). In the presence of 0.01 M glycine this peak shifted to 405 mμ ($\mathcal{E}_{max.}$ 5800). With 0.1 M amino acid a maximum was observed at 413 mμ ($\mathcal{E}_{max.}$ 6600). Metzler (79) reported that in the presence of valine and pyridoxal in aqueous solution, a maximum appeared at 414 mμ; the solution was intensely yellow. The intensity of the maximum increased as the amino acid concentration was increased. The absorption band is apparently due to a form of the imine in which the phenolic group is hydrogen-bonded to the imino nitrogen. Matsuo (80) observed that in the presence of various amino compounds in phosphate buffer at pH 7.5 the absorption peak of pyridoxal phosphate at 388 mμ moved to a higher wavelength of 400 to 415 mμ. Among a large number of amino acids, which caused this type of change, were several alpha-substituted compounds, α-aminoisobutyric acid, α-methyl- α-amino-n-butyric acid, and α-methylglutamic acid. The author proposed that any compound that possesses an unsubstituted amino group can form a Schiff base with PLP. However, the spectrum of a PLP-cysteine reaction product was quite different from that of PLP or the other Schiff bases examined, because a thiazolidine ring was probably formed by the reaction of the formyl group of pyridoxal phosphate with the amino and the sulfhydryl group of cysteine. The absorption peak near 400 mμ had disappeared. The PLP -cysteine complex is much more stable than the ordinary Schiff base.

The presence of small amounts of metal ions shifted the absorption maxima of Schiff bases at 230 mμ and 388 mμ to shorter wavelengths. For

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instance, in the presence of PLP plus glycine and Cu^{++} the maximum was decreased from 408 mμ to 383 mμ. The spectra were not those of the metal and amino acid.

The formation of a Schiff base between PLP and bovine serum albumin was observed by Christensen (81). Maximum absorption occurred at 415 mμ.

Greenberg (34) measured the absorption spectrum of the chromophore, isolated from cystathionase, at pH 7.5. He observed a major peak at 388 mμ and a minor one at 330 mμ. The crystalline holoenzyme showed an absorption peak at 427 mμ.

Equilibria between pyridoxal and amino acids and their imines have been studied by Metzler (79). He showed that the apparent equilibrium constant for imine formation depends on a complex interaction of several factors, namely basicity of the amino group, presence of one or more hydrogen atoms on the alpha-carbon of the amine (hyperconjugation effects), and branching in the β-position of the amine. To this should be added steric factors. The K(apparent) values for some of the amines studied were as follows: ammonia, 0.3; methylamine, 17; ethylamine, 26; n-butylamine, 83; isobutylamine, 119; t-butylamine, 6.0; glycine, 10.2; alanine (R = CH_3-), 9; α-amino-n-butyric acid (R = CH_3CH_2-), 17; valine (R = $(CH_3)_2$>CH-), 44; norvaline (R = $CH_3CH_2CH_2$-), 19.

In non-enzymatic transamination valine reacts very slowly (82), despite the high stability of its imine. Therefore it seems that the extent of imine formation does not play an important role in the determination of the rate of non-enzymatic transamination. Metzler suggests that, since the rates of imine formation and breakdown appear to be rapid, the tautomeric rearrangement of the imine must be the rate-limiting step in non-enzymatic transamination and other similar non-enzymatic

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reactions. Included here should be the cleavage of cystine.

Matsuo (80) determined the stability constants of the Schiff bases formed from PLP and a number of amines and amino acids. It is interesting to note that the orders of magnitude of these stability constants relative to each other, among a series of amino acids, compares with that of the equilibrium constants obtained by Metzler (79). Thus for alanine (R = $-CH_3$), $K \times 10^{-4} = 0.32 \pm 0.01$; for α-amino-n-butyrate (R = CH_3CH_2-), $K \times 10^{-4} = 1.16 \pm 0.02$; for norvaline (R = $CH_3CH_2CH_2-$), $K \times 10^{-4} = 1.2 \pm 0.1$; for valine (R = $\frac{CH_3}{CH_3}{>}CH-$), $K \times 10^{-4} = 4.5 \pm 0.2$, and for norleucine (R = $CH_3CH_2CH_2CH_2-$), $K \times 10^{-4} = 1.1 \pm 0.1$. It is also interesting to note that several alpha-substituted amino acids studied, form a less stable imine than the non-substituted ones. Compare: alanine ($K \times 10^{-4} = 0.32 \pm 0.01$) with α-aminoisobutyrate ($K \times 10^{-4} = 0.07 \pm 0.01$); α-amino-n-butyrate (1.16 ± 0.02) with α-methyl-α-amino-n-butyrate (0.09 ± 0.00); and glutamate (0.42 ± 0.01) with α-methylglutamate (0.03 ± 0.01).

Matsuo (80) tried to draw a correlation between the effectiveness of amino compounds as inhibitors of enzyme systems and the stability constant of their Schiff base. The latter factor should contribute significantly to the overall affinity of the amine to the enzyme. He showed that, with homoserine deaminase, the percent inhibition by the three alpha-substituted amino acids studied is either non-existent or very small, compared to the inhibitory effect of the alpha-hydrogen compounds. However, the limited number of examples studied in which only α-methyl substitution was used does not allow any definite comparison. As the author himself points out the affinity of an amine for the active site of the enzyme is determined also by such factors as the

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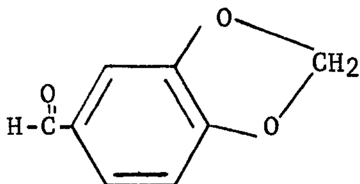
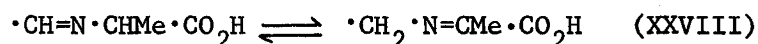
attractive and repulsive forces between the functional groups of the reactant and the apoenzyme as well as steric factors.

The importance of steric factors in Schiff base formation was pointed out by Hill and Crowell (83). These authors studied "Structural Effects in the Reactivity of Primary Amines" with piperonal,



They observed that reaction rates do not parallel the basicity of the amino group, as shown by the following results in which k is expressed as l/mole/sec. at 25°: methylamine, 5.55; ethylamine, 2.88; n-propylamine, 3.15; isopropylamine, 0.895; n-butylamine, 3.37. The rates observed seem to be a result of the basicity of the amine and steric effects of the alkyl groups. One should note in particular the relatively high rate with methylamine and low rate with isopropylamine and compare the results with the stability data discussed in the preceding paragraphs.

Banks *et al*. (84) studied the reaction of pyridoxamine and pyruvate in water at 25° over a pH range of 7 to 10. They showed conclusively that Schiff base formation occurs in a fast and reversible step and that the rate limiting step is the interconversion of this type of Schiff base and its isomer, that is the change



$$\cdot CH{=}N\cdot CHMe\cdot CO_2H \rightleftharpoons \cdot CH_2\cdot N{=}CMe\cdot CO_2H \qquad (XXVIII)$$

The instability of cystine in alkaline solution has been known for a long time. A few years ago the mechanism of this degradation was finally worked out, Swan (9) studied the behavior of α-methylcystine in hot aqueous sodium hydroxide. He found that the stability of the

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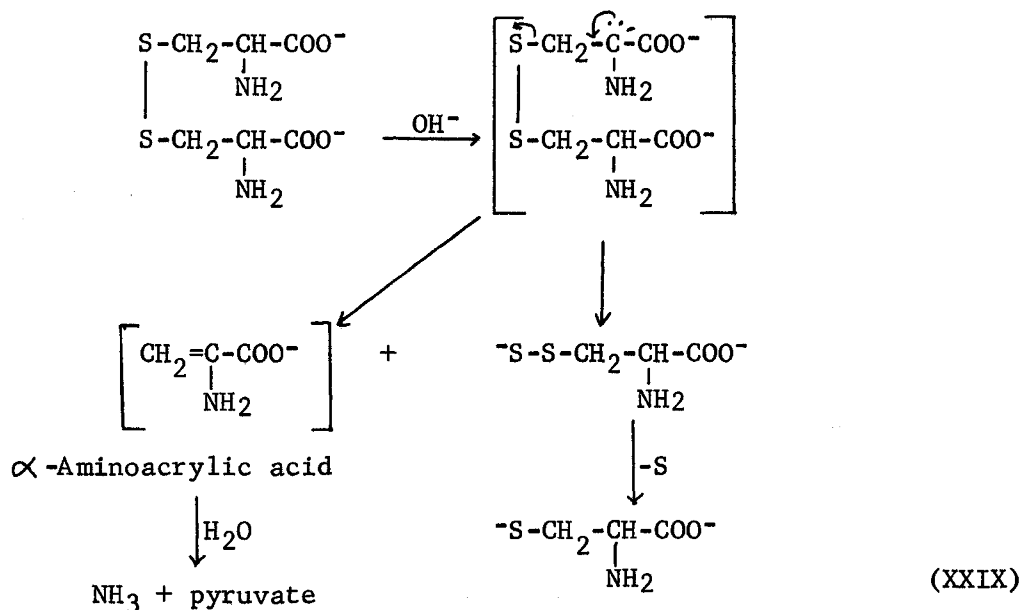
amino acid had been increased tremendously by the replacement of the alpha-hydrogens by methyl groups. Such resistance to base hydrolysis could not be explained by direct hydrolysis of the sulfur-sulfur bond or ionization of a proton adjacent to one of the sulfur atoms as the initial step in the cleavage. Ionization of the amino acid alpha-hydrogen atom, followed by elimination of an unstable disulfide anion, however, would be a mechanism in accordance with the high stability of α-methylcystine. This β-elimination has been described by the following equations by Swan (9) and also Dann and coworkers (85):



(XXIX)

The cleavage of cystine by a pyridoxal model system has been studied by Cavallini and coworkers (38) in the presence of copper ions (Cu^{++}) at a pH of 8.5 and 38°. The mechanism of the degradation of the amino acid was summarized as follows:

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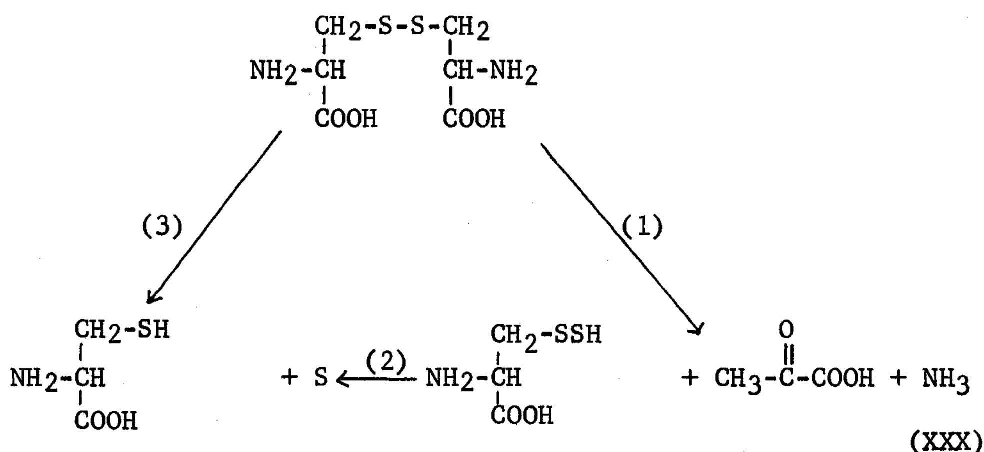

(XXX)

(1) In an α-β-elimination reaction the chelate complex of cystine with pyridoxal and copper ions is cleaved. The products of this reaction are pyruvate, ammonia and thiocysteine. These products are predictable on the basis of Snell's work on pyridoxal phosphate-catalyzed reactions (86) and the mechanism of the alkaline cleavage of cystine (9,85).

(2) Thiocysteine decomposes to cysteine and elementary sulfur.

(3) Cystine is reformed by the reoxidation of cysteine in the presence of oxygen and copper ions.

The cyclic reaction is continued until most of the cystine has been used up. Several side reactions complicate this basic mechanism.

The interaction of cystine, pyridoxal (phosphate), and copper can be pictured as shown in FIG. 4.

Recently Bergel and coworkers (10) have observed that in an enzyme model system consisting of vanadium salts and pyridoxal phosphate, cysteine was degraded. Ammonia, pyruvate, and cystine were among the reaction products. The authors proposed a vanadium-catalyzed hydrolysis of the thiazolidine intermediate formed, followed by the formation of a Schiff base that decomposed rapidly by α-β-elimination. The cysteine

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FIG. 4

NON-ENZYMATIC DEGRADATION OF CYSTINE

+ Cu^{++}

thiocysteine

pyruvate

+ NH_3

+ pyridoxal

H_2O

α-elimination

H_2O

β-elimination

Schiff base of aminoacrylic acid

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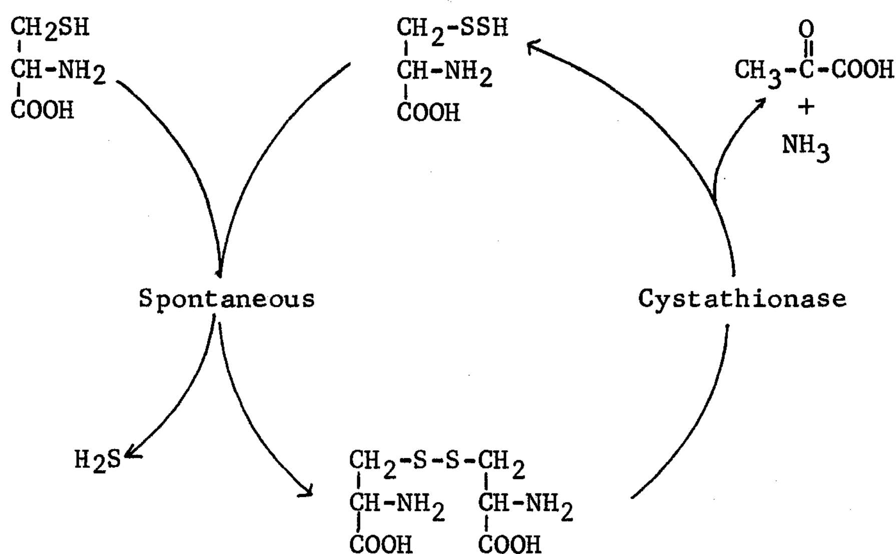
molecule was stabilized by α-or β alkylation. For instance, α-methyl -DL-cysteine was inert.

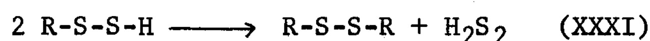
The mechanism of the enzymatic cleavage of cysteine and cystine has been clarified by the work of Mondovi and coworkers (17,18,38,40,87) and Flavin (16). The true substrate of the enzyme is cystine which, even when present only in trace amounts, catalyzes the desulfhydration of large quantities of cystine according to FIG. 5.

FIG. 5

CYSTINE AS THE TRUE SUBSTRATE OF CYSTATHIONASE



In this scheme cysteine reacts non-enzymatically with thiocysteine and cystine is cleaved to pyruvate, ammonia, and thiocysteine by cystathionase. When cystine is the only substrate present, the same three products are formed. Thiocysteine is unstable (18) and decomposes to cystine and polysulfides according to the reaction



$$2\ R\text{-}S\text{-}S\text{-}H \longrightarrow R\text{-}S\text{-}S\text{-}R + H_2S_2 \quad \text{(XXXI)}$$

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Polysulfides then decompose at neutral pH to sulfur and H_2S, while cystine is used again by the enzyme. In this manner the enzymatic cleavage of cystine yields pyruvate, ammonia, sulfur and small amounts of hydrogen sulfide (17). Side reactions account for a lack of stoichiometry among the products. The stoichiometry of the reaction

$$\text{Cystine} + 2\ H_2O \longrightarrow 2\ \text{pyruvate} + 2\ NH_3 + S + H_2S \qquad \text{(XXXII)}$$

is not observed.

The function of the apoenzyme portion of cystathionase is not yet fully understood. Some ideas, however, have been expressed in this regard.

Metzler (79) suggested that the function of pyridoxal-containing enzymes is to aid rearrangement of imine intermediates (this is the rate controlling step) by holding suitable acidic and basic groups of the protein in proper orientation.

Braunstein (67) proposed that in the absence of metal ions a stabilized planary system of double conjugated bonds is set up in the Schiff base, aided by intramolecular hydrogen bonding between the nitrogen atom and the phenolic group. Activation of the Schiff base would occur by the formation of hydrogen bonds or other links between groups of the protein and the nitrogen atom and phenolic hydrogen.

The L-form of the sulfur amino acid substrate of cystathionase seems to be required in the end which is bound (16), but the D-configuration is apparently preferred in the free end. This is suggested by the observation that mesocystine reacts three times faster than L-cystine (16), but yields only one equivalent of pyruvate (88). D-cystine is not a substrate for the γ-cleavage enzyme of _Neurospora_ (60).

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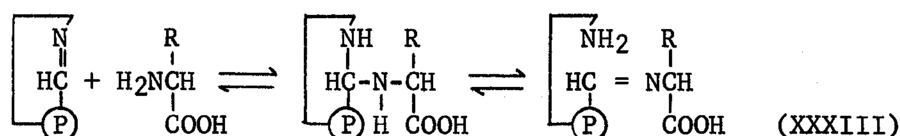
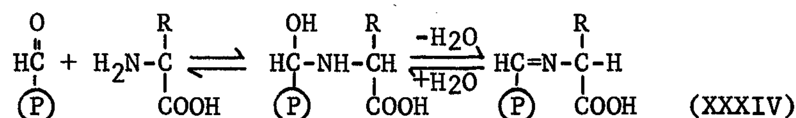
A free carbonyl group has not been observed in any of the pyridoxal phosphate proteins. All of them show spectral bands typical of a Schiff base linkage between coenzyme and protein (6). Fischer and coworkers (89) and Greenberg (33) have reduced the Schiff base form of cystathionase with borohydride. Fischer isolated ε-pyridoxyllysine after hydrolysis; this showed that the carbonyl group is bound to lysine. Schiff base formation cannot involve a reaction between substrate and a free carbonyl group, but rather a process called by Snell "transaldimination" (6). This is shown in the next set of equations:



(XXXIII)

Aldimine formation from pyridoxal and an amino acid can be represented by:



(XXXIV)

Snell proposes that aldimine formation between enzyme and coenzyme enhances the reactivity of the latter (6). This theory is supported by the experimental observation that the semicarbazone of PLP is formed at higher rates by reaction of semicarbazide with an aldimine than with the free carboxyl compound (6,90). After formation of the enzyme substrate complex, a proximal nucleophilic amino group, which has been displaced from the azomethine linkage, may increase the labilization of a proton on the alpha-carbon of an amino acid substrate. The removal of this alpha-hydrogen is necessary for several reactions catalyzed by pyridoxal

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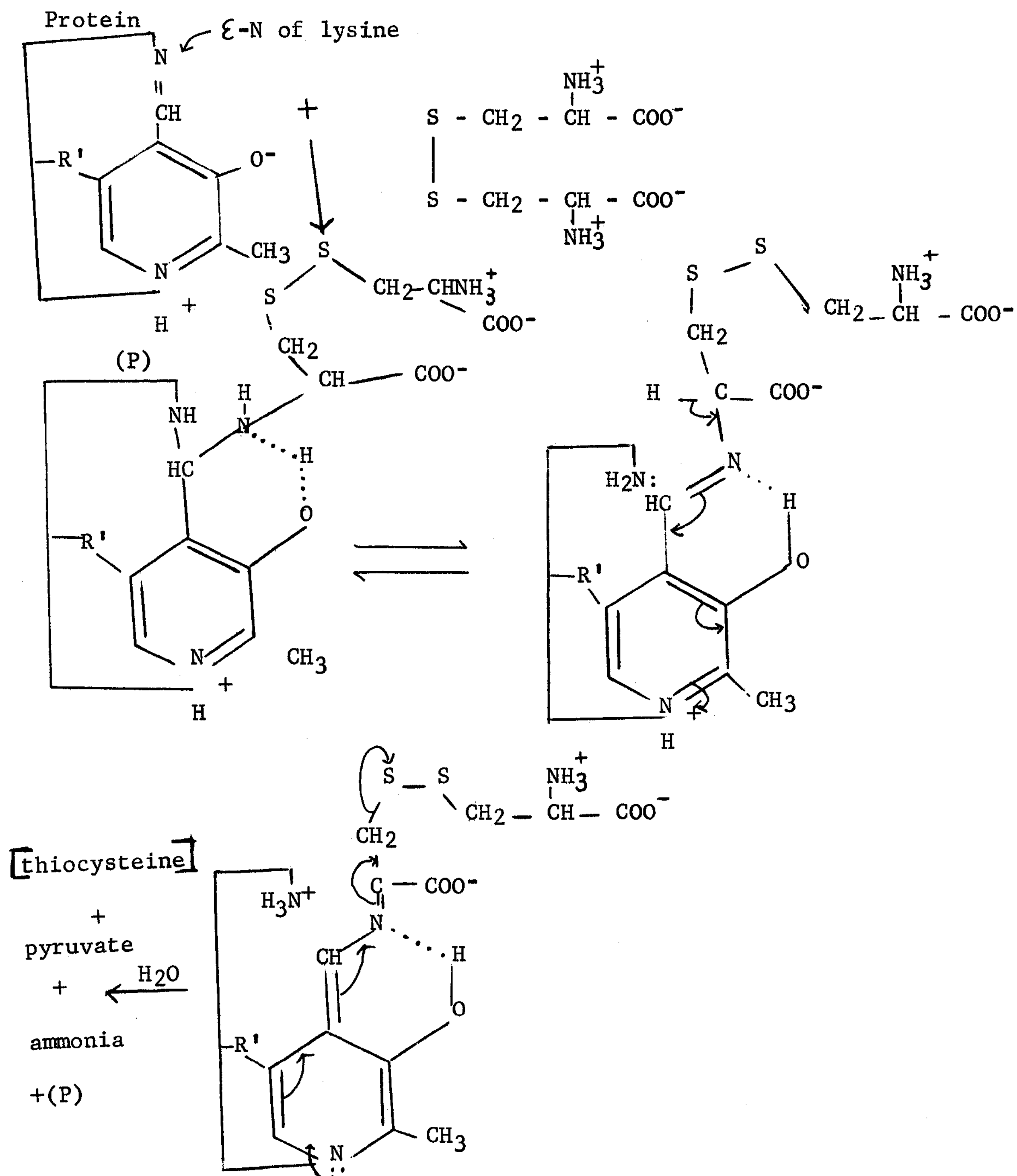
FIG. 6

SPECULATIVE SCHEME FOR THE ENZYMATIC CLEAVAGE OF CYSTINE



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phosphate enzymes. The liberated ϵ-amino group of lysine could thus act as a general acid-base catalyst for the electron shifts catalyzed by pyridoxal phosphate. Snell uses this theory to draw up a scheme for enzymatic transamination (6). However, the general mechanism could also be applied to the cleavage of cystine by cystathionase as shown in FIG. 6.

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CHAPTER II

EXPERIMENTAL

A. MATERIALS AND METHODS

Enzyme Assays and Methods Used with Enzymatic Systems

Several assays were used during the purification of cystathionase, the inhibition studies, and the experiments with alpha-substituted cystines as possible substrates for the enzyme.

1. α-Ketobutyric acid formed from DL-homoserine was determined colourimetrically by the procedure of Friedemann and Haugen (91), modified as described by Sayre and Greenberg (92), and used by Greenberg to assay cystathionase during the enzyme purification (12,14).

2. Pyruvate produced from cystines was determined by the method of Friedemann and Haugen (91), modified by Greenberg and coworkers (39, 92). The incubation mixture for this assay was prepared according to a procedure used by Cavallini _et al_. (18) in their study of the cleavage of cystine by cystathionase.

3. Formation of sulfhydryl compounds from cystines was estimated by the colourimetric ArSSAr assay method of Flavin (16). Slight modifications described by Greenberg (39) were used in some experiments. This assay allowed the determination of the quantity of sulfhydryl produced during long incubations. It was also used in the kinetic assays.

The α-ketobutyrate assay (12,14) is based on the formation of the

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keto acid from L-homoserine as shown in reaction (XXXV).

L-homoserine + H_2O ⟶ α-ketobutyric acid + NH_3 + H_2O (XXXV)

The keto acid produced was determined colourimetrically as the 2,4-dinitrophenylhydrazone. A standard curve was constructed from solutions of α-ketobutyrate. Within the range of 0.1 to 2.0 μ moles of keto acid, the amount of α-ketobutyrate produced was directly proportional to the quantity of enzyme.

During the purification of the enzyme the following assay procedure was used (No. 1(a)): The components of the reaction mixture were prepared in tenfold final concentration, except for the buffer, which was prepared at a concentration of 0.2 M. Incubations were carried out with shaking at 37° in air in test tubes for 30 minutes; for all incubations of this type a Dubnoff Metabolic Shaking Incubator was used. The 1 ml. volumes of reaction mixture contained the following: 1.6×10^{-2} M DL-homoserine, 5×10^{-5} M pyridoxal phosphate, 7.5×10^{-2} M mercaptoethanol, 7×10^{-3} M EDTA, 0.1 M potassium phosphate buffer, pH 7.5, and 0.1 ml. of enzyme solution. The latter was added after a 5 minute preincubation period. The enzyme solution was suitably diluted so that from 0.1 to 1.5 micromoles of the keto acid would be produced.

At the end of the incubation 2.0 ml. of 10% trichloroacetic acid were added to stop the reaction. Any protein precipitate present was removed by centrifugation. Determination of α-ketobutyric acid was carried out on 1.0 ml. aliquots of the supernatant. This was pipetted into a 10-ml. volumetric flask; 1.0 ml. of 0.1% 2,4-dinitrophenylhydrazine solution (in 2 N HCl) was then added. The mixture was shaken and allowed to stand for 5 minutes. Two milliliters of absolute ethanol

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followed by 5 ml. of 2.5 N NaOH were then added quickly with shaking. After the volume had been made up to 10 ml., the mixture was allowed to stand for 10 minutes for complete colour development. The optical density of the solutions was then read at 515 mμ in a Bausch and Lomb Spectronic 20 or a Beckman Model DUR spectrophotometer. The colour of the solution containing the complete reaction mixture was read against that in which the enzyme solution was replaced by water to correct for any spontaneous reaction. Correction was also made for any absorption due to the enzyme.

Protein of crude enzyme preparations (crude homogenate and heated extract) was determined by the biuret method (93). A standard curve was constructed for this purpose from bovine plasma albumin. The protein in all other preparations was estimated spectrophotometrically by measuring light absorption at 280 mμ and 260 mμ (94). The protein concentration was read off from a monograph by E. Adams, as distributed by the California Corporation for Biochemical Research.

An enzyme unit is defined as the amount of enzyme that produces 1 micromole of α-ketobutyric acid per hour. Specific activity is expressed as the number of enzyme units per milligram of protein.

The formation of alpha-ketoacids was also used in the inhibition studies. The α-ketobutyric acid produced from homoserine was determined as the 2,4-dinitrophenylhydrazone, which absorbs strongly at 515 mμ. When L-cystine was also present in the reaction mixture as an inhibitor, the small amount of pyruvate produced from it by the enzyme was not separated from α-ketobutyrate. The 2,4-dinitrophenylhydrazone of pyruvate also absorbs at 515 mμ. Colour production in the presence of L-cystine, therefore, represents the sum of α-ketobutyrate and

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pyruvate formed. However, very little pyruvate was produced compared to α-ketobutyrate in these experiments.

In some experiments concerned with the effects of various concentrations of L-cystine and substituted cystines on the deamination of homoserine by cystathionase the following procedure was used (No. 1(b)): Incubations were performed with shaking at 37° for 1 hour. The reaction mixture contained the following in a total volume of 2 ml., added in the order shown : 0.05 M potassium phosphate buffer, pH 7.5, 3.5×10^{-3} M EDTA, 3×10^{-3} M DL-homoserine, 4.5×10^{-3} M, 3.75×10^{-3} M, 3×10^{-3} M, 2×10^{-3} M, or 1×10^{-3} M L-cystine or substituted cystine, 2.5×10^{-5} M PLP and 4 units of enzyme, added after 5 minutes of preincubation. The reaction was stopped by the addition of 1 ml. of 20% trichloroacetic acid. After the removal of any protein precipitate by centrifugation, the keto acid produced was determined by the standard procedure discussed before, except that, after the addition of 2,4-dinitrophenylhydrazine, the mixture was allowed to stand for 10 minutes.

A similar assay system was used for the determination of inhibition constants and the type of inhibition of homoserine deaminase by various cystines (No. 1(c)). Incubations were performed for 60 minutes or 30 minutes at 37°. The reaction mixture contained in a total volume of 2 ml. (added in the order shown): 0.1 M potassium phosphate buffer, pH 7.5, 3.5×10^{-3} M EDTA, 7.5×10^{-3} M cystine, 2×10^{-2} M, 1.5×10^{-2} M, 1×10^{-2} M, 7.5×10^{-3} M, 5×10^{-3} M, 4×10^{-3} M, 3×10^{-3} M, or 2.5×10^{-3} M DL-homoserine, 2.5×10^{-5} M PLP, and 4 units of enzyme.

L-Cystine was always used as a solution in 0.5 M HCl; to neutralize the acid an equivalent amount of 0.5 N sodium hydroxide was added to the reaction mixtures. In a similar manner α-phenyl-DL-cystine was used as

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a solution in 2 N HCl. When assays No. 1(b) and 1(c) were used spectrophotometric measurements were made with a Beckman Model DUR spectrophotometer, as were the calibration curves for protein and keto acid determination.

The second type of assay used (No. 2) is based on the fact that pyruvate is formed from L-cystine. However, it is difficult to observe the stoichiometry of reaction XXXII experimentally (16). This assay cannot be used for initial rate determinations or to define a unit of enzyme activity. It has been used in this study to check whether substituted cystines are substrates for cystathionase, experiments with relatively long incubation periods and high concentrations of cystines.

A standard curve was prepared using sodium pyruvate. In the concentration range used, 0 - 1.0 micromole of pyruvate, the optical density of the 2,4-dinitrophenylhydrazone solution prepared from the keto acid, was directly proportional to the concentration of pyruvate.

The incubation procedure followed the one used by Cavallini (18) with several minor modifications. Pyruvate was determined by the method of Friedemann and Haugen (91), modified by Greenberg (39,92). Incubations were carried out in thick-walled test tubes of about 15 ml. capacity in air at 38° for a period of 1 hour. The 1 ml. volumes of reaction mixture contained the following in order of addition: 0.1 M potassium phosphate buffer, pH 8, 5 x 10^{-5} M PLP, 7 x 10^{-3} M EDTA, 0.01 M quantities of L-cystine, α-methyl-, α-n-propyl-, or α-isopropyl-DL-cystine, and 2 units of enzyme. Before addition of enzyme the reactants were incubated for 5 minutes. When α-phenyl-DL-cystine was used, the volume of the incubation mixture was 1.5 ml., the amino acid had a concentration of 0.01 M, and the concentration of all other reagents was two thirds of

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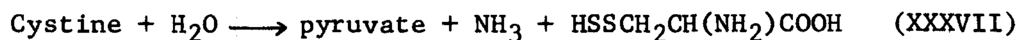
that given above. With the exception of α-phenyl-DL-cystine the amino acids were dissolved in 0.5 N HCl; the reaction mixture was neutralized by the addition of an equivalent amount of 0.5 N NaOH. The phenyl-substituted compound was dissolved in 1.25 N HCl; neutralization was achieved with 0.5 N NaOH. The final pH of the mixtures before incubation was 7.7.

The reaction was stopped by the addition of 2 ml. of 0.1 N HCl.. The colour was developed and read at 515 mμ as in assay No. 1(c), except that all of the liquid was used (3 ml.) and consequently about 4 ml. of sodium hydroxide were added to bring the volume to the 10 ml. mark. The Bausch and Lomb Spectronic 20 spectrophotometer was used for absorbancy determinations.

The third type of assay (No. 3) used is based on the fact that the aromatic disulfide 5,5' dithiobis - (2-nitrobenzoic acid) or ArSSAr (94) reacts with aliphatic thiol compounds near pH 8, a reaction which liberates a strongly coloured aromatic mercaptan (ε=12,000 at 412 mμ)(equation XXXVI).

$$2\ \text{Alkyl-SH} + \text{ArSSAr} \longrightarrow \text{alkyl-SS-alkyl} + 2\ \text{ArSH} \qquad \text{(XXXVI)}$$

ArSSAr is incorporated directly into the reaction mixture. At the pH used the reaction goes instantaneously to completion; this allows a kinetic assay (16). Flavin showed (16) that when ArSSAr is present during the enzymatic cleavage of cystine by cystathionase, equimolar amounts of pyruvate and ArSH were produced according to the reactions:



$$\text{Cystine} + H_2O \longrightarrow \text{pyruvate} + NH_3 + HSSCH_2CH(NH_2)COOH \qquad \text{(XXXVII)}$$

$$\text{RSSH} + \text{ArSSAr} \longrightarrow \text{RSSAr} + \text{S} + \text{ArSH} \qquad \text{(XXXVIII)}$$

In experiments used to determine whether any sulfhydryl groups were

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liberated from L-cystine or the substituted cystines in the presence of cystathionase, incubations were carried out in the presence of ArSSAr in test tubes at 30° for 90 minutes. The procedures used by Greenberg (39) and Flavin (16) were modified as required. The reaction mixtures contained the following in a total volume of 2 ml. (No. 3(a)): 0.1 M potassium phosphate buffer, pH 7.5, 5×10^{-4} M L-cystine, α-methyl-, or α-isopropyl-DL-cystine, 2.5×10^{-3} M PLP, 3.5×10^{-3} M EDTA, 1×10^{-3} M ArSSAr (prepared as a 0.005 M solution in 0.05 M potassium phosphate buffer, pH 7.0), and 10 units of cystathionase. At the end of the incubation 1 ml. quantities of the solution were pipetted into quartz cuvettes (1 cm. light path); and the absorbancy was read at 412 mμ in a Beckman DUR spectrophotometer. The instrument was blanked with a solution containing no enzyme. Solutions of the amino acids were again made up in 0.5 N HCl and neutralized as before.

The rates of formation of aryl mercaptans from cystines were followed continuously (16,39). Production of ArSH with time from ArSSAr and disulfides was followed at 412 mμ for about six minutes (No. 3(b)). Measurements were made with a Beckman Model DUR spectrophotometer and a Gilford Model 2000 Multiple Sample Absorbance Recorder (Beckman Monochromator). The cell compartment was maintained at $37 \pm 0.5°$ by circulating water from a thermostated circulating pump through thermospaces at the ends of the cell compartments. Cuvettes of 1 ml. volume and 1 cm. light path were preincubated for 10 minutes with the following content (in 0.9 ml. total volume): 0.08 M potassium phosphate buffer, pH 7.5, 1×10^{-3} M L-cystine or alpha-substituted cystines, 7×10^{-3} M EDTA, 5×10^{-5} M PLP, and 5×10^{-4} M ArSSAr (prepared as in assay 3(a)). After preincubation, 10 units of enzyme in 0.1 ml. of 0.2 M potassium phosphate

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buffer, pH 7.5, was added with shaking. The change in absorbancy at 412 mμ with time was recorded as quickly as possible after addition of enzyme (about 30 seconds). The appearance of ArSH was also followed in cuvettes which either lacked enzyme, or substrate, or both. Solutions of amino acids were again prepared and neutralized as before. Reaction rates (close to initial rates) were determined from the slopes of the recorded absorbancy curves.

An assay system very similar to 3(b) was used during attempts to determine the Michaelis constant for the cleavage of L-cystine by cystathionase. The use of 0.1 M buffer and aqueous solutions of the amino acid were the only differences.

The last ArSSAr assay system (3(d)) was used at 25° instead of 37°. Changes in the content of the reaction cuvette were as follows: no EDTA was used, the buffer had a pH of 7.2, 1 x 10^{-4} M PLP and 2.5 x 10^{-4} M ArSSAr, 150 units of enzyme, 1.5 x 10^{-5} M L-cystine.

Methods Used in Non-Enzymatic Systems

The first method used to study the behavior of L-cystine and the substituted cystines in an enzyme model system, consisting mainly of the amino acids, PLP, and Cu^{++} in some cases, is based on the production of ArSH from ArSSAr as in the ArSSAr assay (16).

Quartz cuvettes of 1 cm. light path and 1.5 ml. volume contained the following, added in order: 0.1 M potassium phosphate buffer, pH 7.4, 1.7 x 10^{-3} M L-cystine or alpha-substituted cystine, 6.7 x 10^{-5} M cupric chloride (in many experiments copper ions were left out), and 1.2 x 10^{-3} M ArSSAr (prepared in the usual manner). The volume was made up to a total of 1.5 ml. by the addition of 2.6 x 10^{-5} M PLP either after a preincu-

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bation period of 10 minutes at 37° or at once. Amino acids were dissolved in 0.5 N or 2 N HCl (α-phenyl) as usual. Changes in absorbancy at 412 mμ were followed using a Gilford Model 2000 Multiple Sample Absorbance Recorder in conjunction with a Beckman DUR spectrophotometer. The temperature of the cuvette holding the reaction mixture was maintained at 37 $\pm$ 0.5° as described before. Results were represented either as a recording traced by the instrument or a plot of absorbancy against time, made by determining the absorbancy at different time intervals. Rates were obtained from the slopes of the recorded tracings.

Experiments were performed to check, whether there was any production of pyruvate or sulfhydryl groups from L-cystine and the substituted cystines.

Pyruvate was determined as the 2,4-dinitrophenylhydrazone by a slight modification of the usual colorimetric method (39,91,92). Incubations were performed at 37° for one hour. The reaction mixture contained the following in a total volume of 1.5 ml. : 0.1 M potassium phosphate buffer, pH 7.4, 1.7 x 10^{-3} M L-cystine or substituted cystine, 6.7 x 10^{-5} M cupric chloride, and 2.6 x 10^{-5} M PLP added after a 5-minute preincubation period. The reaction was stopped by pipetting 1 ml. of the liquid into 1 ml. of 2.4-dinitrophenylhydrazine solution. The colour was then developed and determined in the usual manner. A blank was run with a solution containing no PLP. All solutions, including the blanks, were also read against water.

Sulfhydryl production was determined using the same incubation procedure. The only difference was the use of 1.2 x 10^{-3} M ArSSAr instead of water. At the end of the incubation period, 1 ml. of solution was pipetted into a cuvette of 1 cm. light path. The absorbancy was deter-

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mined at 412 mμ.

Absorption spectra of Schiff bases formed from PLP and cystines and the spectrum of PLP alone were determined with a Bausch and Lomb Spectronic 505. The spectra were recorded between 300 mμ and 600 mμ or 300 mμ and 450 mμ with a high paper speed. The spectrophotometric cells contained in general in a volume of 1 ml. : 0.09 M buffer, 5×10^{-3} M L-cystine or substituted cystine, and 1.9×10^{-4} M PLP or 2.5×10^{-4} M PLP(when L-cystine was used). The coenzyme was added last with shaking. The spectrum was traced about 30 seconds after addition of PLP. When the effect of time and copper ions was studied, the cuvette contained 0.08 M buffer, 5×10^{-3} M cystine, 1×10^{-4} M cupric chloride, and 2.5×10^{-4} M pyridoxal phosphate.

The rates of formation of Schiff bases from amino acids and PLP were studied by observation of the change in absorbancy with time as recorded with a Gilford Model 2000 Multiple Sample Absorbance Recorder. The wavelength at which the reaction was studied was selected from the spectral data. For a general observation of rates and determination of rate constants a wavelength of 412 mμ was selected. The reaction mixture contained the following reagents in a total volume of 1 ml., added in the order shown: 0.1 M potassium phosphate buffer, pH 7.4, 5×10^{-3} M DL-cystine or substituted cystines or 2.5×10^{-3} M L-cystine, and 2×10^{-4} M PLP. When cupric chloride was added, 1×10^{-4} M reagent was used. Solutions of cystine were prepared in acidic medium, neutralized by sodium hydroxide, as discussed before. The instrument was adjusted to zero absorbancy with the proper blank. For instance, for the complete system above, the blank contained everything but PLP.

The reaction was followed at 25° for time periods of at least 30

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minutes. Results were represented either as variation of absorbancy with time or by a pseudo first order rate constant. The latter were determined graphically by plotting log $\frac{a}{a-x}$ against time, where a is the highest absorbancy recorded (usually that of a steady plateau) and x is the absorbancy at time t. The pseudo first order rate constant equals 2.303 x slope.

Polarographic Measurements

Polarographic methods were used to measure changes in concentration of several cystines during incubation with cystathionase, in order to present further evidence that alpha-substituted cystines are not substrates.

In preliminary experiments with L-cystine as substrate the reaction mixture contained the following in a total volume of 3 ml. (added in the order shown): 3.3×10^{-2} M potassium phosphate buffer, pH 7.4, 2.3×10^{-3} M EDTA, 1×10^{-3} M L-cystine, 1.7×10^{-5} M PLP, 1 ml. of 0.2 N HCl, and 10 units of enzyme. This "stopped blank" was polarographed. The reaction mixture above, minus the mineral acid was incubated at 37° for one hour. The reaction was stopped with the addition of 1 ml. of 0.2 N HCl. Pyruvate produced was determined in the usual manner to check the activity of the enzyme sample. Polarographic measurements were performed on a sample of this type.

When the effect of incubation of α-n-propyl-DL-cystine and α-methyl-DL-cystine with cystathionase was measured polarographically, the following procedure was used: incubations were performed for 1 hr. at 37° on a total volume of 1.6 ml. containing the following: 6.7×10^{-2} M potassium phosphate buffer, 4.4×10^{-3} M EDTA, 1.9×10^{-3} M,

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1.4×10^{-3} M, or 1×10^{-3} M α-n-propyl-DL-cystine or 1.9×10^{-3} M α-methyl-DL-cystine, 3.1×10^{-5} M PLP, and 10 units of enzyme, added after 5 minutes of preincubation. The reaction was stopped with 1 ml. of 0.2 N HCl. The volume was then made up to 3 ml. by the addition of 0.4 ml. of thymol in hydrochloric acid (96), added as a maximum suppressor. For each concentration of amino acid a "blank" in which water replaced the enzyme was also incubated. The diffusion current was determined for the solutions of substituted cystine, incubated in the presence and the absence of the enzyme, with a E. H. Sargent and Co. Model XXI polarograph. Comparison of the diffusion currents allowed one to estimate whether any significant amount of disulfide had been cleaved during the incubation with the enzyme.

The resistance of α-phenyl-DL-cystine to cleavage by the enzyme was studied in a similar manner. In this case 2×10^{-3} M amino acid was incubated in a total volume of 1.8 ml.; 0.2 ml. of thymol were added later. This disulfide had to be dissolved in 2 N HCl, neutralized by 2 N NaOH, while aqueous solutions of the other amino acids could be prepared.

Enzyme Purification

The method described by Greenberg for the purification and crystallization of cystathionase (12,14) was used. Greenberg's procedure was modified slightly in several instances. The details of the purification are presented in the APPENDIX. However, crystallization could not be achieved. The three batches of enzyme were purified partially. During the first two preparations enzyme activity and specific activity were actually reduced during the protamine sulfate treatment. Therefore the

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third purification was ended after the second ethanol fractionation.

Materials

The following materials were obtained from commercial sources: Bovine plasma albumin, crystallized, A grade, DL-cystine, D-cystine, DL-cystathionine (containing some allocystathionine), and EDTA (tetrasodium dihydrate) (California Foundation for Biochemical Research, Los Angeles), L-cystine (The British Drug House Ltd., Poole, England), ArSSAr (Aldrich Chemical Co., Milwaukee, Wisconsin), pyridoxal phosphate, protamine sulfate (Grade II), DL-homoserine, and α-ketobutyric acid (monosodium salt) (Sigma Chemical Co.), Na-pyruvate (Boehringer and Sons, Mannheim, Germany). Frozen rat livers were purchased from Pel-Freez Biologicals, Inc., Rogers, Arkansas. Other Commercial reagent grade chemicals were used without further purification.

Distilled water used for this work was deionized by passage through a column of mixed ion exchange resins supplied by Barnstead Still and Sterilizer Co., Boston Mass. (Model BD-1).

The Synthesis of Alpha-Substituted Cystines

The following substituted amino acids were synthesized: α-methyl-, α-ethyl- (Na_2 salt), α-n-propyl-, α-isopropyl-, α-n-butyl-, and α-phenyl-DL-cystine. The preparation of α-t-butyl-DL-cystine was attempted, but not completed, since the respective hydantoin intermediate could not be hydrolyzed. The methyl-substituted cystine was synthesized by R. M. Ottenbrite in this laboratory using the Strecker synthesis (97). All other amino acids were synthesized via hydantoin intermediates.

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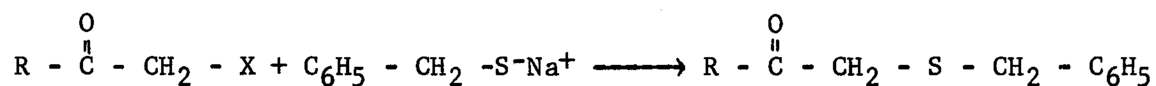
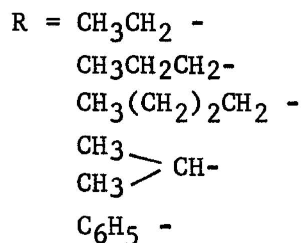
FIG. 7

SYNTHESIS OF ALPHA-SUBSTITUTED CYSTINES



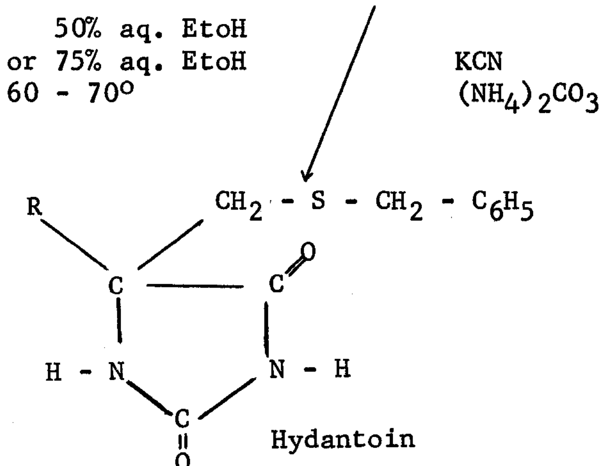
x = Cl or Br



50% aq. EtoH
or 75% aq. EtoH
60 - 70°

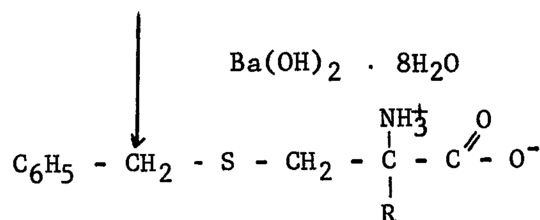
S-benzylmercapto-
methyl alkyl or aryl ketone

KCN
$(NH_4)_2CO_3$



Hydantoin

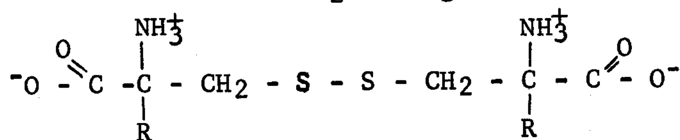
$Ba(OH)_2 \cdot 8H_2O$



S-benzyl amino acid

(1) Na, liquid NH_3

(2) O_2, $FeCl_3$



∝-substituted cystine

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Some theoretical considerations and a description of the synthesis of α-ethyl-, α-n-propyl-, α-n-butyl-, and α-phenyl-DL-cystine have been presented elsewhere (98,99). In this report the synthesis of α-n-propyl -DL-cystine will be shown in detail as a typical example.

With the exception of the methyl-substituted amino acid all cystines discussed here, as well as most of the intermediates prepared during the syntheses, are new compounds, synthesized for the first time in this laboratory.

The reaction scheme shown in FIG. 7 summarizes the synthetic route by which the alpha-substituted cystines were prepared.

Analyses were performed by the Schwarzkopf Microanalytical Laboratory, 56-19 37th Avenue, Woodside 77, New York. Melting points are uncorrected.

Alpha-Haloketones

Either of two standard procedures described in the literature was employed to prepare these compounds (98). 1-Bromo-2-butanone was obtained via the bromination of 2-butanone according to the procedure of Catch *et al.* (98,100). Bromomethyl pinacolone was synthesized from pinacolone by bromination according to the method used by Boyer (101). 1-Chloro-2-pentanone, 1-chloro-2-hexanone, b.p. 173-176°, and 1-chloro -3-methyl-2-butanone, b.p. 149°, were prepared according to Reid (102). The appropriate acid chlorides, butyryl chloride, valeryl chloride, and isobutyryl chloride, respectively, were allowed to react with diazomethane. Decomposition of the diazoketones, formed in this manner, with hydrochloric acid produced the chloromethyl ketones. The preparation of 1-chloro-2-pentanone has been reported in detail (98). The acid chlorides were obtained commercially with the exception of valeryl chloride

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which was prepared from valeric acid following a method used by Brown (103). Phenacyl chloride was a commercial preparation.

Benzylmercaptomethyl Ketones

These compounds were prepared according to the procedure of Wahl (104). The preparation of benzylmercaptomethyl-n-propyl ketone is typical of the method used in the synthesis of these ketones. The sodium salt of α-toluenethiol was prepared by extracting α-toluenethiol (130 g., 1.05 mole) in ether (500 ml.) with 10% sodium hydroxide solution (400 g.). The mixture was shaken intermittantly at room temperature for 2 hours. The aqueous phase was then separated and heated at reflux temperature with 1-chloro-2-pentanone (60 g., 0.50 mole) overnight. The light-yellow top phase was washed with several 25 ml. portions of water until it was free of chloride ions as tested by 10% silver nitrate solution. The heavy oil thus obtained (101 g.) was dried over anhydrous sodium sulfate. Fractional distillation yielded benzylmercaptomethyl -n-propyl ketone as a colourless oil (93.5 g., 90%), b.p. 163-166° (9.5 mm.).

Anal. Calc. for $C_{12}H_{16}OS$: C, 69.19; H, 7.74; S, 15.39

Found: C, 69.17; H, 7.90; S, 15.53.

In a similar manner benzylmercaptomethyl ethyl ketone (b.p. 120-130° (3.5 mm.) was obtained in 98% yield from 1-chloro-2-butanone and sodium benzyl mercaptide (98). Upon fractional distillation a boiling point of 126-128° (3.5 mm.) was obtained.

Anal. Calc. for $C_{11}H_{14}OS$: C, 68.02; H, 7.23; S, 16,51.

Found: C, 68.13; H, 7.19; S, 16.28.

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Benzylmercaptomethyl-n-butyl ketone was obtained in a crude yield of 97% from 1-chloro-2-hexanone and sodium benzylmercaptide. Fractional distillation produced a colourless oil, b.p. 166-167° (6 mm.).

Anal. Calc. for $C_{13}H_{18}OS$: C, 70.21; H, 8.15; S, 14.42.

Found: C, 70.25; H, 8.30; S, 14.50.

Benzylmercaptomethyl phenyl ketone, m.p. 85°, was prepared in 97% yield from phenacyl chloride and sodium benzyl mercaptide. Because phenacyl chloride is insoluble in water an alcoholic reaction medium was used. This compound had been prepared previously by Wahl (104) and his procedure was followed.

Benzylmercaptomethyl isopropyl ketone was prepared in the standard manner with a crude yield of 95% from 1-chloro-3-methyl-2-butanone and sodium benzylmercaptide. Upon fractional distillation a colourless oil, b.p. 147-150° (9mm.), was obtained.

Anal. Calc. for $C_{12}H_{16}OS$: C, 69.19; H, 7.74; S, 15.39.

Found: C, 69.02; H, 7.53; S, 15.23.

Benzylmercaptomethyl t-butyl ketone was obtained in a crude yield of 97% from t-butyl bromomethyl ketone and sodium benzylmercaptide. Fractional distillation produced a faintly yellow oil (218 g., 92%), b.p. 166-170° (13 mm.).

Anal. Calc. for $C_{13}H_{18}OS$: C, 70.21; H, 8.15; S, 14.42.

Found: C, 69.98; H, 8.01; S, 14.47.

Benzylmercaptomethyl Alkyl Hydantoins

These compounds were prepared according to the method of Bucherer

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(105). The preparation of 5-benzylmercaptomethyl-5-n-propyl hydantoin is an example of the method used in the synthesis of the alkylated hydantoins. A mixture of benzylmercaptomethyl-n-propyl ketone (90 g. 0.43 mole), potassium cyanide (40 g., 0.61 mole) and ammonium carbonate (135 g., 1.40 mole) dissolved in 400 ml. of 50% ethanol was heated in a 1-litre, three-necked, round-bottomed flask (equipped with a condenser and mechanical stirrer) on a water bath at 60-70° for seven hours. The dark-yellow solution was allowed to partially evaporate overnight. The residue was then poured on crushed ice. The white precipitate was separated by filtration, washed with water and small quantities of ether, and dried in a vacuum dessicator. This procedure gave 52 g. (97%) of hydantoin, m.p. 148-151°. After one crystallization from hot ethanol white plates, m.p. 154°, were obtained.

Anal. Calc. for $C_{14}H_{18}N_2O_2S$: C, 60.37; H, 6.51; N, 10.11; S, 11.51.
Found: C, 60.50; H, 6.74; N, 10.17; S, 11.70.

Similarly 5-benzylmercaptomethyl-5-ethyl hydantoin, m.p. 125-128°, was obtained in 90% yield from benzylmercaptomethyl ethyl ketone, potassium cyanide, and ammonium carbonate. One crystallization from hot ethanol yielded white needles, m.p. 130-131°.

Anal. Calc. for $C_{13}H_{16}N_2O_2S$: C, 59.03; H, 6.10; N, 10.65; S, 12.12.
Found: C, 59.09; H, 6.05; N, 10.40; S, 11.85.

5-Benzylmercaptomethyl-5-n-butyl hydantoin, m.p. 140-145°, was obtained in 71% yield from benzylmercaptomethyl-n-butyl ketone. One crystallization from hot ethanol gave white needles, m.p. 169°.

Anal. Calc. for $C_{15}H_{20}N_2O_2S$: C, 61.61; H, 6.89; N, 9.58.

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Found: C, 61.56; H, 6.64; N, 9.29.

5-Benzylmercaptomethyl-5-isopropyl hydantoin, m.p. 139-145°, was prepared in 88% yield from benzylmercaptomethyl isopropyl ketone in a similar manner after a reaction period of ten hours. Two crystallizations from hot ethanol yielded a white crystalline solid, m.p. 153-154°.

Anal. Calc. for $C_{14}H_{18}N_2O_2S$: C, 60.37; H, 6.51; N, 10.11; S, 11.51.
Found: C, 60.62; H, 6.67; N, 10.10; S, 11.78.

5-Benzylmercaptomethyl-5-t-butyl hydantoin was synthesized from benzylmercaptomethyl-t-butyl ketone under somewhat different experimental conditions. When standard conditions were used a large proportion of the starting material was recovered.

A solution of potassium cyanide (17.5 g., 0.27 mole) and ammonium carbonate (115 g., 1.19 mole) in 625 ml. of water was added to a solution of the ketone (35 g., 0.16 mole) in 625 ml. of absolute ethanol. The resulting mixture was heated and stirred at 65-75° in the usual manner. After twenty hours a light yellow, faintly cloudy mixture was observed. The reaction was then allowed to proceed for another ten hours. The contents of the reaction vessel was allowed to evaporate partly at room temperature overnight. The solid, which had precipitated, was separated by filtration and dried in a vacuum dessicator. Crystallization from hot ethanol yielded 25.6 g. of white, crystalline plates, m.p. 184-186°. Evaporation of the ethanol filtrate gave another 4.5 g. of the product for a final yield of 66%.

Anal. Calc. for $C_{15}H_{25}N_2O_2S$: C, 61.59; H, 6.89; N, 9.58; S, 10.97.
Found: C, 61.44; H, 6.65; N, 9.44; S, 10.72.

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5-Benzylmercaptomethyl-5-phenyl Hydantoin

The Bucherer-Bergs method was modified slightly; the reaction time was increased and a higher percentage of ethanol was used.

A mixture of benzylmercaptomethyl phenyl ketone (125 g., 0.52 mole) and 1500 ml. of absolute ethanol was stirred at 60° until all the solid had dissolved. A solution of potassium cyanide (50 g., 0.77 mole) dissolved in 500 ml. of water was added with stirring. Ammonium carbonate (300 g., 3.13 mole) was then added. A clear, light-yellow solution was obtained after this mixture had been stirred at 60-70° for about 20 hours. This was allowed to react for another 28 hours, decanted into a beaker, and allowed to stand at room temperature for several hours whereupon a white solid precipitated. This was recovered by filtration, m.p. 164-168°. The filtrate was poured on a mixture of ice and hydrochloric acid. The solid which separated was recovered by filtration.

All of the solid material obtained was crystallized from hot ethanol to yield 110 g. of pure 5-benzylmercaptomethyl hydantoin, m.p. 175-176°. More hydantoin, 5 g., m.p. 175-176°, was recovered by partial evaporation of the combined filtrates. A total yield of 115 g. (71%) of hydantoin, m.p. 175-176°, was thus obtained.

Anal. Calc. for $C_{17}H_{16}N_2O_2S$: C, 65.34; H, 5.17; N, 8.97; S, 10.27.
Found: C, 65.61; H, 5.26; N, 8.63; S, 10.50.

S-Benzyl-α-alkyl-DL-cysteines

The preparation of S-benzyl-α-n-propyl-DL-cysteine is typical of the procedure used, where R = ethyl, n-propyl, or n-butyl. Potts (106) used a similar method in the preparation of S-benzyl-α-methyl-DL-cysteine.

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A mixture of 5-benzylmercaptomethyl-5-n-propyl hydantoin (60 g., 0.27 mole), barium hydroxide (315 g., 1.0 mole) and 1500 ml. of water was heated at reflux temperature for forty hours in a 2-litre, round -bottomed flask. Barium was precipitated as barium carbonate at 100° using carbon dioxide generated from the reaction of marble chips with hydrochloric acid. The barium carbonate was separated by filtration and washed with boiling water and then acetone. When the filtrate was allowed to cool, unreacted hydantoin (13 g.) precipitated and was removed by filtration. The filtrate was then allowed to evaporate at room temperature; and S-benzyl-α-n-propyl-DL-cysteine precipitated after several hours. It was separated by filtration and washed with small amounts of acetone. A crude yield of 72% (39 g.), m.p. 218-220° dec. was obtained. After the product was recrystallized from hot water, washed with acetone and dried over phosphorous pentoxide in a vacuum dessicator, its melting point was 229° dec. The ninhydrin test gave a reddish-purple colour.

Anal. Calc. for $C_{13}H_{19}NO_2S$: C, 61.61; H, 7.56; N, 5.56; S, 12.65.

Found: C, 61.47; H, 7.62; N, 5.56; S, 12.87.

Similarly, S-benzyl-α-ethyl-DL-cysteine, a white powder, m.p. 224-226° dec. was obtained in 74% yield from 5-benzylmercaptomethyl-5-ethyl hydantoin.

Anal. Calc. for $C_{12}H_{17}NO_2S$: C, 60.20; H, 7.15; N, 5.88; S, 13.31.

Found: C, 60.50; H, 7.00; N, 5.93; S, 13.64.

In a like manner from 5-benzylmercaptomethyl-5-n-butyl hydantoin S-benzyl-α-n-butyl-DL-cysteine, m.p. 210-214° dec., in 73% yield was

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obtained. After purification from hot water a white powder, m.p. 229-230° dec. resulted.

Anal. Calc. for $C_{14}H_{21}NO_2S$: C, 62.99; H, 7.89; N, 5.22; S, 11.97.
Found: C, 62.73; H, 7.96; N, 5.05; S, 12.22.

When the standard conditions described above were used to prepare S-benzyl-α-isopropyl-DL-cysteine, more than fifty percent of starting material were recovered. The hydrolysis of the hydantoin was, therefore, carried out under somewhat different conditions.

5-Benzylmercaptomethyl-5-isopropyl hydantoin (30 g., 0.108 mole) was added slowly to a boiling, stirred solution of barium hydroxide (500 g., 1.6 mole) in 750 ml. of water. The mixture was heated at reflux temperature in a 2-litre, round-bottomed flask. After one day a white solid was observed along the walls of the flask. The mixture was allowed to react for a total of four days. Barium hydroxide was then removed in the usual manner. The filtrate thus obtained was allowed to evaporate to a small volume, whereupon 12 g. of a white solid, m.p. 234-238° dec., precipitated. The product was separated by filtration and washed with several small portions of acetone to remove traces of unreacted hydantoin. Further evaporation of the filtrate allowed the recovery of another 1.5 g. of product. In this manner a total crude yield of 13.5 g. (49%) was obtained. By washing the barium carbonate with several portions of acetone, 10.5 g. of unreacted hydantoin were recovered. Purification of the S-benzyl amino acid in the usual manner produced a white, crystalline powder, m.p. 239-241° dec.

Anal. Calc. for $C_{13}H_{19}NO_2S$: C, 61.61; H, 7.56; N, 5.56; S, 12.65.
Found: C, 61.41; H, 7.48; N, 5.68; S, 12.54.

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Attempts to hydrolyze the hydantoin (2 g., 0.007 mole) with 1 N sodium hydroxide solution (100 ml.) by heating the clear solution at reflux temperature for four days were unsuccessful. More than sixty percent of starting material was recovered.

In another experiment hydantoin (2 g., 0.007 mole) was stirred at room temperature with 20 ml. of 90% sulfuric acid for one day. The dark solution was poured on crushed ice; a yellowish solid precipitated, but redissolved when the mixture was stirred. The pH of the solution was raised to about 6 by the slow additon of concentrated ammonium hydroxide. When the solution was allowed to evaporate on the steambath, 1.3 g. of a light yellow solid precipitated. Washing with acetone and determination of melting point showed that the material was a mixture of hydantoin and a small fraction of S-benzyl amino acid.

5-Benzylmercaptomethyl-5-t-butyl hydantoin could not be hydrolyzed to the S-benzyl amino acid with barium hydroxide. Several other reaction conditions were, therefore, tried.

A solution of hydantoin (2 g., 0.007 mole) in 0.25 N NaOH (50 ml.) was stirred and heated at reflux temperature for forty hours. The clear liquid was poured on crushed ice; and the resulting solution was evaporated on a steambath to a volume of about 40 ml. During dropwise addition of concentrated hydrochloric acid 1.8 g. of unreacted hydantoin precipitated.

When a solution of hydantoin (2 g.) in 25 ml. of 95% trifluoroacetic acid was stirred and heated at reflux temperature for twenty-four hours, only unreacted starting material was recovered after evaporation of the acid at room temperature.

In another experiment hydrolysis of the hydantoin (1 g., 0.004 mole)

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was attempted by stirring a solution of the solid in 90% sulfuric acid (30 ml.) at room temperature for about twenty hours. The dark green solution obtained in this way was added dropwise to a mixture of concentrated ammonium hydroxide and crushed ice. When a pH of about 7 was reached, a light-yellow solid began to precipitate out of solution. This material did not dissolve again when the pH was lowered further. It was separated by filtration, washed with several small portions of water and acetone, and allowed to dry at room temperature. In this manner 0.5 g. of a white, amorphous powder, m.p. 314-314° dec. were obtained. It was crystallized from boiling ethanol. During decomposition a pungent, irritating odour plus the odour of hydrogen sulfide were noticed. After ignition of the solid a charcoal residue remained. The analysis did not agree with the composition of the S-benzyl amino acid.

Anal. Calc. for $C_{14}H_{21}NO_2S$: C, 62.88; H, 7.92; N, 5.24; S, 11.99.
Found: C, 47.96; H, 5.98; N, 13.61; S, 15.37.

The analysis agreed fairly well with that of the disulfide which could be formed from two molecules of hydantoin after removal of the benzyl groups as benzyl alcohol.

Anal. Calc. for $C_{16}H_{26}N_4O_4S_2$: C, 47.74; H, 6.51; N, 13.92; S, 15.93.

However, benzyl alcohol could not be detected in the reaction mixture by ether extraction of the filtrate obtained after removal of the solid. Attempts to salt out any alcohol present from the filtrate by addition of sodium chloride also had negative results. Because only small amounts of hydantoin were available, no further experiments were performed to identify conclusively the reaction product as the disulfide.

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The presence of the bulky t-butyl group on the hydantoin ring makes hydrolysis very difficult, if not impossible. Alkyl substituents, β to the carbonyl, are known to be very effective "steric hinderers" (107) in this type of hydrolysis reaction. Newman's rule of six (108) is very applicable here. However, a sealed-tube reaction similar to the ones used by Elks (109), for instance, to hydrolyze hydantoins, might lead to conversion of the hydantoin to the S-benzyl amino acid.

S-Benzyl-α-phenyl-DL-cysteine

A mixture of 5-benzylmercaptomethyl-5-phenyl hydantoin (42 g., 0.13 mole), and 860 ml. of water was heated at reflux temperature with stirring. Only part of the hydantoin dissolved. After a reaction time of seven days a large quantity of a white solid appeared in the reaction flask. This was recovered by filtration. The barium in the filtrate was precipitated as barium carbonate and was separated by filtration. The filtrate was partially evaporated on a hot plate whereupon some S-benzyl-α-phenyl-DL-cysteine precipitated. This was separated by filtration and washed with acetone, (m.p. 236-240° dec.). However, only a few grams of material were obtained in this manner. The solid removed previously from the reaction flask was added with stirring into 3 l. of boiling water. Carbon dioxide was then passed into the mixture to precipitate barium as barium carbonate. The latter was then removed by filtration. Evaporation of the filtrate yielded a few grams of desired product, m.p. 235-239° dec. Most of the amino acid was apparently adsorbed to the barium carbonate. This could be recovered by stirring the mixture of inorganic and organic materials with several 3-l. quantities of boiling water, followed by filtration and evaporation of the filtrate. This process yielded a total of 30.5 g. (79%) of the cysteine, m.p.

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236-240° dec.

The cysteine is very insoluble in hot water. In order to purify it, it was dissolved in 3 N sulfuric acid. Insoluble impurities were removed by filtration. Dropwise addition of concentrated ammonium hydroxide caused the amino acid to precipitate. Precipitation began at about pH 2. The final pH was 5. The material melted at 242-243° dec.

Anal. Calc. for $C_{16}H_{17}NO_2S$: C, 66.80; H, 5.96; N, 4.88; S, 11.16.
Found: C, 67.12; H, 5.67; N, 5.09; S, 11.13.

α-Alkyl Cystines

A method used by Arnstein (110) and Wood and du Vigneaud (111) was followed with slight modifications. The preparation of α-n-propyl-DL-cystine is typical of the procedure used. Sodium metal (7.5 g., 0.33 mole), cut into small pieces and freshly dried S-benzyl-α-n-propyl-DL-cysteine (25 g., 0.077 mole) were added alternatively in small amounts with stirring to about 500 ml. of liquid ammonia over a period of thirty minutes. After one hour the blue colour of the mixture was discharged with 3 g. of ammonium chloride. The reaction mixture was left at room temperature overnight to allow the ammonia to evaporate. The white paste which remained was dissolved in 350 ml. of cold water. The insoluble material was removed by filtration. The filtrate was then extracted with 35 ml. of ether. The pH of the filtrate was adjusted to 7.5 with concentrated hydrochloric acid and a trace (about 5 mg.) of ferric chloride was added. The dark-violet solution was aerated for thirty hours. After this period no sulfhydryl groups were present as tested by sodium nitroprusside. The pH was then adjusted to 6. When this solution was evaporated on a steam bath to a volume of about 200 ml.,

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the α-n-propyl-DL-cystine precipitated as a yellow, amorphous powder. After recrystallization from boiling water which contained a small amount of decolourizing charcoal, a white solid was obtained, m.p. 260° dec. The melting point was found to vary with variation in rate of heating. A yield of 11.6 g. (72%) was obtained.

Anal. Calc. for $C_{12}H_{24}N_2O_4S_2$: C, 44.42; H, 7.46; N, 8.63.
Found: C, 44.11; H, 7.54; N, 8.50.

In a similar manner α-ethyl-DL-cystine was obtained as the disodium salt from S-benzyl-α-ethyl-DL-cysteine in 72% yield by a final precipitation at a pH of 7.5. The material precipitated as a white solid, which began to turn dark in colour at about 250° and decomposed at 254-258°.

Anal. Calc. for $C_{10}H_{20}N_2O_4S_2Na_2$: C, 35.06; H, 5.87; N, 8.18; S, 18.73.
Found: C, 34.64; H, 6.15; N, 8.13; S, 18.64.

α-n-Butyl-DL-cystine was obtained from S-benzyl-α-n-butyl-DL-cysteine in 70% yield. The white solid started to darken slowly at about 220°. Decomposition took place at 245-249°.

Anal. Calc. for $C_{14}H_{28}N_2O_4S_2$: C, 47.70; H, 8.01; N, 7.95; S, 18.16.
Found: C, 47.52; H, 7.86; N, 7.84; S, 17.89.

In the synthesis of α-isopropyl-DL-cystine from S-benzyl-α-isopropyl-DL-cysteine, the solution obtained in the usual manner after oxidation and adjustment of the pH to six, was allowed to evaporate to dryness on a steambath. The white, amorphous powder thus obtained was stirred with a little water. Separation by filtration yielded 4.35 g. of a white solid, m.p. 281-284° dec. The filtrate was allowed to

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evaporate to dryness. The residue was again stirred with a little water. After separation by filtration,another 1.9 g. of product were recovered. Crystallization of the total amount of product, 6.25 g., 51%, from hot water yielded 5.9 g. of a white, crystalline powder, m.p. 286-289° dec.

Anal. Calc. for $C_{12}H_{24}O_4N_2S_2$: C, 44.42; H, 7.46; N, 8.63; S, 19.77.
Found: C, 44.18; H, 7.62; N, 8.50; S, 19.94.

α-Phenyl-DL-cystine

This amino acid was isolated in 57% yield as a light-yellow solid, m.p. 241-243° dec. by following a procedure similar to that used for the alkyl-substituted cystines. A brown colouration was observed at 210°. The cystine was further purified by dissolution in hot hydrochloric acid (2 N) followed by the addition of a small amount of Norit. Filtration, followed by dropwise addition to the filtrate of concentrated ammonium hydroxide (pH 5) produced colourless crystals, m.p. 243-245° dec.

Anal. Calc. for $C_{18}H_{20}N_2O_4S_2$: C, 55.08; H, 5.14; N, 7.10; S, 16.34.
Found: C, 54.84; H, 4.94; N, 7.33; S, 16.92.

B. RESULTS

Enzyme Purification

The isolation and partial purification of homoserine deaminase was performed three times. In the first two purification procedures attempts were made to get a crystalline preparation. Both times, however, activity was lost in the protamine sulfate treatment, apparently during dialysis. Therefore, the third preparation was stopped after the second ethanol fractionation step. This time a high specific activity was

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obtained in the enzyme sample which was used for the following experiments.

During the first preparation's protamine sulfate step the enzyme solution was dialyzed against 15 volumes of 0.2 M potassium phosphate buffer, pH 7.2, for a total of 24 hours at 5°. The buffer was changed every 8 hours. During this treatment the activity decreased from 13,300 units to 8100 units; the specific activity went down from 61 to 24. Dialysis against 15 volumes of cold 0.02 M potassium phosphate buffer, pH 7.2, brought the number of units down to 3600 and the specific activity to 15.

The final preparation of enzyme in 0.2 M potassium phosphate buffer, pH 7.2, was used as such in experiments concerned with the substrate behavior of the substituted cystines. This solution contained 6.5 mg. protein/ml. and 95 units of enzyme per millilitre. Its specific activity was 15. It was not used for inhibition studies.

In the second purification the specific activity changed from 64.8 to 17 during the protamine sulfate step. The enzyme solution used in subsequent experiments contained 160 units/ml. and 8.3 mg. protein/ml. Specific activity was found to be 19.1. This sample was stored in the deep freeze for 50 days. During this time the activity decreased from 158 units/ml. to 124 units/ml. The specific activity went down from 19 to 15. The process of thawing and refreezing resulted in losses of activity. However, the third preparation of enzyme lost little activity during two months of storage in the deep freeze.

The purification of the enzyme sample used for most of this study is summarized in TABLE 1. Note that after the second ethanol treatment the yield of units is 70% of that obtained by Greenberg (12), but the

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LEGEND TO TABLE 1

The enzyme was prepared from 950 g. of rat livers. Greenberg used 1500 g. (12). Values presented in brackets are the ones obtained by Greenberg (12). Protein was determined as described under methods. One unit of enzyme produces 1 micromole of α-ketobutyric acid per hour. Specific activity is the number of enzyme units per milligram of protein.

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TABLE 1

SUMMARY OF PURIFICATION OF HOMOSERINE DEAMINASE

Fraction	Protein (mg.)	Yield of Protein (%)	Total Activity (units)	Yield of Units (%)	Specific Activity
Crude Homogenate	2.78×10^5 (5.22×10^5)	100 (100)	2.22×10^5 (3.27×10^5)	100 (100)	0.8 (0.6)
Heated Extract	5.70×10^4 (2.43×10^5)	20.5 (47)	1.90×10^5 (2.62×10^5)	86 (80)	3.3 (1.1)
Ammonium Sulfate Fraction	1.45×10^4 (1.94×10^5)	5.2 (7.2)	1.21×10^5 (1.94×10^5)	54.5 (60)	8.3 (5.1)
First Ethanol Fraction	8.1×10^2 (5.65×10^3)	0.3 (1.1)	8.50×10^4 (1.42×10^5)	38.3 (43.4)	105 (25)
Second Ethanol Fraction	2.87×10^2 (8.20×10^2)	0.1 (0.16)	6.17×10^4 (1.30×10^5)	28 (40)	215 (159)

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specific activity is higher. Greenberg obtained a crystalline enzyme sample with a specific activity of 346 in a yield of 14%.

The purified enzyme was divided into 1 ml. volume samples and stored in the deep freeze. It contained 4.6 mg. protein/ml. of 0.2 M potassium phosphate buffer, pH 7.5 and 995 units/ml. For use the sample was diluted usually 1:25 with the same buffer.

Assays

The assay 1(a), based on the production of α-ketobutyrate from homoserine, was applied conveniently to follow the purification of the enzyme preparation. With this assay the activity could be determined rapidly. It showed that mercaptoethanol has little effect on the activity of the enzyme, but that the addition of EDTA and PLP was required for full activity.

The pyruvate assay, No. 2, could be applied only to large scale incubations at long time intervals. It was not sensitive or accurate enough to detect small changes in keto acid production. The colour developed tended to be unstable; timing was, therefore, very critical. Tubes containing the complete system and their blanks had to have their colour developed and read at the same time.

The ArSSAr assay was found to be most useful for the determination of sulfhydryl group production during incubation for half an hour or more and to get comparative data for rates of reaction of L-cystine and substituted cystines. When it was used in kinetic studies of the rates of reaction of L-cystine, it was found to be inadequate, not because it was not sensitive enough, but, because of interference by impurities in the enzyme sample (preparation 1 or 2).

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Study of the Enzymatic System

Substrate Behavior of Cystines

The effect of cystathionase on L-cystine and alpha-substituted cystines is summarized in TABLE 2. Pyruvic acid and sulfhydryl groups are produced only when L-cystine is the substrate. When the hydrogen atom is replaced, no keto acid or ArSH is formed during incubation; and the rate of formation of sulfhydryl is zero.

Attempts to determine the Michaelis constant for the enzyme with L-cystine using the ArSSAr assay were not successful. It was not possible to determine initial rates from the slopes of the absorbancy at 412 mμ vs. time plots. The lines were not straight, but curving; therefore, the drawing of tangents to determine the initial rate could not be accomplished. The reaction was complete within about a minute and had been underway for about thirty seconds, before a reading could be taken. The change in rate when the concentration of amino acid was increased or decreased was small and impossible to determine accurately, because of the speed of the reaction, the curvature of the line, and the interference by the enzyme. The enzyme protein itself reacted with ArSSAr. The rate of formation of sulfhydryl from this source was one half the highest rate observed in the presence of L-cystine. With more dilute enzyme this interference disappeared, but the rates were too small to be measured now.

When the enzyme was preincubated with ArSSAr, in order to eliminate the rate due to the enzyme protein, the catalyst lost its activity.

Polarographic studies were made in order to determine, whether there was any appreciable decrease in the concentration of a substituted cystine after incubation with cystathionase. The results of these

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LEGEND TO TABLE 2

The table contains the result of three separate experiments. Therefore pyruvate and mercaptan production cannot be compared. Experimental conditions varied between the sets of experiments.

Pyruvate was determined as described under methods. Mercaptan, produced during an incubation period of 90 minutes, was determined with assay 3(a). The rates of mercaptan formation were followed with assay 3(b).

The quantities of ArSH produced were calculated using a molar absorbancy index of 12000 (16).

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TABLE 2

EFFECT OF CYSTATHIONASE ON ALPHA-SUBSTITUTED CYSTINES

Amino Acid	μ mole/60 min./ml. of Pyruvic Acid Produced	μ mole/90 min./ml. of ArSH Produced [a]	Rate of Formation of ArSH	
			$\frac{O.D._{412} \times 10^3}{min.}$	μ mole/min./ml. $\times 10^3$
L-Cystine	0.19	0.087	91	7.6
α-Methyl-DL-Cystine	0.0	0.0	0.0	0
α-Ethyl-DL-Cystine			0.0	0
α-n-Propyl-DL-Cystine	0.0		0.0	0
α-n-Butyl-DL-Cystine			0.0	0
α-Isopropyl-DL-Cystine	0.0	0.0	0.0	0
α-Phenyl-DL-Cystine	0.0		0.0	0

[a] A comparatively large amount of sulfhydryl was liberated from the enzyme protein itself during incubation (about 0.02 μ moles).

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TABLE 3

POLAROGRAPHIC DETERMINATION OF THE EFFECT OF CYSTATHIONASE ON ALPHA-SUBSTITUTED CYSTINES

Amino Acid	Concentration (m M)	i_D (μ amp.) Complete System	Blank[a]
	1.0	6.02	6.13
α-n-Propyl-DL-Cystine	0.75	4.66	4.75
	0.50	3.21	3.25
α-Methyl-DL-Cystine	1.0	6.30	6.53
α-Phenyl-DL-Cystine	1.0	5.70	5.89

[a] The "blank" contained amino acid incubated in the absence of enzyme - see METHODS.

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experiments are summarized in TABLE 3. It is apparent that there is no significant change in the concentration of α-n-propyl-DL-cystine when enzyme is present. Similarly, samples of α-methyl-DL-cystine or the phenyl amino acid, incubated in the absence and presence of enzyme, produced diffusion currents with only small differences in magnitude.

Preliminary experiments were performed to find an incubation system with no interfering polarographic waves. Trichloroacetic acid produced a large wave in the neighborhood of the cystine reduction wave. Therefore hydrochloric acid was used to stop the enzymatic reaction. The effect of the enzyme on the concentration of L-cystine could not be determined with the polarograph, since pyruvate, a reaction product, produced an interfering wave. When pyruvate itself was studied with the polarograph, a similar wave was observed. This interference was not observed, when the substituted cystines were incubated with enzyme and reduced polarographically.

Inhibition of Homoserine Deaminase

The effects of L-cystine and alpha-substituted cystines as well as cystathionine on the deamination of homoserine by the enzyme studied are summarized in TABLE 4 and 5 and FIGURES 8, 9, 10.

Formation of α-ketobutyric acid from homoserine was studied as a function of time. With 5×10^{-3} M amino acid the response was linear for at least 60 minutes. Lower concentrations of homoserine were not used. However, in FIG. 9, a plot of $1/v_o$ vs. $1/s$ with homoserine as substrate is linear for all concentrations of the amino acid used. K_m values were the same, whether an incubation period of 30 minutes or 60 minutes was used.

FIG. 8 shows the effect of different concentrations of L-cystine

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FIG. 8

INHIBITION OF HOMOSERINE DEAMINASE

Legend

The rates of production of alpha-keto acid were determined by assay 1(b). All concentrations used are presented under Methods except for the concentrations of L-cystine which were as follows: 4.5 mM, 3 mM, 2 mM, 1.5 mM, and 1 mM.

The rates (v) are expressed as μ moles of α-keto acid produced per 60 minutes incubation. The inhibiting sulfur amino acid concentrations I, are expressed as μ moles per ml. (mM).

The cystines used are:

(○) α-methyl-DL-cystine,

(□) α-ethyl-DL-cystine,

(▲) α-butyl-DL-cystine,

(•) α-phenyl-DL-cystine,

(■) L-cystine.

The inhibition by α-n-propyl- and α-isopropyl-DL-cystine was also studied, but is not represented here, because the effect of these two amino acids was very much like that of the ethyl or butyl compound.

Since experiments were performed on different days and with differences in the activity of the enzyme samples used, values of v in the figure are normalized.

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FIG. 8

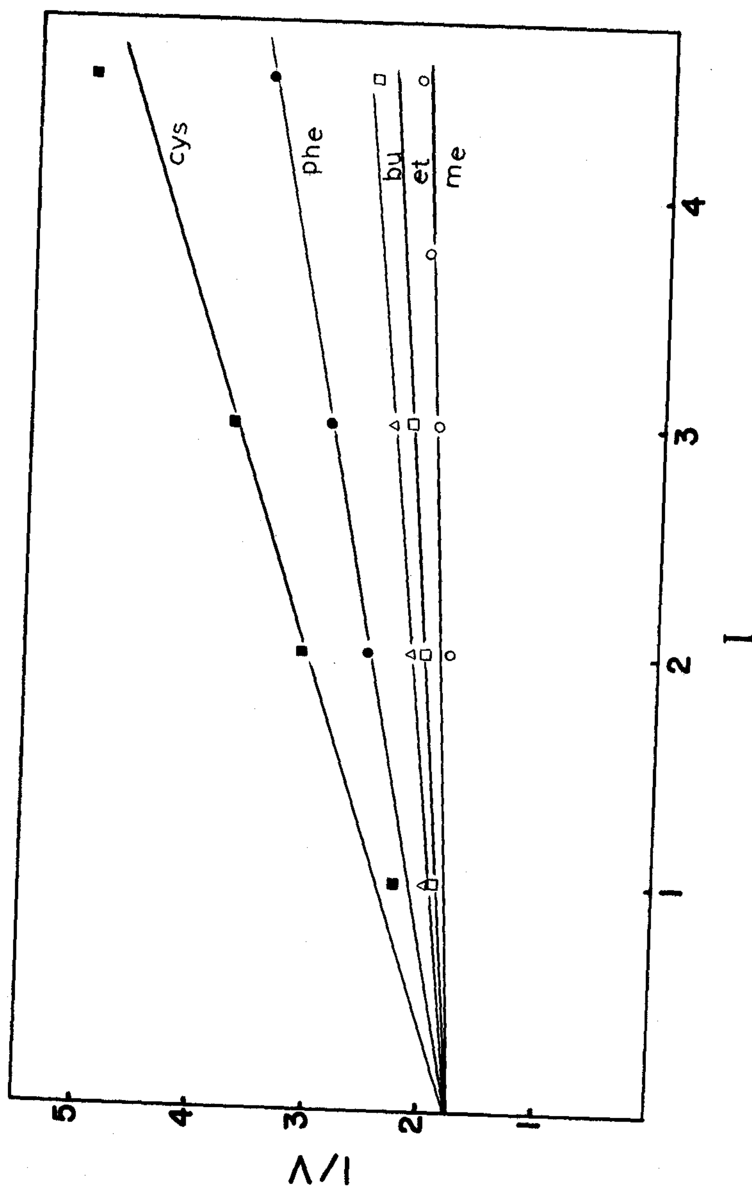

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FIG. 9

COMPETITIVE INHIBITION OF HOMOSERINE DEAMINASE

Legend

Assay 1(c), was used in the inhibition studies with results in this figure. The rates (v_o) are expressed as μ moles of keto acid produced per 60 minutes of reaction. Concentrations of DL-homoserine (s) are expressed as moles/liter (M). Because the experiments were performed on different days, a fact which effected enzyme activity to a small detree, the values of v_o were normalized.

The following cystines were used: (▵) L-cystine, (▫) α-phenyl-, (○) α-ethyl-, α-methyl-, α-n-propyl-, α-isopropyl-, and α-n-butyl -DL-cystine.

The figure represents a summary of data obtained in separate experiments in which rates of production of keto acid were measured in the presence of DL-homoserine alone (•) and DL-homoserine plus sulfur amino acid (▵,▫,○). The data from each experiment was used to determine the K_m and K_i values summarized in TABLE 5. The method of determining K_m, the Michaelis constant, is described in the Legend to TABLE 5. K_i, the inhibitor constant (see Legend to TABLE 5), was determined using the fact that, for competitive inhibition, the slope of the line obtained by a $1/v_o$ vs. 1/s plot in the presence of substrate and inhibitor equals $\left(1 + \frac{i}{K_i}\right) \frac{K_m}{V_{max}}$ (112)

where i = molar concentration of inhibitor, V_{max} = maximum velocity at infinite concentration of substrate. V_{max} was obtained by extrapolation of the $1/v_o$ vs. 1/s plots (Lineweaver - Burk (113)).

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FIG. 9

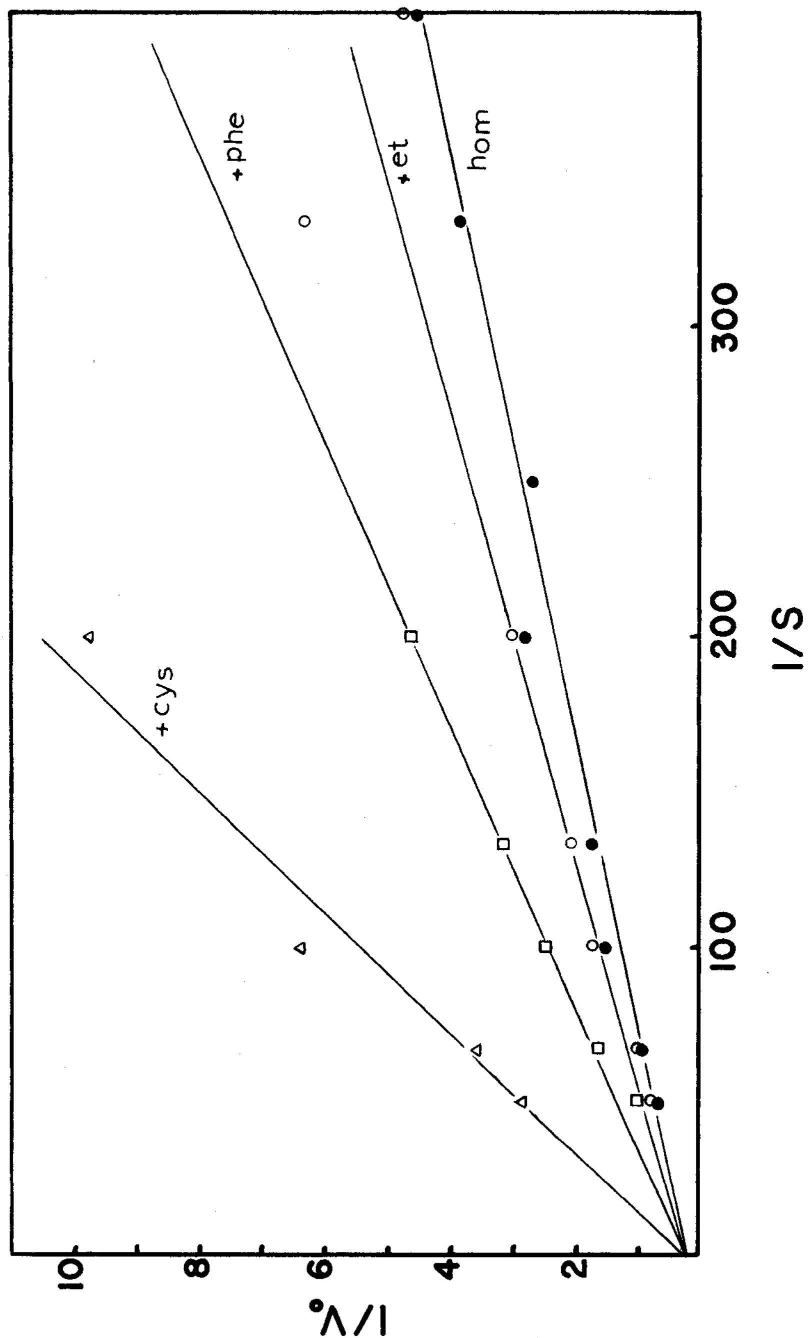

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FIG. 10

DEAMINATION OF HOMOSERINE IN THE PRESENCE OF CYSTATHIONINE

Legend

Assay conditions were the same as those used for the studies of the inhibition of homoserine deaminase by various cystines (No. 1(c)).

v is expressed as μ moles of keto acid produced per 30 minutes of incubation. Concentrations of DL-homoserine are in millimoles/liter (mM). The rate of keto acid formation in the absence (•) and presence (○) of cystathionine is recorded in the graph.

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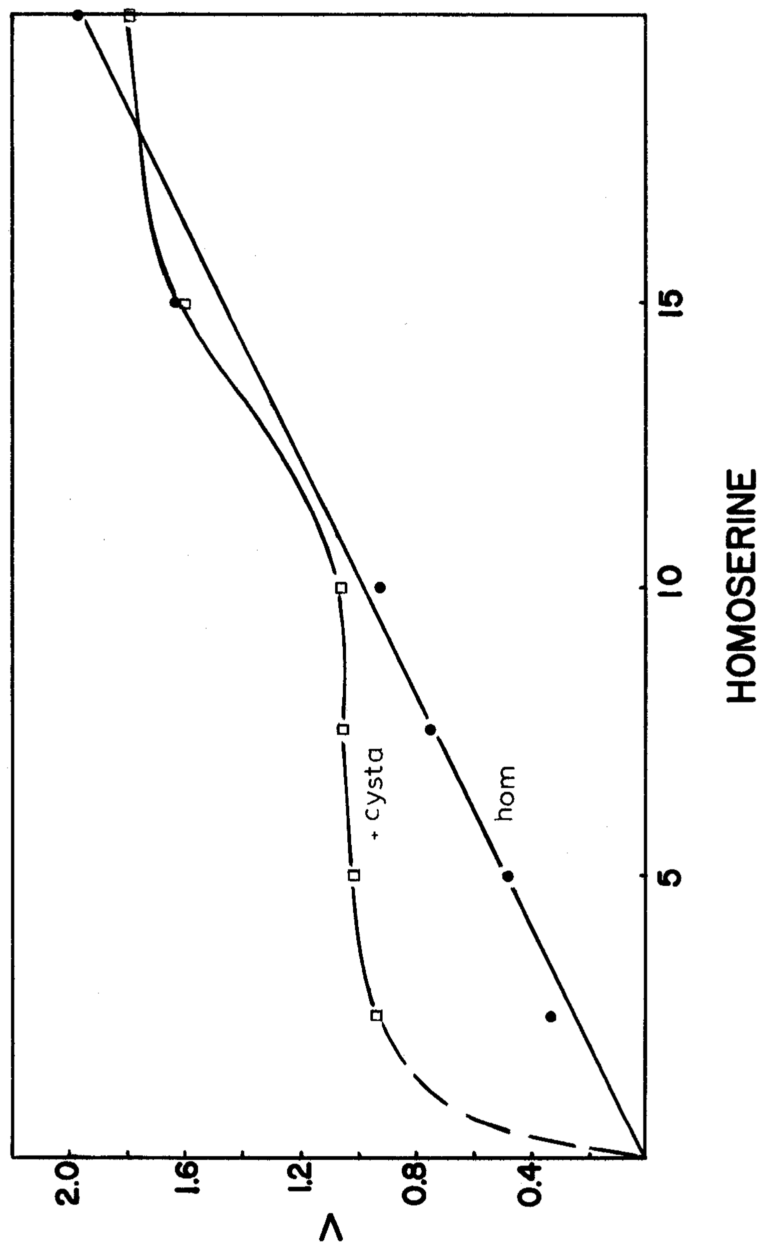
FIG. 10



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LEGEND TO TABLE 4

For experiments a,b,c, assay No. 1(b) was used. Keto acid produced in experiments 1,2,3 was determined by assay No. 1(c). The value obtained for percent inhibition is only an approximation, since pyruvic acid produced was not subtracted from the total keto acid determined.

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TABLE 4

INHIBITION OF HOMOSERINE DEAMINASE BY L-CYSTINE AND α-METHYL-DL-CYSTINE

Expt. No.	Amino Acids Incubated	Concentration of Amino Acids (m M) Homoserine	Cystine	μ moles/60 min. of α-Keto Acid*	Inhibition (%)
a	Homoserine	2		0.090	
	Homoserine + Methyl-Cystine**	2	5	0.053	41
b	Homoserine	2		0.096	
	Homoserine + Methyl-Cystine	2	2.5	0.067	30
c	Homoserine	2		0.073	
	Homoserine + Methyl-Cystine	2	1	0.055	25
1	Homoserine	200		2.70	
	L-Cystine		5	0.120	
	Homoserine + L-Cystine	200	5	0.510	81
2	Homoserine	10		0.819	
	L-Cystine		0.02	0.000	
	Homoserine + L-Cystine	10	0.02	0.726	11
3	Homoserine	10		0.660	
	L-Cystine		5	0.105	
	Homoserine + L-Cystine	10	5	0.045	93

* When DL-homoserine and L-cystine are incubated together, α-ketobutyric acid and pyruvic acid are produced.

** Methyl Cystine = α-Methyl-DL-Cystine.

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LEGEND TO TABLE 5

The Michaelis constants and inhibitor constants summarized in the table were obtained from Lineweaver-Burk (113) plots($1/v_o$ vs. 1/(substrate)), where

v_o = the initial velocity of the reaction.

K_m (moles liter^{-1}) = the Michaelis constant; this is the slope/intercept value taken from the $1/v_o$ vs. 1/(substrate) plots. It is equal to the substrate concentration which gives half-maximal velocity.

K_i (moles liter^{-1}) = inhibitor constant,is the equilibrium constant of the reaction $E + I \rightleftharpoons EI$, where I represents an inhibitor.

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TABLE 5

INHIBITOR CONSTANTS FOR THE INHIBITION OF HOMOSERINE DEAMINASE BY CYSTINES

Amino Acid	Ki (M)	Ki average (M)	Km* (M)	Ki/Km
L-Cystine	$1.6x10^{-3}$	$1.6x10^{-3}$	$4.5x10^{-2}$	0.04
α-Phenyl-DL-Cystine	$1.0x10^{-2}$	$1.0x10^{-2}$	$5.5x10^{-2}$	0.2
α-n-Propyl-DL-Cystine	$2.1x10^{-2}$	$2.1x10^{-2}$	$5.5x10^{-2}$	0.4
α-Methyl-DL-Cystine	$2.9x10^{-2}$ $3.1x10^{-2}$	$3.0x10^{-2}$	$5.6x10^{-2}$ $5.4x10^{-2}$	0.5 0.6
α-Ethyl-DL-Cystine	$2.5x10^{-2}$ $3.3x10^{-2}$	$2.9x10^{-2}$	$5.5x10^{-2}$ $5.6x10^{-2}$	0.5 0.6
α-n-Butyl-DL-Cystine	$2.4x10^{-2}$ $3.1x10^{-2}$	$2.8x10^{-2}$	$5.5x10^{-2}$ $6.0x10^{-2}$	0.4 0.5
α-Isopropyl-DL-Cystine	$3.2x10^{-2}$ $3.4x10^{-2}$	$3.3x10^{-2}$	$5.5x10^{-2}$ $5.4x10^{-2}$	0.6 0.6

* The average of the Km values presented in the table is 5.4×10^{-2} M.

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and substituted cystines on the deamination of DL-homoserine by homoserine deaminase. For each amino acid tested, the rate of reaction of the substrate, homoserine, decreases as the sulfur amino acid concentration increases. Note that the reaction is inhibited most by L-cystine, which is itself a substrate; α-phenyl-DL-cystine has the largest inhibitory effect among the substituted cystines. With the other sulfur amino acid inhibition increases a little from α-methyl-, to α-ethyl-, to α-n-butyl-DL-cystine. The cystines not shown on the graph are about as effective as the butyl compound. This graph does not show conclusively that the small differences between α-methyl-, α-ethyl-, α-n-propyl-, α-n-butyl-, and α-isopropyl-DL-cystine are significant and not caused by experimental error. Further experimental evidence is needed, before one can assign the following order of inhibition: L-cystine $>$ α-phenyl- $>$ α-n-propyl- $=$ α-isopropyl- $=$ α-n-butyl- $>$ α-ethyl- $>$ α-methyl-DL-cystine.

TABLE 4 presents the results of experiments performed to obtain some idea of the degree of inhibition of homoserine deaminase by α-methyl -DL-cystine and L-cystine. In the latter case concentrations of DL-homoserine above (200 mM) and below (10 mM) the saturation point of the enzyme (100 mM, $K_m = 5.4 \times 10^{-2}$, were used (see TABLE 5). Similarly, L-cystine was used above (5 mM) and below (0.02 mM) the concentration which causes maximum velocity of the reaction ($K_m = 9.3 \times 10^{-5}$ M (50)).

As expected, the percent inhibition by α-methyl-DL-cystine increases as the ratio of inhibitor to substrate increases.

L-cystine is a very effective inhibitor of the enzyme. When both substrates are at a concentration above the saturation point of homoserine deaminase, the deamination of homoserine is inhibited by at least 81%. With the concentration of homoserine at 1/5 K_m and that of L-cystine

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at 1/5 K_m the inhibition is 11%. When an excess of amino acid is used, the reaction is arrested nearly completely.

The competitive nature of the inhibition by the cystines becomes apparent from an examination of FIG. 9. The plots of $1/v_o$ vs. 1/s have different slopes, but converge on the same point of the $1/v_o$ axis. The inhibition by L-cystine is considerably larger than that by α-phenyl-DL-cystine, the best inhibitor among the substituted cystines. Inhibition by the other sulfur amino acids is relatively small and about the same for each substituent.

The inhibitor constants obtained from plots of the type shown in FIG. 9 are presented in TABLE 5. These figures permit a better evaluation of inhibition than FIG. 8. The inhibitor constant for L-cystine is about six times larger than that for α-phenyl-DL-cystine. Inhibition by the methyl, ethyl, and butyl amino acids seems about the same, although it is tempting to assign an order of butyl > ethyl > methyl. Inhibition by α-isopropyl-DL-cystine is the lowest. The following increasing order of inhibition could be tentatively assigned: L-cystine > α-phenyl- > α-n-propyl- = α-n-butyl- = α-ethyl- = α-methyl- > α-isopropyl-DL-cystine.

The effect of a saturating quantity of cystathionine (7.5×10^{-3} M, $K_m = 3.7 \times 10^{-3}$ (22)) on the deamination of DL-homoserine is shown in FIG. 10. The concentrations of the latter varied from 2.5×10^{-3} M (1/20 K_m) to 20×10^{-3} M (1/3 K_m). (Keto acid is produced from both substrates.) When cystathionine is present, pyruvic acid (from cysteine) may be formed in addition to α-ketobutyric acid. Therefore, the graph allows only a qualitative interpretation. Since the K_m for L-cysteine is 5.2×10^{-4} M (22) and cysteine is produced from cystathionine, little

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pyruvate should be produced compared to α-ketoburyrate. At low concentrations the amount of keto acid produced in the presence of the sulfur amino acid is considerably larger than in its absence. When the homoserine concentration is about 10 x 10^{-3} M, addition of cystathionine has little effect. At a homoserine concentration of 20 x 10^{-3} M, the amount of keto acid produced seems to be lowered by the addition of the other substrate.

Study of a Model System

Behavior of Cystines

According to FIG. 11, cystine reacts in a model system consisting of pyridoxal phosphate and cupric ions. Sulfhydryl groups are formed at different rates, depending on the time after addition of pyridoxal phosphate. One should note the biphasic nature of the curve.

It is interesting that there is such a pronounced increase in the absorbancy at 412 mμ after about 25 minutes.

To check whether ArSSAr itself was affected by the reaction mixture, it was incubated in the absence of cystine. No significant increase in optical density was observed.
In the absence of cupric ions a change in absorbancy of 0.034 units/min. /1.5 ml. took place for the 4 minutes of observation. When cupric chloride was added, the rate increased to 0.061 units/min./1.5 ml.

Copper ions affected the rates of production of ArSH from L-cystine at different times significantly. In the absence of added metal this rate remained steady at 3.5 x 10^{-3} μ moles/min./ml. for about three minutes; then there occurred a steady increase for about 15 minutes, until a plateau was reached at 6 x 10^{-3} μ moles/min./ml. During 15 minutes

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FIG. 11

CLEAVAGE OF L-CYSTINE IN AN ENZYME MODEL SYSTEM

Legend

The absorbancy, A, at 412 mμ, which is due to the formation of ArSH, was determined in a manner described in the section on methods.

"Time" is the period in minutes, elapsed after the zero time reading. The latter was taken 0.5 minutes after addition of PLP. Pyridoxal phosphate was added after 10 minutes of preincubation at 37°.

Δ A(412 mμ) is the difference in the absorbancy at 412 mμ between a reading at time t and the initial absorbancy at "zero time". The absorption by PLP or the other components did not change with time.

The system contained 6.7 x 10^{-5} M cupric chloride.

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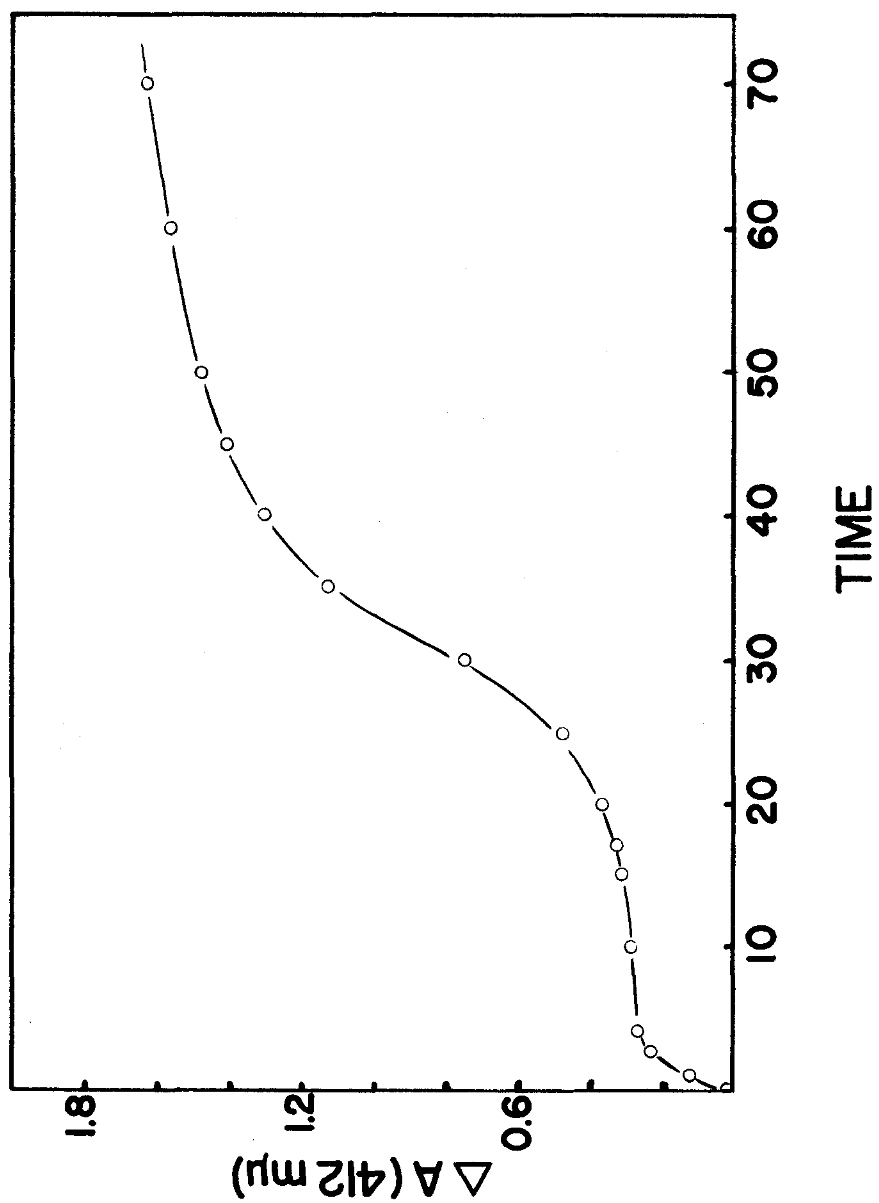
FIG. 11



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LEGEND TO TABLE 6

Procedures used to determine the amounts of pyruvate or ArSH produced after incubation of cystines with pyridoxal phosphate are described under the section on methods. The way in which mercaptan formation was followed is also presented there.

In the kinetic assay the rates were corrected for spontaneous production of mercaptan or absorbancy due to PLP. These were negligible in most cases. The results obtained in the incubation experiments were corrected also for spontaneous reaction and absorbancy due to pyridoxal phosphate.

For the calculation of the amounts of ArSH produced a molar absorbancy index of 12000 at 412 mμ was used (16).

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TABLE 6

THE BEHAVIOR OF CYSTINES IN AN MODEL SYSTEM

Amino Acid	μ mole/60 min./ml. of Pyruvic Acid Produced	μ mole/60 min./ml. of ArSH Produced	Rate of Formation of ArSH	
			$\frac{O.D._{412} \times 10^3}{min.}$	μ mole/min./ml. x 10^3
L-Cystine	0.40	0.11	49	4.1
α-Methyl-DL-Cystine	0.00	0.00	1	0.1
α-Ethyl-DL-Cystine	0.00	0.00	0.0	0.0
α-n-Propyl-DL-Cystine	0.00	0.00	0.0	0.0
α-Isopropyl-DL-Cystine	0.01	0.00	0.0	0.0
α-n-Butyl-DL-Cystine	0.00	0.00	0.0	0.0
α-Phenyl-DL-Cystine	0.00	0.006	3	0.3

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the rate remained steady. Finally a steady decrease was observed for the next 15 minutes till a rate of 3 x 10^{-3} μ moles/min./ml. was reached at the end of the experiment.

In the presence of cupric chloride a steady rate of 7.5 x 10^{-3} μ moles/min./ml. occurred for the first 2 minutes, followed by a rapid decrease to nearly zero, reached after about ten minutes. Then a rapid increase in the rate took place until 9 x 10^{-3} μ moles of ArSH were produced per min./ml. Finally a fast decrease in sulfhydryl production to about 2 x 10^{-3} μ moles/min./ml., reached after 45 minutes, was observed.

The behavior of the substituted cystines in the model system is compared with that of L-cystine in TABLE 6. L-cystine is the only amino acid from which significant amounts of sulfhydryl groups or pyruvate are produced. The behavior of all cystines is similar in the enzymatic system - see TABLE 2.

Absorption Spectra of Schiff Bases

The maxima in the spectra between 300 mμ and 500 mμ of pyridoxal phosphate and pyridoxal phosphate plus L-cystine or α-methyl-DL-cystine are recorded in TABLE 7(a). With PLP a small absorption peak is observed at 328 mμ and a much more pronounced one at 388 mμ. In the presence of L-cystine the position of the peak shifts to 412 mμ and the absorbancy decreases. When α-methyl-DL-cystine is used, the change to a longer wavelength is not as pronounced; and the decrease in absorbancy is less. Similar results were obtained with the other substituted cystines as well as DL- or D-cystine. These results are summarized in TABLE 7(a). The absorbancy peak occurs at about the same wavelength for all substituted amino acids. The absorbancy is practically the same for all cystines, except for α-phenyl-DL-cystine. With L-cystine the optical density is

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LEGEND TO TABLE 7(a)

Details of the procedures used to obtain the spectra are presented under methods. Spectra were corrected for absorption due to the amino acids themselves or the other components of the solution. PLP was always added last and spectra were traced rapidly, as quickly as possible after addition of PLP. Copper ions were not added to these systems.

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TABLE 7 (a)

SCHIFF BASE FORMATION WITH CYSTINES AND PYRIDOXAL PHOSPHATE. POSITION AND MAGNITUDE OF THE MAXIMUM NEAR 400 mu.*

System	λ (mμ)	Optical Density
PLP in 0.5 N HCl neutralized with 0.5 N NaOH	388	0.71
PLP in 2 N HCl neutralized with 2 N NaOH	388	0.65
D-, DL-, or L-cystine + PLP	412	0.61
α-Methyl-DL-Cystine + PLP	396	0.63
α-Ethyl-DL-Cystine + PLP	393	0.64
α-n-Propyl-DL-Cystine + PLP	396	0.63
α-Isopropyl-DL-Cystine + PLP	393	0.64
α-n-Butyl-DL-Cystine + PLP	396	0.63
α-Phenyl-DL-Cystine + PLP	396	0.55

* A smaller peak at 328 mu was also observed for all cystines except α-phenyl-DL-cystine. The height of this maximum was 0.44 for PLP and varied from 0.45-0.50 units of optical density in the presence of cystines and co-enzyme. The cystines themselves showed maximum absorption near 300 mu, but absorbed very little near 400 mu.

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FIG. 12

ABSORPTION SPECTRA OF SCHIFF BASES

Legend

The spectra were obtained as described in the section on methods.

When the spectra of the buffer and the solution of L-cystine were determined, the instrument was blanked with water and buffer, respectively. Solutions of PLP and PLP plus amino acid were read against buffer. Thus, in the latter case absorption due to the amino acid is not compensated for. (However, this is very small near 400 mμ). The figure shows the absorption spectra in the range of 300 mμ - 500 mμ of:

(●) L-cystine,

(○) pyridoxal phosphate,

(▫) pyridoxal phosphate plus L-cystine,

(▵) pyridoxal phosphate plus α-methyl-DL-cystine.

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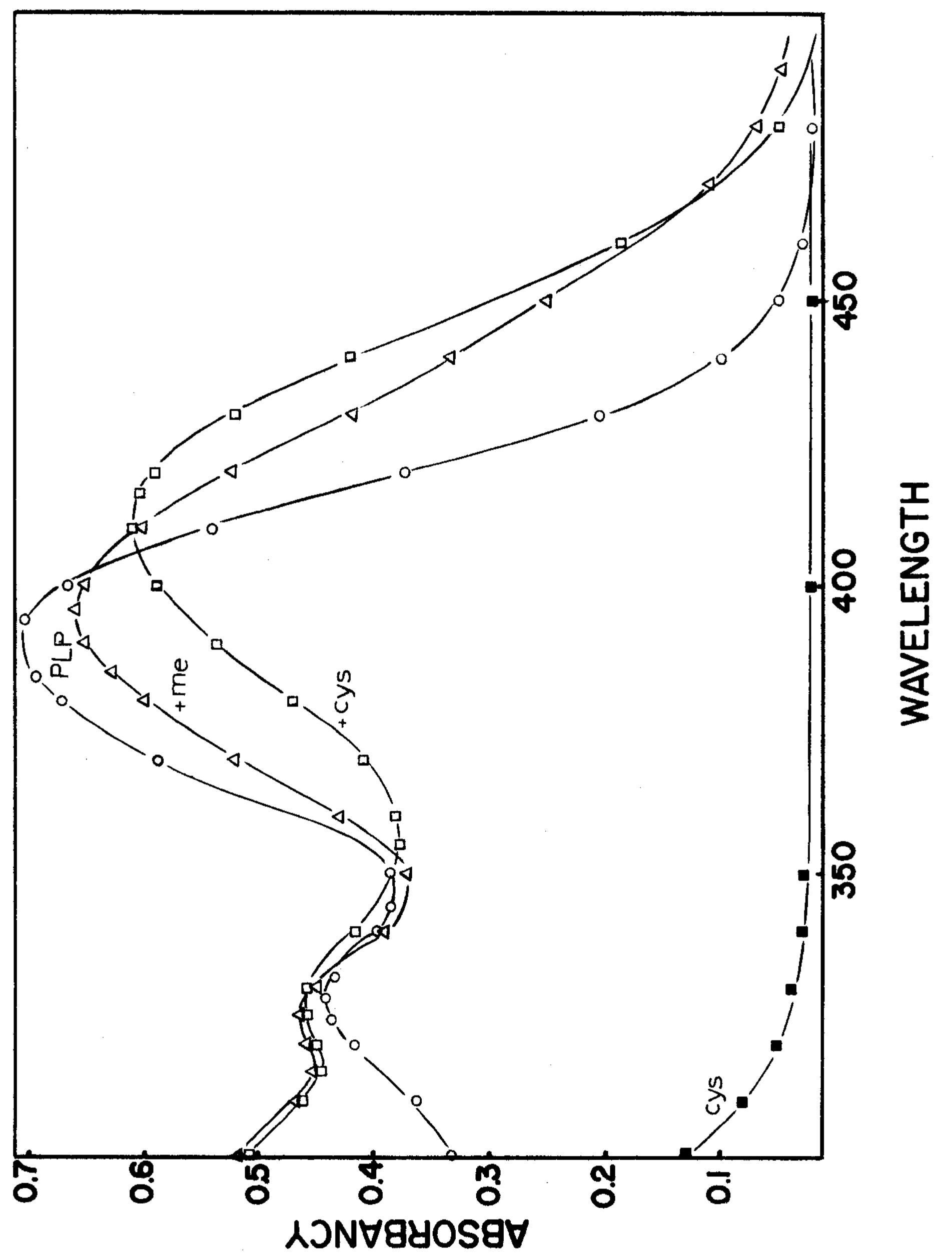
FIG. 12



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LEGEND TO TABLE 7(b)

Absorbancy of the complete system was not corrected for absorption by the amino acids. The maximum absorption of the latter was near 300 mμ and was very small near 400 mμ.

Time was measured after the addition of PLP. At 300 mμ no maximum was observed, but changes in optical densities occurred with time when L-cystine was a reactant.

When the latter amino acid was used, a maximum formed at 310 mμ after the addition of PLP.

In the presence of 2.5 x 10^{-3} M L-cystine, but with no copper ions added, a maximum was observed near 400 mμ. It changed from 402 mμ to 405 mμ within a time period of 20 minutes. However, no significant changes occurred at 300 mμ or 310 mμ within this time.

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TABLE 7 (b)

SCHIFF BASE FORMATION WITH CYSTINES AND PYRIDOXAL PHOSPHATE.
EFFECT OF TIME AND COPPER IONS.

System	Time (min.)	$O.D._{300}$	$O.D._{310}$	Absorbancy near 400 mμ (mμ)	O.D.
PLP + Cu^{++}	0.5	0.46		388	0.41
	20	0.46		388	0.41
L-cystine + Cu^{++}	0.5	0.06			
	15	0.06			
	0.5	0.51	0.48	392	0.38
	5	0.57	0.55	392	0.37
L-cystine + PLP + Cu^{++}	10	0.65	0.66	392	0.39
	20	0.83	0.93	400	0.52
	40	0.83	0.93	400	0.52
α-Methyl-DL-Cystine + Cu^{++}	0.5	0.12			
	20	0.12			
α-Methyl-DL-Cystine+PLP+Cu^{++}	0.5	0.52		391	0.40
	20	0.55		393	0.40
α-Isopropyl-DL-Cystine+PLP+Cu^{++}	0.5	0.54		391	0.46
	20	0.54		399	0.39
α-n-Butyl-DL-Cystine+PLP+Cu^{++}	0.5	0.57		390	0.37
	20	0.57		396	0.39
α-Phenyl-DL-Cystine+Cu^{++}	0.5	0.33			
	20	0.33			
α-Phenyl-DL-Cystine+PLP+Cu^{++}	0.5	0.67		392	0.40
	20	0.67		397	0.37

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lowered a little. When PLP in 2 N HCl, neutralized by 2 N NaOH, is used, the peak height is lower than in 0.5 N HCl, neutralized by 0.5 N NaOH. This might explain the lowered absorbancy for α -phenyl-DL-cystine.

The absorbancy caused by PLP or cystines in the presence of cupric ions does not change with time. Neither do the positions of the absorption peaks. This is shown in TABLE 7(b). When alpha-substituted amino acids are added to PLP and Cu^{++}, the absorption maxima shift to a slightly higher wavelength in a 20 minute time period. The behavior of the system containing L-cystine is quite different. The absorbancy at 300 mμ increases markedly, particularly between 10 and 20 minutes. At 310 mμ an absorption peak builds up. The changes here are also most pronounced in the 10 minute to 20 minute interval. The position of the maximum near 400 mμ shifts from 392 mμ to 400 mμ during this same time period; and the absorbancy increases significantly.

Rates of Formation of Schiff Bases

After the spectral studies had given an indication of the wavelengths of maximum absorbancy, the rates of the reactions of PLP with cystines were followed spectrophotometrically. The changes in absorbancy with time observed in these experiments are shown in FIG. 13. The change in absorbancy is particularly fast with L- or DL-cystine and α -phenyl-DL -cystine. With α -isopropyl-DL-cystine it is relatively slow. With most amino acids a steady plateau in absorbancy is reached, except for DL- and L-cystine and the α -phenyl amino acid. In these cases a decrease in absorbancy is observed after a few minutes.

With α -methyl-DL-cystine in the presence of PLP at 396 mμ, a constant absorbancy of 0.924 was observed. There occurred no rapid changes as noted at 412 mμ.

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FIG. 13

SCHIFF BASE FORMATION

Legend

The details of the procedure used to follow the change of optical density at 412 mμ with time are recorded under methods.

At zero time PLP was added to the cuvette. Time is measured in minutes.

The curves were used to obtain pseudo first order rate constants for the formation of Schiff bases as described under methods.

The following systems were examined:

(▲) PLP alone,

(■) L-cystine + PLP,

(●) DL-cystine + PLP,

(△) α -methyl-DL-cystine + PLP,

(□) α -ethyl-DL-cystine + PLP,

(○) α -n-propyl-DL-cystine + PLP,

(◐) α -isopropyl-DL-cystine + PLP,

(◨) α -n-butyl-DL-cystine + PLP,

(×) α -phenyl-DL-cystine + PLP.

The concentration of L-cystine was 2.5 x 10^{-3} M compared with 5 x 10^{-3} M for all the other amino acids.

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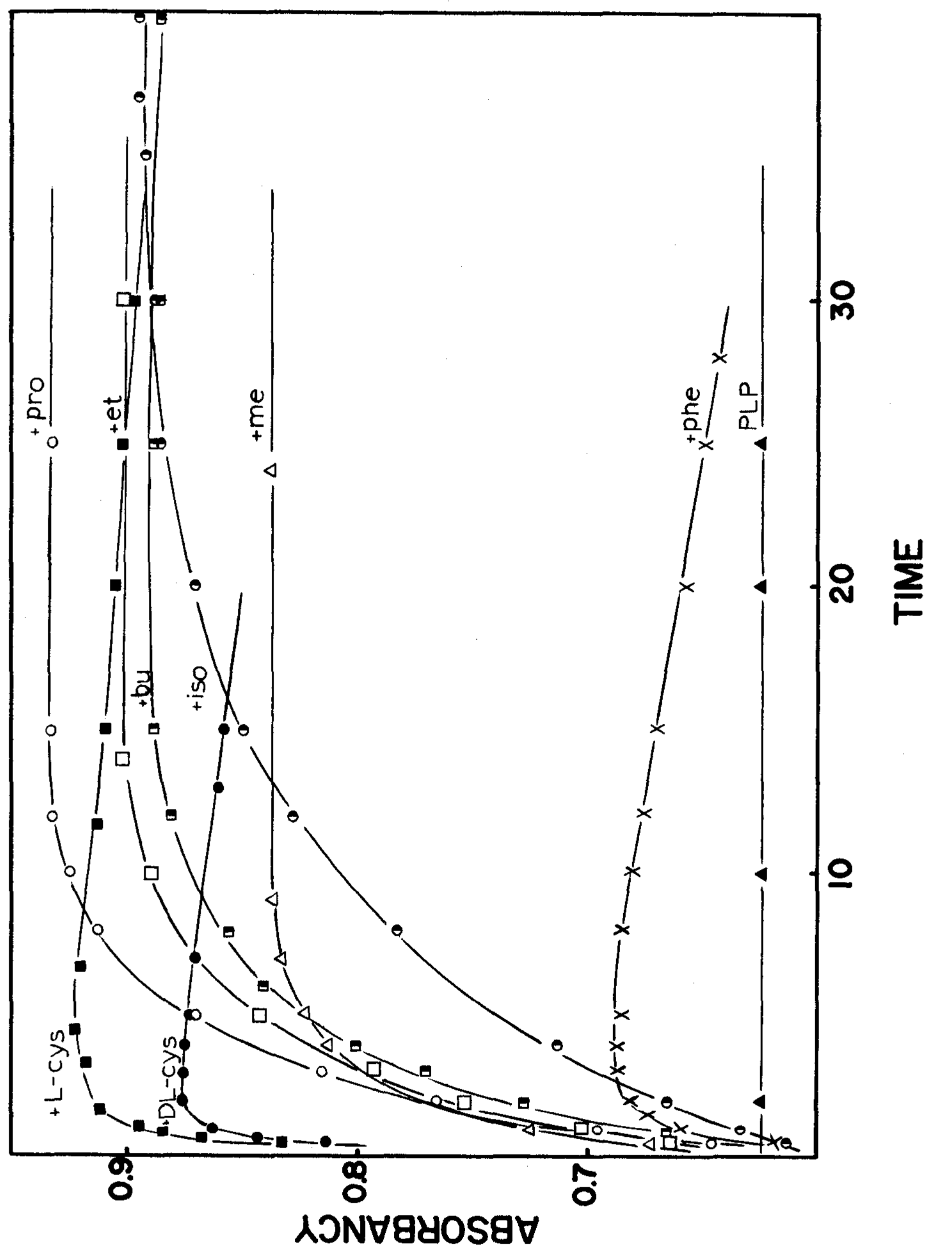
FIG. 13



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LEGEND TO TABLE 7(c)

The initial absorbancy was determined about 0.5 minutes after the addition of PLP.

The initial rate was obtained from the slope of the line recorded.

The concentration of L-cystine was 2.5×10^{-3} M instead of the usual 5×10^{-3} M.

Rate constants were determined as described under methods.

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TABLE 7 (c)

SCHIFF BASE PRODUCTION WITH CYSTINES AND PYRIDOXAL PHOSPHATE. RATES OF FORMATION

System	Maximum Absorbancy at 412 mμ	Time to Reach Maximum Absorbancy (min.)	Initial Rate (ΔO.D.$_{412}$/min.)	k* (min^{-1})
PLP	0.625	0.5		
DL-Cystine+PLP	0.877	2.5	0.2	3.07
L-Cystine+PLP	0.923	4.5	0.3	1.89
α-Methyl-DL-Cystine+PLP	0.837	9	0.1	0.535
α-Ethyl-DL-Cystine+PLP	0.903	14	0.07	0.300
α-n-Propyl-DL-Cystine+PLP	0.932	12	0.09	0.345
α-Isopropyl-DL-Cystine+PLP	0.895	37	0.03	0.123
α-n-Butyl-DL-Cystine+PLP	0.888	15	0.07	0.313
α-Phenyl-DL-Cystine+PLP	0.688	4	0.09	1.74

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This was also observed with the other substituted cystines. Therefore the wavelength of 412 mμ was used to follow rates. TABLE 7(c) shows the results of these rate studies. The important feature of this table is, of course, the list of pseudo first order rate constants. The rate with DL-cystine is considerably higher than that of the substituted cystines. (The concentration of L-cystine is one half that of DL-cystine). Among the latter the high rates with the phenyl and methyl compounds as well as the low rate with α-isopropyl-DL-cystine stand out. The rates appear to be in the following order of decreasing magnitude (min^{-1}): DL-cystine (L-cystine) $\gg$ α-phenyl- $\gg$ α-methyl- $\gg$ α-n-propyl- $>$ α-n-butyl- $>$ α-ethyl- $\gg$ α-isopropyl-DL-cystine.

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CHAPTER III

DISCUSSION

The Enzymatic System

Substrate Behavior of Cystines

Substituted cystines, in which the alpha-hydrogen atom is replaced by various alkyl groups or phenyl, are not cleaved by cystathionase to yield pyruvic acid or sulfhydryl groups (equation III). The importance of the alpha-hydrogen atom in desulfuration reactions has been pointed out by Snell and coworkers (70) and by Braunstein (67) (see also INTRODUCTION). The data in TABLE 1 is in accordance with the mechanism proposed for the cleavage of cystine by either a model system or the enzymatic system (38,16). No detectable amount of ArSH or keto acid is formed when there is no hydrogen atom on the alpha-carbon.

The alpha-substituted cystines do not undergo any appreciable reaction when incubated with the enzyme. The polarographic studies summarized in TABLE 3 indicate no significant change during incubation in the concentration of the sulfur amino acids examined. The small decrease in the diffusion current shown in the table might be caused by some effect by the enzyme protein on the reduction process. In any case these diffusion current differences are insignificant compared to the changes observed when the concentration of α-n-propyl-DL-cystine, for instance, is decreased from 1 mM to 0.75 mM.

Although lack of a hydrogen atom on the alpha-carbon makes the

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cystines studied inert in desulfhydration reactions, these amino acids might be reactive in other enzymatic systems where an alpha-hydrogen atom is not required, for instance, decarboxylation reactions (6).

Inhibition of Homoserine Deaminase

The deamination of homoserine by the enzyme studied is inhibited by L-cystine and, to a smaller degree, by the substituted cystines. With every cystine used competitive inhibition is observed, that is, the inhibitor combines with the coenzyme. Homoserine and the sulfur amino acid compete for pyridoxal phosphate, which is present in cystathionase in the form of an internal Schiff base (6, 14). The reversibility of the inhibition and its dependence on both the substrate and the inhibitor concentration is shown by the results presented in TABLE 4 and FIGURES 8 and 9. L-cystine is a much more potent inhibitor than α-methyl-DL-cystine, see TABLE 4. As the concentration of sulfur amino acid increases, the rate of production of keto acid from DL-homoserine decreases in a manner shown in FIG. 8. These results, as well as those presented in FIG. 9, indicate competitive inhibition of the deamination of homoserine by the substituted cystines, as well as L-cystine.

The affinity of the enzyme for DL-homoserine is relatively small as indicated by the large Michaelis constant of 5.4×10^{-2} M(TABLE 5). Kato (19) reports a K_m of 3×10^{-2} M for L-homoserine, Greenberg one of 2×10^{-2} M (14). L-cystine ($K_m = 9.3 \times 10^{-5}$ M (19)) is bound much tighter by cystathionase.

Among the cystines examined the natural substrate of the enzyme, L-cystine, forms the most stable enzyme-inhibitor complex, when one regards DL-homoserine as the substrate. The affinity of the enzyme for

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α-phenyl-DL-cystine is also relatively high. The amino acid with the branched substituent, α-isopropyl-DL-cystine, is removed most easily from the enzyme. Reasons for these variations in the inhibitor constants will be examined more closely in a general discussion after all of the experimental results have been interpreted briefly.

The K_m for L-cystathionine is 3.7×10^{-3} M compared to 3×10^{-2} M for L-homoserine (19). This difference explains the results shown in FIG. 10. At low concentrations of homoserine,cystathionine and the hydroxyl amino acid are cleaved to produce keto acid. As the concentration of homoserine increases, competition between it and the sulfur amino acid slows down the rate of α-ketobutyrate formation until some of the homoserine molecules are prevented from reaching the active site and a small degree of inhibition is observed. With concentrations of homoserine higher than those used, a higher degree of inhibition should be observed.

The Non-Enzymatic System

L-cystine is cleaved in a model system, consisting of pyridoxal phosphate and cupric ions, in a reaction in which sulfhydryl groups are liberated and keto acid is formed. The alpha-substituted cystines are inert, because of the absence of an alpha-hydrogen. The model system therefore, resembles closely the enzymatic reaction. TABLE 6 shows that very small quantities of ArSH could have been produced from α-phenyl -DL-cystine. This could be explained by a decarboxylation of the type which Snell (8) observed with some alpha-substituted amino acids (equation XVIII - XXVI). After decarboxylation and replacement of CO_2 by a hydrogen, the Schiff base could undergo an elimination reaction with the

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production of thiocysteine(FIG. 4). The extend of the reaction, however, would be small, because of the low temperature used and the presence of metal ions, which inhibits decarboxylation (8).

In the experiments concerned with the behavior of cystines in the model system (TABLE 6, FIG. 11), spectrophotometric measurements were made at 412 mμ. At this wavelength ArSH, produced by the cleavage of cystines, and the Schiff bases formed from the amino acids and PLP, absorbs strongly. However, the molar absorbancy index for the imine formed is much smaller than that of the mercaptan; when 0.1 M glycine was used, this constant was 6600 at 413 mμ, for 0.01 M amino acid it was observed to be 5800 at 405 mμ; with PLP alone the value obtained was 5700 at 388 mμ (78). The molar absorbancy index of ArSH, on the other hand, is 12,000 (16). In the one hour incubation experiments reported in TABLE 6, no significant absorbancy differences were observed between tubes containing amino acid plus PLP and others having no PLP, when the substituted cystines were used. The concentrations of coenzyme and amino acid used were too small to contribute significantly to the absorbancy at 412 mμ, although substituted cystines also form Schiff bases as shown in FIG. 12 and TABLE 7(c). This also applies to experiments in which a continual change in absorbancy at 412 mμ was followed. When substituted cystines were incubated in the presence of PLP and copper ions, no appreciable increase of the absorbancy at 412 mμ was observed. Consequently the changes observed with L-cystine were caused by ArSH formation.

These conclusions are supported by the data of TABLE 7(b). With a fivefold increase in the pyridoxal phosphate concentration and a larger amount of L-cystine, the absorbancy at 400 mμ did not change for

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at least 10 minutes after the addition of PLP.

During the enzymatic studies of sulfhydryl production, Schiff base formation could not contribute to the absorbancy at 412 mμ, because the spectrophetometer tubes containing PLP, enzyme, and amino acid were read against cuvettes with only enzyme missing.

As shown in TABLE 7(b) the formation of a chelate from L-cystine, PLP, and Cu^{++}, with maximum absorbancy at 400 mμ, becomes pronounced between the tenth and twentieth minute after addition of PLP. During ArSH production from L-cystine, PLP and Cu^{++}, experiments described under RESULTS, the rate of mercaptan production reached nearly zero around the ten minute mark, before a fast increase in rate was observed. This reflects the changes in copper chelate formation shown in TABLE 7(b). In the absence of copper a hydrogen-bonded Schiff base is formed from PLP and amino acid, which breaks down at a steady rate. The sulfhydryl groups produced react with ArSSAr; and a steady increase in absorbancy is observed. The diphasic curve presented in FIG. 11 probably reflects the cyclic nature of the non-enzymatic cleavage of L-cystine suggested by Cavallini (38), a reaction in which thiocysteine is produced first. This unstable intermediate then decomposes to cysteine, which is oxidized to cystine. Both thiocysteine and cysteine can react with ArSSAr.

The absorbancy increases at 300 mμ and 310 mμ with time, shown in TABLE 7(b), are caused by the formation of pyruvate. This keto acid has an absorption maximum in this region (114).

The data of TABLE 7(a) and the spectra in FIG. 12 show that Schiff bases are formed from cystine or substituted cystines and PLP. An absorption maximum at 388 mμ is typical of pyridoxal phosphate (78,79). Imines formed from PLP and amino acids absorb at a characteristic wave-

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length of 414 mμ (6). Absorption peaks near 400 mμ have been reported for the reaction products of PLP and various amino acids (78,80). The position of the maximum depends on the concentration of the amino acid used and the rate of the imine formation. The substituted cystines apparently react at a slower rate than cystine. The spectra were recorded within a few minutes after addition of PLP. A shift of the maximum to a higher wavelength with time is observed on examination of TABLE 7(b). This is not as pronounced as expected, because copper ions present in the system tend to shift the absorption peak of the imine to a shorter wavelength (80). The imine of L-cystine absorbs at 400 mμ in the presence of copper ions, compared to 412 mμ in their absence (TABLE 7(a).

The maximum absorbancy at 412 mμ (TABLE 7(c)) is larger in the presence of PLP and amino acid than with the former alone. The amino acids themselves absorb little at this wavelength. These observations reflect Schiff base formation. A certain amount of time is required to reach the maximum absorbancy. The reaction followed at 412 mμ is relatively fast with L-cystine, DL-cystine, or α-phenyl-DL-cystine. α-Isopropyl-DL-cystine and PLP react at a relatively slow rate. In the non-enzymatic reaction either the D- or the L-form of cystine can form a Schiff base, while in the enzymatic cleavage of cystine the L-form is required for the subsequent rearrangement of the Schiff base formed, a process which leads to the cleavage of the amino acid and which is catalyzed by the apoenzyme (6). Among the substituted cystines studied, α-phenyl-DL-cystine forms a Schiff base with PLP at a surprisingly fast rate, when one considers the steric hindrance by the phenyl group. In addition the basicity of the amino group should be decreased, because of the electron-withdrawing

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phenyl side group. Perhaps the rate is increased, because of resonance stabilization.

The importance of steric factors is pointed out by the low rate with α-isopropyl-DL-cystine and the higher rate with the methyl compound. There should be a correlation between the rate of Schiff base formation in the non-enzymatic reaction and the rate of reaction of the amino acids with the coenzyme part of cystathionase, and hence the inhibition of the enzyme. This question will be examined in the general discussion.

FIG. 13 indicates the rate of formation of the Schiff bases studied and their stability. The imine of L-cystine is not stable, but decomposes into products and PLP. Very little sulfhydryl is formed from α-phenyl-DL-cystine as pointed out in TABLE 6. The absorbancy decrease observed with the Schiff base of this compound, as shown in FIG. 13, is probably due to the formation of the non-hydrogen-bonded form described by Christensen (81). This product does not absorb strongly at 412 mμ. The phenyl group apparently is a sufficiently strong electron-attracting group to lower the electronegativity of the imine-nitrogen and prevent strong hydrogen bonding with the phenolic hydrogen (see FIG. 3).

General Discussion

When Hill and Crowell studied the reaction rates of primary amines with piperonal, they obtained the following rate constants, expressed as $1\ mole^{-1}\ sec.^{-1}$ at 25° C (83): methylamine, 5.55; n-butylamine, 3.37; n-propylamine, 3.15; ethylamine, 2.88; isopropylamine, 0.895. These results should be compared with the rates of formation of Schiff bases from PLP and cystines ($min.^{-1}$): DL-cystine, 3.07; α-phenyl-, 1.74; α-methyl-,

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0.535; α-n-propyl-, 0.345; α-n-butyl-, 0.313; α-ethyl-, 0.300; and α-isopropyl-DL-cystine, 0.123. If one neglects DL-cystine and the α-phenyl amino acid for the moment, one observes that the rates decrease in basically the same order. The rate is determined by the basicity of the amino group and steric factors. For instance, although the amino group is less basic with a methyl side group than with an isopropyl, the steric effect of the latter slows down the reaction rate. DL-cystine is expected to react at a fast rate, since there is only a hydrogen attached to the alpha-carbon. The fast rate with α-phenyl-DL-cystine, however, cannot be explained in this manner.

When one attempts to compare the order of the first order rate constants obtained with the following stability constants of Schiff bases measured by Matsuo (80): valine (R = isopropyl), 4.5; norvaline (R = propyl), 1.2; α-amino-n-butyrate (R = ethyl), 1.16; norleucine (R = n -butyl), 1.1; and alanine (R = CH_3), 0.37; one observes that the imine of alanine is least stable, although with α-methyl-DL-cystine a relatively fast rate of Schiff base formation is obtained. The Schiff base of valine is stable, but α-isopropyl-DL-cystine reacts at the slowest rate among the compounds examined. Therefore the extent of imine formation does not seem to have much influence on the rate of its formation.

The following inhibitor constants were obtained (M x 10^2): α-isopropyl-, 3.3; α-methyl-, 3; α-ethyl-, 2.9; α-n-butyl-, 2.8; α-n-propyl-, 2.1; α-phenyl-DL-cystine, 1.0; and L-cystine, 0.16.

If one compares these values with the rate constants for Schiff base formation, one observes a nearly perfect reversal in the sequences from one to the other. Apparently, the higher the rate constant in the model system, the lower the inhibitor constant in the enzymatic system,

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the higher the stability of the enzyme-inhibitor complex. One might be able, therefore, to use a study of the rate of Schiff base formation in an enzyme model system, to predict the relative effectiveness of the amino acid used as an inhibitor of the enzyme.

Matsuo (80) stressed the importance of the stability of the Schiff base to the overall affinity of the amine to the enzyme. Amino acids with an α-methyl group had low stability constants and were poor inhibitors compared to the corresponding unsubstituted amino acids. This study has shown the importance of the rate of Schiff base formation determined in a non-enzymatic system.

In an enzyme model system simulating the effect of cystathionase, L-cystine forms a Schiff base with PLP at the fastest rate. In the corresponding enzyme system the same amino acid forms the most stable Schiff base with the imine of cystathionase and is the best inhibitor. On the other hand α-isopropyl-DL-cystine, probably because of the steric effect of the branched side group, reacts with PLP at a relatively low rate. In the enzyme system it is the poorest inhibitor.

The importance of the rate of Schiff base formation, determined in a non-enzymatic system, with regard to enzyme inhibition, is somewhat surprising. One would expect the apoenzyme to play a more determining role. However, cystathionase acts on a wide variety of substrates of different size and functional groups. The apoenzyme is thought to function mainly in the rearrangements of the Schiff base that lead to cleavage of the substrate (6). This tautomerism cannot occur with the substituted cystines, which lack the alpha-hydrogen. The functional groups on the apoenzyme in the vicinity of the enzyme's active centre apparently do not offer much steric hindrance to the approach of the substituted

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cystines. Even α-phenyl-DL-cystine reaches the active centre. When it has blocked this site the bulky phenyl group probably prevents molecules of homoserine from reaching its vicinity.

The reports of the inhibition of PLP enzymes by amino acids or amines (67), particularly Matsuo's observation of the inhibitory effects of various amino acids on homoserine deaminase (80), show rather convincingly that inhibition occurs by a reaction of the amino acid with the coenzyme. The results of this study lend support to this proposal.

The inhibition of homoserine deaminase by L-cystine was observed in this laboratory shortly after Kato had reported it (50). As was mentioned before,cysteine arises from the cleavage of cystathionine. In the presence of the enzyme and small amounts of cystine, it is converted to the disulfide and then cleaved by cystathionase (cystine desulfurase). Cystine inhibits the enzyme considerably. We have, therefore, an example of negative feedback control of the activity of an enzyme. This control is particularly important with regard to the breakdown of cystathionine, an important intermediate in sulfur amino acid metabolism. The process of transsulfuration in the conversion of the essential amino acid, methionine, to the important sulfur amino acid, cysteine, apparently requires careful control (50). Similar examples of feedback control have been reported in _E_. _coli_ in the enzyme system converting cysteine to methionine (58,63).

The substituted cystines could be prepared only in a low total crude yield, since the number of intermediate synthetic steps is considerable. The total yield decreased from 47% for the ethyl or n-propyl compounds, to 35% for α-n-butyl-DL-cystine, to 31% when the substituent was phenyl.

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Isopropyl-DL-cystine could be prepared only in 22% total yield, because of the low yield of hydantoin and S-benzyl amino acid. As the size of the side group increased, the yield decreased. The t-butyl compound could not be prepared, because of the steric hindrance by the alpha-substituent.

Considerable time was required to prepare the substituted cystines, because of the number of intermediate steps; and since several reactions, involving dangerous reagents, could only be allowed to proceed with small quantities of reagents. The synthesis of these compounds, therefore, took a considerable part of this study.

Several interesting studies with these alpha-substituted cystines offer themselves for the future: one could study the kinetics of their alkaline degradation to get an idea of the stabilizing effects of the side groups. In a model system the effect of high temperature should be of interest, particularly with regard to decarboxylation reactions. With α-phenyl-DL-cystine one might get an appreciable rate of reaction, because the bonds on the alpha carbon atom are further weakened by the electron withdrawing side group.

The behavior of cystines in other enzymatic systems should be of interest. For instance, a cystine reductase has been purified to some extent (115). The enzyme, a flavoprotein, catalyzes the reduction of cystine to cysteine in the presence of DPNH. Studies with an amino acid oxidase should also be fruitful.

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CHAPTER IV

SUMMARY

Several alpha-substituted cystines were synthesized via hydantoin intermediates. These compounds, in which the hydrogen on the alpha -carbon is replaced by larger groups, are inert in enzymatic desulfhydration reactions by cystathionase, as well as a non-enzymatic model system, compared to L-cystine which is cleaved under both conditions. All the cystines studied inhibited the deamination of homoserine by the enzyme. The most efficient inhibitor was L-cystine, a natural substrate of the enzyme. This inhibition seems to be a means of controlling the level of cystathionine in the rat liver. Schiff base formation between substituted cystine and pyridoxal phosphate, bound to the apoenzyme by an internal Schiff base, causes the inhibition. The relative rate of formation of the Schiff base from the (substituted) cystine and PLP in a non-enzymatic system, allows a prediction about the degree of inhibition by the sulfur amino acid in the enzyme system.

During the study the formation of Schiff bases and their spectra were studied spectrophotometrically.

The enzyme cystathionase was isolated from rat liver and purified to some extent.

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APPENDIX

ENZYME PURIFICATION

Preparation of Liver Extract

A total of 125 rat livers with a net weight of 950 g. were cut into small pieces, added to 2 volumes of 1% KCl solution containing 10^{-4} M EDTA, and homogenized in a Waring blendor for 3 minutes at low speed at room temperature (crude homogenate, see TABLE 1).

Controlled Heat Denaturation

The crude homogenate (2700 ml. total volume) was poured in 1-litre batches into a 2-l. Erlenmeyer flask, equipped with a thermometer, and shaken in a water bath, maintained at 65 ± 1°. When the temperature of the mixture had reached 60°, the flask was transferred to a second water bath with a temperature of 60°. This temperature was maintained for 5 minutes. The flask was then cooled rapidly in an ice-water bath to bring the temperature of its content to about 20°. Centrifugation for 20 minutes at 2000xg yielded about 600 ml. of a clear supernatant solution from each litre of the crude homogenate. The voluminous sediment was extracted once with 400 ml. of the KCl solution containing EDTA. This extract was added to the supernatant from the first centrifugation. In this manner a total volume of 3130 ml. of clear reddish liquid were obtained (heated extract, see TABLE 1.

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Ammonium Sulfate Fractionation

The solution obtained after heat denaturation was adjusted to pH 6.5 by the dropwise addition of concentrated ammonium hydroxide. A Fisher Accumet pH Meter was used for pH measurements. The solution was brought to 55% ammonium sulfate saturation by the addition of 351 g./l. of salt over a 10 - 15 minute time period with constant stirring. All operations were carried out in the cold room (4°). The temperature of the solution was kept at 0 - 2°; and the pH was not allowed to vary by more than 0.2 units from 6.5. The mixture was stirred for six hours; and the precipitate formed was removed by centrifugation at 2000xg for 20 minutes at 0° and discarded. Ammonium sulfate, 131 g./l., was added to the supernatant liquid in the manner described above to bring it to 73% saturation. The mixture was allowed to equilibrate for 30 minutes. The dark red precipitate was collected by centrifugation at 2000xg for 20 minutes at 0°. It was then suspended in about twice its volume of 0.2 M potassium phosphate buffer, pH 7.5. A red, insoluble precipitate was removed by centrifugation in the usual manner. A dark-red supernatant solution (210 ml.) was thus obtained (ammonium sulfate fraction, see TABLE 1).

First Ethanol Fractionation

To bring the enzyme concentration to about 200 units/ml., 315 ml. of 10% saturated ammonium sulfate solution, buffered at pH 7.2 with 0.1 M potassium phosphate buffer, were added. In this manner a total of 525 ml. of a red, clear solution, pH 7.1, were obtained. The solution was cooled to about -1° in an ethanol-dry ice bath and 0.95 volumes (499 ml.) of cold, absolute ethanol were added dropwise with rapid

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stirring from a separatory funnel. The alcohol was cooled to at least -20° in a dry ice-acetone bath before addition. The speed of addition of alcohol was controlled, in order to maintain the temperature of the mixture at -4 to 2°. The alcohol was added over a period of about half an hour.

The mixture was stirred at -3° for 30 minutes after all alcohol had been added. A red sediment was separated from a red supernatant by centrifugation at 2000xg for 20 minutes at -3° and taken up in about 3 volumes of cold (5°) potassium phosphate buffer, pH 7.5. The mixture was homogenized in a Waring blendor at half speed for 2 or 3 minutes. The suspension formed was then centrifuged for 20 minutes at 0° and 7000xg. The precipitate separated in this manner was extracted once with the buffer and discarded. This extract was added to the previous supernatant. The combined liquids were then brought to 75% saturation with ammonium sulfate by the addition of 515.8 g./1. of solid salt. During this addition over a period of 10 minutes and at temperature of 0°, the pH was maintained at 7 using ammonium hydroxide solution as in the ammonium sulfate fractionation step. The mixture was stirred for 90 minutes at 0°. Centrifugation at 7000xg for 20 minutes at 0° yielded a precipitate. This was dissolved in about 3 volumes of 0.2 M potassium phosphate buffer, pH 7.5. A small amount of insoluble material was removed by centrifugation at 7000xg for 20 minutes at 0°. The final 120 ml. of this solution were used for the next step.

Second Ethanol Fractionation

After the dilution of the solution with 250 ml. of 10% saturated ammonium sulfate solution, buffered at pH 7.2 (dissolved in 0.2 M

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potassium phosphate buffer, pH 7.5), to get a concentration of about 230 units/ml., it was again submitted to the ethanol fractionation step. The final precipitate was dissolved in 0.2 M potassium phosphate buffer, pH 7.5. A total volume of 62 ml. of clear, yellow solution obtained in this manner was divided into about fifty test tubes and stored frozen at about -20°.

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