The Oxidative Deamination of Sulfur Amino Acids by a Bacterial L-Amino Acid Oxidase

Susan S. Chen
THE OXIDATIVE DEAMINATION OF SULFUR AMINO ACIDS

BY A BACTERIAL L-AMINO ACID OXIDASE

by

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Bachelor of Science, Chung-Hsing University, 1968

A Thesis
Submitted to the Faculty
of the
University of North Dakota
in partial fulfillment of the requirements
for the degree of
Master of Science

Grand Forks, North Dakota
August
1970
This thesis submitted by Susan S. Chen in partial fulfillment of the requirements for the Degree of Master of Science from the University of North Dakota is hereby approved by the Faculty Advisory Committee under whom this work has been done.

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BACTERIAL L-AMINO ACID OXIDASE

Department Microbiology

Degree Master of Science

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ACKNOWLEDGEMENTS

The author wishes to express sincere gratitude to Dr. John A. Duerre for his suggestions, encouragement and guidance throughout this investigation. The author also expresses gratitude to Dr. James R. Waller, Dr. Robert C. Nordlie and Dr. John W. Vennes for their helpful suggestions.

The technical assistance of Mrs. Jean H. Walgate is also acknowledged.
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ABSTRACT

The oxidative deamination of sulfur amino acids by a particulate L-amino acid oxidase obtained from an atypical *Proteus rettgeri* has been investigated. In addition to several sulfur amino acids tested here the enzyme catalyzes the oxidation of L-leucine, L-phenylalanine, L-tryptophan, L-arginine, L-tyrosine, L-histidine, L-ornithine, L-citrulline, L-lysine and L-isoleucine with a pH optimum at 7.5. Specific activity, pH optimum, $K_m$ and $V_{max}$ values were determined for each sulfur amino acid. The ordered rate of reaction at pH 7.5 for these amino acids were L-homocystine, L-methionine, S-adenosyl-L-homocysteine, S-adenosyl-L-methionine, L-djenkolic acid, S-ribosyl-L-homocysteine, S-adenosyl-L-homocysteine sulfoxide, L-homocysteine, methionine sulfoxide, L-cysteine and L-cystine.

The particulate fraction contained sufficient catalase to catalyze the complete reaction as follows:

$$\text{R-CH-COOH} + \frac{1}{2} \text{O}_2 \rightarrow \text{R-CO-COOH} + \text{NH}_3$$

The products obtained with all these substrates, except for homocysteine and cysteine, were ammonia and keto acid. With the free sulfhydryl compounds oxygen consumption was disproportionately high and in addition to ammonia and keto acids, hydrogen sulfide was liberated. No activity
was observed under anaerobic conditions, nor did pyridoxal phosphate affect the rate of the reaction; therefore it appeared that hydrogen sulfide is a product of the reaction and not due to the presence of sulfhydralase. The crystalline snake venom L-amino acid oxidase from Crotalus terrificus terrificus was studied in comparison and found to catalyze a similar reaction.

The pH optimum for each substrate was determined either by measuring the rate of oxygen consumption or keto acid formation. The pH optimum for all sulfur amino acids with both enzymes appeared in the alkaline range. The shape of the pH curve was dependent on the individual substrate and enzyme used.

The keto acids produced from the various substrates with both enzymes were identified by thin-layer chromatography of the free keto acid or their dinitrophenylhydrazones. Except for homocysteine or cysteine all the resultant keto acids were found to correspond to the parent compound. Homocysteine was found to be completely oxidized to 4,4'-dithio-bis(2-ketobutyric acid) by chemical and elemental analysis. S-Adenosyl-L-methionine which is resistant to attack by most enzymes including snake venom and rat kidney L-amino acid oxidase was readily oxidized to S-adenosyl-α-keto-γ-methiobutyrate by the bacterial enzyme.

The keto acids derived from homocysteine and cysteine by both enzymes were α-ketobutyrate and pyruvate respectively.
INTRODUCTION

It has been established that S-adenosyl-L-homocysteine is a direct product of numerous transmethylase reactions involving S-adenosyl-L-methionine. Studies involving the metabolic fate of S-adenosyl-L-homocysteine have shown that the compound is metabolized in at least three ways. The first is catalyzed by S-adenosyl-L-homocysteine hydrolase (EC 3.3.1.1.) found in mammalian liver and yeast. This enzyme catalyzes the hydrolytic cleavage of S-adenosyl-L-homocysteine to yield adenosine and L-homocysteine. This reaction is reversible with the equilibrium far in the direction of synthesis. The second pathway involves two separate enzymes found in Gram negative bacteria. The first enzyme, S-ribosyl-L-homocysteine nucleosidase, catalyzes the hydrolysis of the glycosyl bond of S-adenosyl-L-homocysteine to yield adenine and S-ribosyl-L-homocysteine. Cleavage of the thioether bond of the latter compound is then catalyzed by a second enzyme, S-ribosyl-L-homocysteine cleavage enzyme, to yield free L-homocysteine and a C\textsubscript{5} compound. Neither of these enzymes have been found in mammalian systems; however, we have recently found S-adenosyl-L-homocysteine nucleosidase present in kidney and liver extract from birds.

Recently S-adenosyl-L-homocysteine was reported to be oxidized to S-adenosyl-\textgammathio-\textalpha-ketobutyrate by the rat kidney L-amino acid oxidase (L-amino acid:oxygen oxidoreductase(deaminating), EC 1.4.3.2.).
Although the oxidative deamination of naturally occurring L-amino acids by L-amino acid oxidase from rat kidney, chicken and turkey liver, molluscs, snake venom and microorganisms, e.g. *Proteus vulgaris*, *Neurospora crassa* etc., has been demonstrated, the information concerning the action of this enzyme on sulfur amino acids is scarce. The present study demonstrates the action of a particulate L-amino acid oxidase from *Proteus rettgeri* on S-adenosyl-L-homocysteine, S-adenosyl-L-methionine, S-ribosyl-L-homocysteine and other sulfur amino acids. This particulate enzyme was shown previously to oxidize several amino acids including L-leucine, L-phenylalanine, L-tryptophan, L-arginine, L-tyrosine, L-histidine, L-orthinine, L-citrulline, L-lysine and L-isoleucine. Substrate specificity, pH optima, $K_m$ and $V_{max}$ values for various sulfur amino acids were studied to further characterize the enzyme. An L-amino acid oxidase from *Crotalus terrificus terrificus* venom was also studied for comparison.
HISTORY

The first detailed study concerning the enzymatic oxidation of L-amino acid oxidase was initiated in 1935 by Krebs (1). He reported the oxidation of L- and D-amino acids by homogenates of rat liver and kidney. At the same time Bernheim et al. (2) observed that the bacterium Proteus vulgaris was capable of oxidizing practically all the natural amino acids. These studies stimulated a general study of the enzyme involved in the oxidative deamination of amino acids which resulted in the characterization of an L-amino acid:oxygen oxidoreductase (deaminating) EC 1.4.3.2. The enzyme has been observed in a variety of organisms including mammals (3,4), birds (5,6), snakes (7,8) invertebrates (9,10), fungi (11,12,13) and bacteria (14,15). The L-amino acid oxidase from Neurospora crassa, Mytilus edulis, turkey liver and chicken liver readily oxidizes the basic amino acids, such as L-lysine and L-ornithine while the mammalian, ophidian and Proteus vulgaris oxidases are not active towards these amino acids. Of all the naturally occurring amino acids, L-serine and L-threonine have not been found to serve as substrates for L-amino acid oxidases of known sources. Also the snake venom L-amino acid oxidase from Crotalus adamanteus was reported capable of oxidizing amines and peptides (16). The enzyme from various sources differs with respect to specificity. A particular L-amino acid may be oxidized by the L-amino acid oxidase from each of the known sources, but the rates of oxidation may differ (17).
The pH optima of L-amino acid oxidases have been shown to be in the alkaline range; for example, the optimum for snake venom enzyme (Crotalus adamanteus) is pH 7.5 (18,19); pH 8.8 to 9.2 for the rat kidney enzyme (20); pH 8.0 for the enzyme from Penicillium notatum and Aspergillus niger (12).

L-Amino acid oxidase have been shown to contain riboflavin as a prosthetic group (21). The mammalian enzyme has been found to contain flavin mononucleotide (FMN), whereas certain microorganisms and snake venom enzymes contain flavin adenine dinucleotide (FAD) (22). The structural characteristics of the L-amino acid oxidase from the venom of Agkistrodon piscivorus (mocassin) was found to contain one mole of flavin adenine dinucleotide per mole of enzyme protein with the molecular weight of 62,000 (21). The L-amino acid oxidase from the venom of Crotalus adamanteus (eastern diamondback rattle snake) possesses a molecular weight of 133,000 and two moles of FAD per mole enzyme protein (18). In contrast, the mammalian enzyme from rat kidney with a molecular weight of 88,900 contains two moles of FMN (23).

The reaction mechanism is thought to involve the oxidation of the amino acid to the imino acid with the reduction of the flavin prosthetic group, hydrolysis of the imino acid to the corresponding keto acid and ammonia, and reoxidation of the reduced flavin by molecular oxygen (24,25), as shown in the following reactions (E is enzyme):

\[
R-\text{CH(NH}_2\text{)}\text{COOH} + E-\text{FAD} \rightarrow E-\text{FADH}_2 + R-\text{CH(NH)}\text{COOH} \quad (1)
\]

\[
R-\text{CH(NH)}\text{COOH} + H_2O \rightarrow R-\text{CO-COOH} + \text{NH}_3 \quad (2)
\]
E-FADH₂ + O₂ ➝ E-FAD + H₂O₂  \hspace{1cm} (3)

which give the overall reaction as follows:

R-CH(NH₂)COOH + O₂ + H₂O ➝ H₂O₂ + R-CO-COOH + NH₃ \hspace{1cm} (4)

In the presence of catalase, the hydrogen peroxide is decomposed to water and molecular oxygen (Reaction 5):

H₂O₂ ➝ H₂O + 1/2 O₂ \hspace{1cm} (5)

and in the overall reaction (6), 0.5 moles of oxygen is consumed when one mole of substrate is utilized.

R-CH(NH₂)COOH + 1/2 O₂ ➝ R-CO-COOH + NH₃ \hspace{1cm} (6)

In the absence of catalase, hydrogen peroxide reacts nonenzymatically with the α-keto acid (Reaction 7):

R-CO-COOH + H₂O₂ ➝ R-COOH + CO₂ + H₂O \hspace{1cm} (7)

resulting in the production of an acid and release of carbon dioxide as shown in the overall reaction (8):

R-CH(NH₂)COOH + O₂ ➝ R-COOH + CO₂ + NH₃ \hspace{1cm} (8)

where one mole of oxygen is utilized for each mole of amino acid oxidized.

The actual involvement of the two prosthetic groups is not fully understood. The substrate inhibition phenomenon found in snake venom from Crotalus adamanteus (7) and Neurospora crassa (11) L-amino acid oxidases stimulated the study of the mechanism of enzyme action. In the study of Crotalus adamanteus venom L-amino acid oxidase which contains two molecules of FAD per molecule of enzyme, two different mechanisms were suggested. A tentative mechanism was suggested by...
Meister and Wellner (25) in which the active site of the enzyme was thought to contain two prosthetic groups. The amino acid forms a complex with the enzyme and donates two hydrogen atoms, one to each FAD, yielding a dihydro enzyme and an imino acid which is hydrolyzed to ammonia and the corresponding keto acid. The half-reduced enzyme is rapidly reoxidized by oxygen. It is also possible that oxygen reacts with the fully reduced enzyme by removing the hydrogen atoms from the same FAD to yield an enzyme containing one reduced and one oxidized FAD. The intermediate reacts with oxygen to yield a fully oxidized enzyme. Scheme I shows the tentative mechanism.

Another mechanism was proposed by Massey and Curti (26) in which the enzyme was thought to contain two active sites, each of which contains one molecule of FAD. During the reduction of the enzyme-flavin complex with the substrate (amino acid), a semiquinone intermediate is formed by transfer of one electron from substrate to flavin (Reaction 9). The half-reduced enzyme may be reduced further by an additional electron transfer or may be directly reoxidized by molecular oxygen (Reactions 10 and 11).

\[
\begin{align*}
-FAD & \xrightarrow{+AA} -FAD + AA \\
-FADH & \xrightarrow{-AA} -FADH + AA \\
-FADH & \xrightarrow{-IA} -FADH + IA \\
-FADH_2 & \xrightarrow{+IA} -FADH_2 + IA \\
\end{align*}
\]

(semiquinone form)

\[
\begin{align*}
-FADH & \xrightarrow{+O_2} -FADH + O_2 \\
-FADH & \xrightarrow{-O_2} -FADH + O_2 \\
-FADH & \xrightarrow{-IA} -FAD + IA \\
-FAD & \xrightarrow{+H_2O_2} -FAD + H_2O_2 \\
\end{align*}
\]
Tentative mechanism of L-amino acid oxidase.
\[ -\text{FADH}_2 + \text{IA} + \text{O}_2 \rightarrow -\text{IA} + \text{H}_2\text{O}_2 \] (11)

The structure of FAD and its semiquinone is shown in Formula I.

The first bacterial L-amino acid oxidase was isolated and characterized by Stumph and Green in 1944 (14). Among the bacteria investigated, *Pseudomonas pyocyaneus* and *Aerobacter aerogenes* were found to be sources of the enzyme. Gram positive bacteria including *Staphylococcus aureus*, *Streptococcus hemolyticus*, *Diplococcus pneumoniae*, *Sarcina lutea*, *Bacillus subtilis* and Gram negative rods including *Escherichia coli* and *Salmonella paratyphi* are without L-amino acid oxidase activity. The L-amino acid oxidase from *Proteus vulgaris* was found to be associated with particles that sedimented at a centrifugal force of 3,000 x g. Most unsubstituted monocarboxylic, monoamino amino acids except valine and alanine were oxidized. For each molecule of amino acid oxidized, one atom of oxygen was taken up and one molecule of keto acid and ammonia formed as shown in Reaction 6 in the absence of catalase. The pH optima were 7.1.

Krishnamurthi, Buckley and Duerre (15) have reported the presence of an L-amino acid oxidase in a Gram negative bacteria. This particular microorganism had all the characteristics outlined in Burgey's Manual of Determinative Bacteriology of an Achromobacter species (27) except its ability to ferment glucose and other sugars. Further examination of its properties indicated that it might be better classified as an atypical *Proteus rettgeri* (Duerre,
unpublished data). The L-amino acid oxidase from this organism is a particulate enzyme which sediments at a centrifugal force of 100,000 x g. The enzyme catalyzed the oxidative-deamination of L-leucine, L-phenylalanine, L-tryptophan, L-arginine, L-tyrosine, L-histidine, L-ornithine, L-citrulline, L-lysine and L-isoleucine. The particles also contained catalase and catalyzed the overall reaction as shown above. The specificity of this enzyme differed somewhat from that observed for previously studied enzymes, particularly in its activity towards the basic amino acids arginine, ornithine and lysine. Since this bacterial L-amino acid oxidase has a low pH optimum, 7 - 7.5, we decided to use this enzyme to study the oxidative-deamination of sulfur amino acids.

In contrast to Proteus, this microorganism is non-motile, has a lower optimum growth temperature (24°C), produces hydrogen sulfide from cysteine, and gives a negative test for indole on standard peptone or tryptophan medium. However, the organism gives a positive indole test on nitrate medium (28).
EXPERIMENTAL PROCEDURES

Materials

L-Homocysteine, L-cystine, L-methionine, L-lanthionine, L-methionine sulfoxide and L-homocysteic acid were obtained from Calbiochem, Los Angeles, California; L-cystathionine, L-djenkolic acid from Sigma Chemical Company, St. Louis, Missouri; L-cysteine (free base) from Nutritional Biochemicals Corporation, Cleveland, Ohio. L-Amino acid oxidase of Crotalus terrificus terrificus venom and bovine liver catalase were purchased from Boehringer Mannheim Corporation, New York, New York. 1,2,3-Triketohydrindene was purchased from Eastman Organic Chemicals, Rochester, New York; 2,4-dinitrophenylhydrazine was from Matheson Coleman and Bell, East Rutherford, New Jersey. General chemicals were purchased from Fisher Scientific, Minneapolis, Minnesota, and Mallinckrodt Chemical Works, St. Louis, Missouri. The Dowex, Amberlite, Sephadex, and ion retardation resins were purchased from the J. T. Baker Chemical Company, Phillipsburg, New Jersey; Mallinckrodt Chemical Works; Pharmacia, Uppsala, Sweden and Bio Rad Laboratories, Richmond, California, respectively.

Preparation of Substrates

Preparation of S-Adenosyl-L-homocysteine

S-Adenosyl-L-homocysteine was enzymatically synthesized from adenosine and L-homocysteine by the procedure outlined by Duerre,
Salisbury and Miller (29). Rat livers were homogenized in 3 volumes of 0.01 M acetic acid for 30 seconds in a Waring blender at maximum speed. Cellular debris was removed by centrifugation at 18,000 x g for 20 minutes. Solid ammonium sulfate was added with constant stirring to the supernatant fluid to 40% saturation and the resultant precipitate was removed by centrifugation at 15,000 x g for 10 minutes. The supernatant fluid was raised to 50% saturation with solid ammonium sulfate and mechanically stirred for 15 minutes. The precipitate was collected as before by centrifugation and dissolved in 0.05 M potassium phosphate buffer, pH 7.0. This fraction prepared at 0°C served as the source of S-adenosyl-L-homocysteine hydrolase. The specific activity was around 0.3 μmoles of homocysteine utilized per minute per mg of protein.

For each mmole of adenosine, 3.0 mmoles of DL-homocysteine and 18.0 units of the partially purified enzyme were incubated in 0.1 M phosphate buffer, pH 6.5, at 37°C under an atmosphere of nitrogen. After an incubation period of 90 minutes, thiodiglycol (0.025 ml per 100 ml reaction mixture) was added to maintain S-adenosyl-L-homocysteine in the reduced state. The reaction mixture was deproteinized by heating in a boiling water bath for 5 minutes, cooling on ice and centrifuging for 10 minutes at 10,000 x g. S-Adenosyl-L-homocysteine was isolated from the supernatant fluid by column chromatography. Amberlite CG-120, 200 to 400 mesh, was charged with 6.0 N H₂SO₄ and equilibrated with 1.0 N H₂SO₄. A column 15 cm² in cross-sectional area with a resin bed 10 cm deep was sufficient for purification of 4 mmoles of S-adenosyl-L-homocysteine. After application of the sample, 1.5 liters of 3.0 N H₂SO₄ was required to remove the undesired compounds. Elution
was followed by ultraviolet absorption at 260 μm and when the column was free of impurities, S-adenosyl-L-homocysteine was eluted with 2.5 to 3.0 liters of 6.0 N H₂SO₄. S-Adenosyl-L-homocysteine was precipitated from solution by the addition of phosphotungstic acid. The supernatant fluid was removed by centrifugation at 500 x g for 5 minutes. The precipitate was washed twice with 0.05 N H₂SO₄ and dissolved in 6 volumes of a 1:1 mixture of acetone and water. The phosphotungstate complex was dissociated by the method of Schlenk and DePalma (30). The solution was extracted with 4 volumes of an isoamyl alcohol, ether (1:1) mixture. S-Adenosyl-L-homocysteine remained in the aqueous phase. Residual isoamyl alcohol and acetone were removed by 4 extractions with three volumes of ether. Nitrogen was bubbled through the solution to remove residual ether, and the aqueous solution was adjusted to pH 4.0 with freshly prepared BaCO₃. The resulting precipitate was removed by filtration and washed with a small amount of water which was combined with the original filtrate. The filtrate was lyophilized, dissolved in water (45-50 μmoles/ml), frozen, and stored at 4°C for four days to effect crystallization. Crystals were collected by filtration, washed with a small amount of cold water, and stored under vacuum until used. The total yield of S-adenosyl-L-homocysteine calculated from the amount of adenosine in the initial reaction mixture ranged from 40 to 50%. Samples of the purified compound were chromatographed on Whatman No. 1 paper to check for ultraviolet and ninhydrin-positive impurities. No ultraviolet or ninhydrin-positive impurities were detected.
Preparation of S-Adenosyl-L-methionine

The method of Schlenk et al. (31) utilized the synthetic capabilities of yeasts to form S-adenosyl-L-methionine from exogenously supplied L-methionine and endogenously formed ATP. Saccharomyces cerevisiae, NRL strain, was cultured in Snell's medium (Appendix 1) supplemented with L-methionine (0.75 g per liter). After incubation of the culture for two days at 30°C with moderate aeration, the cells were harvested by continuous-flow centrifugation and washed twice with demineralized water. The cells were extracted with 4 volumes of 1.5 N perchloric acid for one hour at room temperature with constant stirring. Cellular debris was removed by centrifugation at 10,000 x g for 10 minutes and discarded. The S-adenosyl-L-methionine was precipitated from the supernatant fluid by the addition of 1/2 volume of a cold freshly prepared saturated solution of ammonium reineckate. After storage at 4°C for 8 hours, the precipitate was collected by suction filtration, washed with a small amount of cold water and dissolved in 10 to 20 volumes of a 1:1 mixture of acetone and 1 N H₂SO₄. The reineckate-S-adenosyl-L-methionine complex was applied to a column of Dowex 50 W-X8 resin and the column was eluted by the same procedures used for the preparation of S-adenosyl-L-homocysteine. The compound was precipitated with phosphotungstic acid and separated from the phosphotungstate with organic solvents by those procedures described for the purification of S-adenosyl-L-homocysteine. The lyophilized compound was diluted with water to a desired concentration and checked for purity by chromatography on Whatman No. 1 paper. The preparations were found to contain trace amounts of an ultraviolet
light absorbing impurity.

**Preparation of S-Ribosyl-L-homocysteine**

S-Ribosyl-L-homocysteine was enzymatically prepared from S-adenosyl-L-homocysteine by the action of S-adenosyl-L-homocysteine nucleosidase by the method of Duerre and Miller (32). *Escherichia coli*, strain W, was cultured at room temperature in four 20 liter carboys containing 15 liters of M-9 medium (Appendix 2). To each carboy was added an inoculum of 1% by volume of a 12-hour culture grown on the same medium. Vigorous aeration was maintained by forcing filtered air through the medium. After 12 hours the cells were harvested by continuous-flow centrifugation at 35,000 x g, washed twice with 0.05 M phosphate buffer, pH 7.8, and resuspended in the same buffer at a concentration of about 400 mg of wet cells per ml. The cells were disrupted in a nitrogen-cooled pressure cell at 26,000 psi and the remaining whole cells and cellular debris were removed by centrifugation at 20,000 x g for 15 minutes. The extract was incubated with commercial deoxy-ribonuclease and ribonuclease, 1 mg each per 100 ml of extract, for one hour at 37°C. Denatured protein was removed by centrifugation at 10,000 x g for 15 minutes. Bases, nucleotides, nucleosides, and other small molecular weight materials were removed by passing the extract through a column of Sephadex G-25. A column (4 x 44 cm) previous equilibrated with 4 liters of 0.05 M phosphate buffer, pH 7.8, was suitable for 170 to 200 ml of extract. Elution of the protein using the same buffer was followed with the Biuret reagent. This preparation was denoted as Fraction I. Ammonium sulfate solution saturated at 0°C containing $1 \times 10^{-3}$ M EDTA and adjusted to pH 7.0 was
added dropwise to crude extract to 40%. After mechanical stirring for 15 minutes, the precipitate was removed by centrifugation at 15,000 x g for 15 minutes and discarded. The supernatant fluid was raised to 61% saturation and mechanically stirred for 15 minutes. The precipitate was collected as before and dissolved in 0.05 M phosphate buffer, pH 7.8, containing $3 \times 10^{-3}$ M 2-mercaptoethanol to yield of a protein concentration of 35 to 50 mg per ml as determined by the method of Warburg and Christian (Fraction II) (33). Fraction II was fractioned on a column of Sephadex G-150 resin. The fractions containing the enzyme were pooled and the protein concentrated by the dropwise addition of saturated ammonium sulfate solution to 60%. The precipitate was collected by centrifugation at 15,000 x g for 15 minutes, dissolved in 0.05 M phosphate buffer, pH 7.8, containing $3 \times 10^{-3}$ M 2-mercaptoethanol, and dialyzed against 4 volumes of the same buffer for 7 hours at 4°C with three changes. This represented Fraction III. Fraction III was applied to a column of DEAE-Sephadex A-50 resin and fractionated. Enzyme activity and protein concentration were determined on selected 10 ml fractions to locate the enzyme. The fractions containing the enzyme were pooled and used for the preparation of S-ribosyl-L-homocysteine. S-Adenosyl-L-homocysteine, prepared as described before, was incubated with the partially purified nucleosidase at a concentration of one µmole per unit of enzyme in the presence of 0.1 M phosphate buffer, pH 6.5, for two hours at 37°C. The reaction mixture was deproteinized by heating in a boiling water bath for 4 minutes, and the clear supernatant fluid obtained by centrifugation at 2,500 x g for 5 minutes was placed on a column of Dowex 50 W-X8-H+.
resin, 200-400 mesh, previously washed with 6.0 N HCl and equilibrated with 0.1 N HCl. The surface area of the column was 1.0 cm$^2$ for each 350 μmoles of S-ribosyl-L-homocysteine. Phosphate and mercapto-ethanol were eluted from the column by development with 0.1 N HCl. The S-ribosyl-L-homocysteine was then eluted with 1.0 N HCl. The effluent containing S-ribosyl-L-homocysteine was adjusted to pH 6.0 by the addition of Dowex 2-X8-OH$^-$. The resin was removed by filtration and washed with demineralized water until free of orcinol-positive material. The wash was combined with the filtrate and the solution was lyophilized to dryness and immediately dissolved in water to a desired concentration as determined by the orcinol reaction. The yield of S-ribosyl-L-homocysteine ranged from 70 to 80%.

**Preparation of S-Adenosyl-L-homocysteine Sulfoxide and S-Ribosyl-L-homocysteine Sulfoxide**

These two compounds were prepared by the method of Duerre, Salisbury and Miller (34). The sulfoxide derivatives of S-adenosyl-L-homocysteine and S-ribosyl-L-homocysteine were prepared by incubating one mole of the parent compound with three moles of hydrogen peroxide at 25°C, pH 5.0 for 12 hours. Residual hydrogen peroxide was decomposed by the addition of one unit crystalline beef liver catalase per μmole hydrogen peroxide used. After 10 minutes of incubation, the reaction tubes were immersed in a boiling water bath for 5 minutes, cooled in ice, filtered and lyophilized. S-Adenosyl-L-homocysteine sulfoxide and S-ribosyl-L-homocysteine sulfoxide were dissolved in water (50 to 60 μmoles/ml) and crystallization effected by the addition
of 5 to 10 volumes of ethanol and acetone, respectively, and stored in vacuo until used. Samples of both compounds were checked by paper chromatography on Whatman No. 1 paper and found to be free of detectable impurities.

**Preparation of Homocysteine from Homocysteine Thiolactone**

The method described by Duerre and Miller (35) was used for cleavage of the thiolactone ring of either DL- or L-homocysteine thiolactone. The free sulfhydryl form was prepared by incubating 0.1 mmole of the thiolactone with 0.3 ml of 3.0 N NaOH for 5 minutes at room temperature. The solution was immediately neutralized with 0.9 ml of 1.0 M KH$_2$PO$_4$ and diluted with water to yield the desired concentration of homocysteine.

**Preparation of Enzyme**

The bacterial L-amino acid oxidase was prepared by the method of Krishnamurthi, Buckley and Duerre (15). *Proteus rettgeri* was maintained on nutrient agar slants (Difco) pH 7.0. Cells were cultured in a medium containing 3 g Bacto beef extract (Difco) and 15 g Bacto-peptone (Difco) per liter at pH 7.0. One percent of inoculum of cells were grown on the same medium in a 20 liter carboy. The carboys were incubated at 24°C for 18 hours under forced aeration. Cells were separated from the medium by continuous flow-centrifugation using the Szent-Georgi-Blum continuous flow attachment to the Sorvall RC-2 refrigerated centrifuge. The cells were washed twice in 0.05 M potassium phosphate buffer, pH 7.5, and resuspended in the same buffer. The cells were disrupted in a nitrogen-cooled pressure cell at 25,000 psi. Cell-free extracts were centrifuged for 20 minutes at 37,000 x g
to remove remaining whole cells and cellular debris. After centri-
fugation of the resultant extract for 2 hours at 100,000 x g, the
supernatant fluid was decanted and discarded. The sediment was
suspended in 0.05 M potassium phosphate buffer, pH 7.5, washed once,
and resuspended in the same buffer. The specific activity of the
particulate fraction was about 0.1 μmoles leucine/minute/mg protein.

**Analytical Methods**

**Determination of α-Keto Acids**

The 2,4-dinitrophenylhydrazine method of Friedemann and Haugen
(36) was used to measure α-keto acids. Samples containing 0.05 to 0.3
μmoles keto acid were diluted to 1.0 ml with water and 0.05 ml of 100%
trichloroacetic acid was added. After mixing, 0.25 ml of a 0.1%
solution of 2,4-dinitrophenylhydrazine in 20% HCl was added and the
tubes incubated at room temperature for 5 minutes. Two ml of 4.0 N
NaOH was added with immediate mixing. After the tubes were incubated
at room temperature for 10 minutes, the absorbance of the samples was
determined at 515 μμ with a Coleman Jr. spectrophotometer. A standard
curve showing the relationship observed between absorbancy and μmoles
of pyruvate and α-ketobutyrate is given in Fig. 1.

**Determination of Protein**

Protein concentration was determined by the method of Lowry et al.
(37). Samples of the particulate enzyme containing 10-200 μl were
diluted to 0.6 ml with water. Three ml of reagent D (Appendix 3) was
added to each sample with vigorously shaking and allowed to stand at
room temperature for 10 minutes then 0.3 ml of Folin-Ciocalteu Phenol
reagent was added with immediate mixing. The tubes were allowed to stand
Fig. 1. -- Reference curve for the determination of pyruvic acid (●—●) and α-ketobutyric acid (○—○) by the 2,4-dinitrophenylhydrazine method (36). Both compounds were obtained commercially.
at room temperature for 30 minutes and read at 690 m\(\mu\) in a Coleman Jr. spectrophotometer. The reaction remained linear up to 50 \(\mu\)g protein per ml. (Fig. 2).

**Determination of Ammonia Nitrogen**

Ammonia was measured using the method of Braganca, Quastel, and Schucher (38). The oxidation of L-amino acid oxidase was carried out by the Warburg manometric technique (39). One ml saturated \(K_2CO_3\) was placed in one of the side arms of the cup and 0.2 ml of 10 N \(H_2SO_4\) on filter paper was placed in the center well to absorb liberated ammonia. At the end of oxidation reaction time the saturated \(K_2CO_3\) was tipped into the main vessel to stop the reaction. The reaction mixture was shaken for 3 hours and the contents of the center well, including filter paper were transferred to a tube. The center well was rinsed with water which was then added to original sample. The volume was brought to 2.0 ml and nitrogen was measured quantitatively by the Nessler's method as outlined by Johnson (40). A portion of the sample was diluted to 2.0 ml with water. Two ml of Nessler's reagent (Appendix 4) was added to the sample, and after mixing, 3.0 ml of 2 N NaOH was added. The reaction mixture was allowed to stand for 15 minutes and read at 490 m\(\mu\) in a Coleman Jr. spectrophotometer. A standard curve was prepared using \((NH_4)_2SO_4\) (Fig. 3).

**Determination of Hydrogen Sulfide**

Hydrogen sulfide was measured quantitatively by the method of Delwiche (41). At the end of the enzymic action, one ml of 2 N NaOH was added to stop the reaction. The sample volume was brought to 2.0 ml with water, and 2.0 ml of aqueous reagent containing 1.0 g of
Fig. 2. — Reference curve for the determination of protein by the method of Lowry et al. (37). A beef albumin was used.
Fig. 3. — Reference curve for the determination of ammonia nitrogen by the method of Johnson (40) using Nessler's reagent. Ammonia sulfate was used.
crystalline lead acetate, 2.5 ml of glacial acetic acid and 2.5 g of gum arabic per liter were added. The tubes were allowed to stand at room temperature for 15 minutes and read at 490 μ in a Coleman Jr. spectrophotometer. A standard curve was prepared by using crystalline lead acetate as a primary standard and sodium sulfide as the precipitating reagent. Two ml of aqueous reagent without lead acetate was added to a 2.0 ml sample containing a known concentration of lead acetate. One ml of 2 N NaOH and an excess of Na₂S was added and the mixture was allowed to stand at room temperature for 15 minutes. The linear relationship between absorbancy and the concentration of hydrogen sulfide is shown in Fig. 4.

**Chromatography**

Ascending thin layer paper chromatography was performed in glass jars. Samples were applied to the paper in aqueous solution. Duplicate chromatograms were developed using either 1) ethanol-acetic acid-water (65:1:34) or 2) propanol-acetic acid-water (65:4:31).

The method of Dancis, Hutzler and Levitz (42) for the identification of keto acids by thin layer chromatography was used. The hydrazine derivatives of keto acids were prepared by the addition of a 20% molar excess of 0.01 M 2,4-dinitrophenylhydrazine in 2.0 N HCl to the reaction mixture. After approximately 4 hours the crystals were collected by centrifugation, washed three times with cold water, dried and dissolved in ethanol. Aliquots were applied to thin layer silica gel plates which had been previously activated at 110°C for 1 hour. The solvent systems used were isoamyl alcohol-0.25 N ammonium hydroxide (20:1) and butanol saturated with 4.0% ammonium hydroxide.
Fig. 4. -- Reference curve for the determination of hydrogen sulfide by the method of Delwiche (41). Hydrogen sulfide was obtained by reacting different quantities of lead acetate with excess of sodium sulfide in alkaline solution.
Enzyme Assays

The oxidations of the various substrates were measured by determining the rate of oxygen consumption manometrically (39). The vessels contained 15 μmoles of substrate, 300 μmoles phosphate buffer, pH 7.5, and 9.6 mg bacterial protein in a final volume of 3.0 ml. When snake venom L-amino acid oxidase was used, it was necessary to add 300 units beef liver catalase, 250 μmoles KCl and 25 μg crystalline enzyme. After 30 minutes of incubation at 30°C the reaction mixtures were deproteinized with 0.1 ml of 100% trichloroacetic acid and the amounts of keto acid, hydrogen sulfide and ammonia liberated were measured.

The pH optima of the enzyme was determined by measuring the rate of oxygen consumption using phosphate buffer at various pH values.

K<sub>m</sub> values and maximum velocities for all substrates were determined from Lineweaver-Burk double reciprocal plots (43) of initial reaction velocities as a function of the rate of keto acid formation and/or oxygen consumption. The rate of oxygen consumption was also determined polarographically using a Gilson oxygraph Model KM equipped with a YSI Clark oxygen electrode. The electrode chamber with a reaction mixture volume of 1.5 ml contained enzyme, 7.5 μmoles of substrate, 150 μmoles phosphate buffer, pH 7.5, 150 units of catalase and 125 μmoles of KCl when snake venom enzyme was used. The temperature of the chamber was kept at 30°C. The percent oxygen utilized per minute was calculated from the slope of the recorded line from the oxygraph operating at a given speed. This value was multiplied by the number of μmoles of dissolved oxygen present in
1.5 ml of water at 30° C and divided by the total mg protein present in the reaction mixture. The resulting value is the specific activity of the enzyme in μmoles of oxygen utilized per minute per mg protein.
RESULTS

The ability of the particulate bound bacterial L-amino acid oxidase to catalyze the oxidative-deamination of sulfur amino acids was tested. No measurable activity was observed with cysteic acid, homocysteic acid, lanthionine or cystathionine. The rates of oxygen consumption and product formation from those sulfur amino acids oxidized are shown in Table 1.

The rate of product formation versus oxygen consumption followed the characteristic pattern observed for a general L-amino acid oxidase, i.e. the rate of oxygen consumption to keto acid and ammonia formed was approximately 1:2:2, except with the free sulfhydryl compounds, homocysteine and cysteine. With these compounds the rate of oxygen consumption was greater than one. The excess oxygen consumption may be due to auto-oxidation of the sulfhydryl compound or the products formed.

Of considerable interest was the observation that large amounts of hydrogen sulfide were liberated from cysteine and homocysteine (Table 1). No hydrogen sulfide was liberated under anaerobic conditions, nor did pyridoxal affect the rate of formation of hydrogen sulfide; hence it was unlikely that the particulate fraction from the bacteria contained a desulfhydrase.

Product Identification

Reaction mixtures incubated as outlined under Table 1 were deproteinized with trichloroacetic acid which was later removed by
### TABLE 1
Rates of Oxygen Consumption and Product Formation from **Sulfur Amino Acids** by a **Bacterial L-Amino Acid Oxidase**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Specific Activity</th>
<th>0₂</th>
<th>Keto Acid Produced</th>
<th>NH₃</th>
<th>H₂S</th>
</tr>
</thead>
<tbody>
<tr>
<td>S-Adenosyl-L-methionine</td>
<td>11.6</td>
<td>23.8</td>
<td>22.5</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>S-Adenosyl-L-homocysteine</td>
<td>9.3</td>
<td>19.7</td>
<td>17.4</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>S-Adenosyl-L-homocysteine sulfoxide</td>
<td>0.5</td>
<td>1.1</td>
<td>1.0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>S-Ribosyl-L-homocysteine</td>
<td>4.0</td>
<td>8.4</td>
<td>7.9</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>L-Methionine</td>
<td>13.3</td>
<td>26.5</td>
<td>24.1</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>L-Methionine sulfoxide</td>
<td>0.9</td>
<td>1.8</td>
<td>1.8</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>L-Homocysteine</td>
<td>8.8</td>
<td>5.8</td>
<td>6.0</td>
<td>12.8</td>
<td></td>
</tr>
<tr>
<td>L-Homocystine</td>
<td>10.0</td>
<td>22.0</td>
<td>24.6</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>L-Cysteine</td>
<td>3.3</td>
<td>2.4</td>
<td>2.4</td>
<td>1.2</td>
<td></td>
</tr>
<tr>
<td>L-Cystine</td>
<td>1.2</td>
<td>3.8</td>
<td>2.5</td>
<td>trace</td>
<td></td>
</tr>
<tr>
<td>L-Djenkolic acid</td>
<td>2.4</td>
<td>4.5</td>
<td>4.1</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

*a* Oxygen consumption was measured manometrically. The vessels contained 15 μmoles substrate, 300 μmoles phosphate buffer, pH 7.5, and 9.6 mg enzyme protein in a final volume of 3.0 ml. After 30 min incubation at 30°C the reaction mixtures were deproteinized with TCA and keto acids and hydrogen sulfide measured as outlined under "Methods". Ammonia was measured in a duplicate vessel by the diffusion technique of Braganca *et al.* (38). There was no oxygen consumption or product formation in endogenous controls. Oxygen consumption remained linear throughout the experiment.

*b* Activity is expressed as μmoles of oxygen consumed or products produced/min/mg protein.
### TABLE 2

**Rf Values of Keto Acids Derived from Sulfur Amino Acids by Bacterial L-Amino Acid Oxidase**

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>S-Adenosyl-L-methionine</td>
<td>0.76</td>
<td>0.77</td>
</tr>
<tr>
<td>S-Adenosyl-L-homocysteine</td>
<td>0.62</td>
<td>0.55</td>
</tr>
<tr>
<td>S-Ribosyl-L-homocysteine</td>
<td>0.79</td>
<td>0.69</td>
</tr>
<tr>
<td>L-Methionine</td>
<td>0.60 0.81</td>
<td>0.40 0.73</td>
</tr>
<tr>
<td>L-Djenkolic acid</td>
<td>0.57 0.72</td>
<td>0.17 0.53</td>
</tr>
<tr>
<td>L-Homocystine (1 hr)</td>
<td>0.23 0.39 0.60</td>
<td>0.23 0.41 0.53</td>
</tr>
<tr>
<td></td>
<td>(4 hr)</td>
<td></td>
</tr>
<tr>
<td>L-Homocysteine (1 hr)</td>
<td>0.60</td>
<td>0.55</td>
</tr>
<tr>
<td></td>
<td>(4 hr)</td>
<td></td>
</tr>
<tr>
<td>L-Homocysteine (1 hr)</td>
<td>0.13 0.36 0.88</td>
<td>0.21 0.46 0.82</td>
</tr>
<tr>
<td></td>
<td>(4 hr)</td>
<td>0.85</td>
</tr>
<tr>
<td>α-Ketobutyrate (control)</td>
<td>0.92</td>
<td>0.88</td>
</tr>
<tr>
<td>L-Cysteine (1 hr)</td>
<td>0.46 0.76</td>
<td>0.45 0.65</td>
</tr>
<tr>
<td></td>
<td>(4 hr)</td>
<td>0.81 0.66</td>
</tr>
<tr>
<td>Pyruvate (Control)</td>
<td>0.78</td>
<td>0.63</td>
</tr>
</tbody>
</table>

*One ml of each reaction mixture from the experiment outlined in Table 1 was deproteinized with TCA and chromatographed in quadruplicate on thin layer cellulose plates. Residual substrates were located on one set of plates with ninhydrin, keto acids on another with 2,4-dinitrophenylhydrazine, thioether and sulfoxide on another with iodoplatinate and carbohydrate on a fourth with ammonical silver nitrate.*
three extractions with an equal volume of ether. After chromatography on thin layer cellulose plates, keto acids were located with 2,4-dinitrophenylhydrazine. Duplicate sets of plates developed in the same solvent system were sprayed with ninhydrin, platinic iodide, or ammonical silver nitrate (Table 2). The keto acids derived from S-adenosylmethionine, S-adenosylhomocysteine and S-ribosylhomocysteine, all reacted with iodoplatininate and ammonical silver nitrate indicating that the sulfur atom and carbohydrate moiety remained associated with the keto acid derivative. In addition, the keto acid derived from S-adenosylhomocysteine and S-adenosylmethionine absorbed ultraviolet light indicating the presence of the purine moiety. In the reaction involving S-adenosylmethionine as substrate, it was found that methylthioadenosine and homoserine were present. However, no activity was observed when L-homoserine was incubated with enzyme, thus eliminating this compound as the active substrate.

The end product from the homocysteine reaction was studied quite intensively to determine in either one or the other or both of the amino groups were eliminated. Results of chromatographs with reaction products from time course studies revealed that mixed products resulted early in the reaction, whereas only one keto acid was observed after prolonged incubation (Table 2).

The 2,4-dinitrophenylhydrazone of early and late reaction products from homocystine also were prepared. As with free keto acids, more than one product appeared to result in the first few minutes, whereas only one product appeared on chromatograms after 4 hours incubation (Table 3).
Sufficient quantities of the dinitrophenylhydrazone from the 4 hour reaction mixture were prepared for chemical analysis. Result of molecular weight determination in acetone was 687, some 10% higher than a value of 626 calculated using the formula for a diketo derivative of dinitrophenylhydrazine. However, these results do not overlap with a molecular weight of 446 calculated for a monoamino-monoketo derivative. The melting point of the dinitrophenylhydrazone was 164°C. Results of chemical analysis are also in agreement with the presence of two keto groups in the parent compound. Therefore, the ultimate product from homocysteine was confirmed to be 4,4'-dithio-bis(2-ketobutyric acid). (Formula II).

Results of chromatographic studies with homocysteine as substrate were somewhat amigous. L-Homocysteine tends to streak, having $R_f$ values of 0.66-0.71, and 0.43 in ethanol-acetic acid-water, and propanol-acetic acid-water respectively. Moreover, other reaction products also were present, some of them appearing to have the same $R_f$ values and chemical properties as homocysteine and homocysteic acid. At least two keto acids also were formed.

Chromatography of the dinitrophenylhydrazones was more successful. Mixed products appeared early in reaction, whereas after 4 hours incubation the $R_f$ values corresponded to the two isomers of the dinitrophenylhydrazone of $\alpha$-ketobutyrate (Table 3).

With cysteine the results were similar to those obtained with homocysteine. The final reaction product appeared to be pyruvate.
FORMULA II

\[
\text{COOH} \quad \text{-} \quad \text{C} \quad \text{-} \quad \text{CH}_2 \quad \text{-} \quad \text{CH}_2 \quad \text{-} \quad \text{S} \quad \text{-} \quad \text{S} \quad \text{-} \quad \text{CH}_2 \quad \text{-} \quad \text{CH}_2 \quad \text{-} \quad \text{C} \quad \text{-} \quad \text{COOH}
\]

\[
\text{N} \quad \text{-} \quad \text{NH} \quad \text{N} \\
\text{C}_6\text{H}_3(\text{NO}_2)_2 \quad \text{NH} \\
\text{C}_6\text{H}_3(\text{NO}_2)_2
\]

\[
\text{C}_{20} \quad \text{H}_{18} \quad \text{O}_{12} \quad \text{N}_8 \quad \text{S}_2
\]

CALculated: \quad C \ 38.4 \quad H \ 2.9 \quad O \ 30.6 \quad N \ 17.9 \quad S \ 10.3

Found: \quad C \ 39.4 \quad H \ 3.7 \quad O \ 29.2 \quad N \ 15.2 \quad S \ 11.6
<table>
<thead>
<tr>
<th>Keto Acid Derived From</th>
<th>( R_f ) Isoamyalcohol:0.25 N NH(_4)OH(20:1)</th>
<th>( R_f ) Butanol saturated with 4.0% NH(_4)OH</th>
</tr>
</thead>
<tbody>
<tr>
<td>S-Adenosyl-L-methionine</td>
<td>0.47</td>
<td>0.81</td>
</tr>
<tr>
<td>S-Adenosyl-L-homocysteine</td>
<td>0.02</td>
<td>0.21</td>
</tr>
<tr>
<td>S-Ribosyl-L-homocysteine</td>
<td>0.16 0.35</td>
<td>0.59</td>
</tr>
<tr>
<td>L-Methionine</td>
<td>0.17 0.36</td>
<td>0.52 0.69</td>
</tr>
<tr>
<td>L-Djenkolic acid</td>
<td>0.22 0.55</td>
<td>0.38 0.65</td>
</tr>
<tr>
<td>L-Homocystine (1 hr)</td>
<td>0.09 0.21 0.44</td>
<td>0.06 0.17 0.26 0.64</td>
</tr>
<tr>
<td>(4 hr)</td>
<td>0.10 0.19</td>
<td>0.19 0.27</td>
</tr>
<tr>
<td>L-Homocysteine (30 min)</td>
<td>0.03 0.12 0.27</td>
<td>0.17 0.26 0.34 0.45 0.68</td>
</tr>
<tr>
<td>(4 hr)</td>
<td>0.13 0.27</td>
<td>0.48 0.65</td>
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</table>
TABLE 3 -- continued

<table>
<thead>
<tr>
<th></th>
<th>0.13</th>
<th>0.26</th>
<th>0.47</th>
<th>0.64</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-ketobutyrate (control)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L-Cysteine (1 hr)</td>
<td>0.04</td>
<td>0.11</td>
<td>0.24</td>
<td>0.45</td>
</tr>
<tr>
<td></td>
<td>0.12</td>
<td>0.23</td>
<td>0.22</td>
<td>0.37</td>
</tr>
<tr>
<td>Pyruvate (Control)</td>
<td>0.09</td>
<td>0.21</td>
<td>0.25</td>
<td>0.36</td>
</tr>
</tbody>
</table>

*One ml of each reaction mixture from the experiment outlined in Table 1 was deproteinized with TCA and dinitrophenylhydrazone prepared as outlined under "Methods". The resultant dinitrophenylhydrazones were dissolved in alcohol and chromatographed on silica gel.*
Snake Venom L-Amino Acid Oxidase

Due to the possibility that other enzymes were associated with the particulate bacterial L-amino acid oxidase, we compared the results with the bacterial oxidase to those obtained with pure crystalline snake venom L-amino acid oxidase. The snake venom enzyme had a more limited specificity than the bacterial enzyme (Table 4). No appreciable activity was observed with any of the sulfoxides, nor was there any activity with S-adenosyl-L-methionine, homocysteic acid, cysteic acid, lanthionine, or cystathionine.

As with the bacterial enzyme, the stoichiometric relation of 1:2:2 for oxygen consumption to ammonia and keto acid formation held for all substrates except homocysteine and cysteine. Here again oxygen consumption was disproportionately high. However, there was a definite one to one stoichiometric relation between the amount of keto acid produced and amount of ammonia and hydrogen sulfide liberated.

Optimal pH for Enzymatic Reactions

The effect of pH on the rate of oxidation of sulfur amino acids by the bacterial enzyme is shown in Fig. 5. The pH optimum with S-ribosyl-L-homocysteine, S-adenosyl-L-homocysteine, S-adenosyl-L-methionine and L-methionine was between 7.4 and 7.8, whereas the pH optimum for L-homocystine and L-djenkolic acid was 8.5 to 8.8. The pH optimum for sulfhydryls could not be measured as a function of oxygen consumption, since auto-oxidation of these compounds increased markedly with increasing pH. The rate of keto acid formation from these compounds was also found to increase throughout the pH range tested (Fig. 5A).
TABLE 4

Rate of Oxygen Consumption and Product Formation from Sulfur Amino Acids by Snake Venom L-Amino Acid Oxidase

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Specific Activity&lt;sup&gt;b&lt;/sup&gt;</th>
<th>O&lt;sub&gt;2&lt;/sub&gt; Produced</th>
<th>Keto Acid Produced</th>
<th>NH&lt;sub&gt;3&lt;/sub&gt;</th>
<th>H&lt;sub&gt;2&lt;/sub&gt;S</th>
</tr>
</thead>
<tbody>
<tr>
<td>S-Adenosyl-L-homocysteine</td>
<td></td>
<td>1.2</td>
<td>2.8</td>
<td>2.5</td>
<td>0</td>
</tr>
<tr>
<td>S-Ribosyl-L-homocysteine</td>
<td></td>
<td>2.1</td>
<td>4.4</td>
<td>4.2</td>
<td>0</td>
</tr>
<tr>
<td>L-Methionine</td>
<td></td>
<td>5.6</td>
<td>11.4</td>
<td>11.6</td>
<td>0</td>
</tr>
<tr>
<td>L-Homocysteine</td>
<td></td>
<td>4.1</td>
<td>1.4</td>
<td>1.4</td>
<td>1.0</td>
</tr>
<tr>
<td>L-Homocystine</td>
<td></td>
<td>1.4</td>
<td>2.5</td>
<td>2.1</td>
<td>0</td>
</tr>
<tr>
<td>L-Cysteine</td>
<td></td>
<td>5.7</td>
<td>1.5</td>
<td>1.4</td>
<td>1.3</td>
</tr>
<tr>
<td>L-Cystine</td>
<td></td>
<td>4.1</td>
<td>6.0</td>
<td>3.9</td>
<td>trace</td>
</tr>
<tr>
<td>L-Djenkolic acid</td>
<td></td>
<td>1.3</td>
<td>2.9</td>
<td>3.0</td>
<td>trace</td>
</tr>
</tbody>
</table>

<sup>a</sup>Oxygen consumption was measured manometrically. The vessels contained 15 μmoles substrate, 300 μmoles phosphate buffer, pH 7.5, 300 units catalase, 250 μmoles KCl and 25 μg crystalline snake venom L-amino acid oxidase. After 30 min incubation at 30°C the reaction mixtures were deproteinized with TCA and hydrogen sulfide and keto acid measured as outlined under methods. Ammonia was measured in a duplicate vessel by diffusion technique of Braganca et al. (38). No endogenous activity was observed.

<sup>b</sup>Activity is expressed as μmoles oxygen consumed or products produced/ min/mg protein.
The effect of pH on the rate of oxidation of sulfur amino acids by snake venom enzyme is shown in Fig. 6. The shape of the curves and pH optimum varied with respect to the individual amino acids. With some of the compounds, i.e., L-cysteine and S-adenosyl-L-homocysteine, there appeared to be two optima, one in the lower region between pH 7.2 and 7.5 and the other 7.7 for cysteine and above 8.5 for S-adenosyl-L-homocysteine. Homocysteine, cystine and methionine exhibited single pH optimum at pH 8.0, 8.4 and 7.3 respectively. The curves with S-riboyl-L-homocysteine, djenkolic acid and homocystine increased beyond pH 8.5; however, it was difficult to extend these curves beyond this point since most of the compounds are alkaline labile.

**Determination of Michaelis Constants**

A Lineweaver-Burk double reciprocal plot of initial reaction velocities of bacterial L-amino acid oxidase against S-adenosyl-L-homocysteine concentrations is shown in Fig. 7. The $K_m$ value for S-adenosyl-L-homocysteine was $1.4 \times 10^{-2}$ M and $V_{\text{max}}$ was 0.26 μmoles keto acid formation per minute per mg protein. When measured as a function of the rate of oxygen consumption, the $K_m$ value was the same and the $V_{\text{max}}$ was exactly one half of the value obtained when measured as a function of keto acid formation. This relationship held for both enzymes with all sulfur amino acids tested except homocysteine and cysteine.

A summation of the $K_m$ and $V_{\text{max}}$ values obtained with both enzymes at pH 7.5 is given in Table 5. The $K_m$ values for cysteine and homocysteine are nearly the same for both enzymes, whereas the $K_m$
Fig. 5. -- The effect of pH on the rate of oxidation of sulfur amino acids by a bacterial L-amino acid oxidase. Oxygen consumption was measured polarographically. The vessel contained 5 μmoles substrate, 100 μmoles phosphate buffer, pH 7.5, and 3.0 mg enzyme protein per ml. S-Riboyl-L-homocysteine interfered with its operation of the Clark electrode, and hence was measured manometrically. Keto acids were measured by a discontinuous assay. Vessels containing substrate and other components as described above were incubated for 30 minutes. After deproteinization keto acids were determined with 2,4-dinitrophenylhydrazine assay and the rates plotted as a function of time.

a Measured by keto acid formation.
**A**

- **L-Homocysteine**
- **L-Cysteine**
- **L-Homocystine**
- **L-Djenkolic Acid**

**B**

- **L-Methionine**
- **S-Ribosyl-L-Homo-cysteine**
- **S-Adenosyl-L-Homo-cysteine**
- **S-Adenosyl-L-Methionine**

**Final pH**

<table>
<thead>
<tr>
<th>pH</th>
<th>7.0</th>
<th>7.5</th>
<th>8.0</th>
<th>8.5</th>
<th>9.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-Methionine</td>
<td>0.12</td>
<td>0.10</td>
<td>0.08</td>
<td>0.06</td>
<td>0.04</td>
</tr>
<tr>
<td>L-Cysteine</td>
<td>0.12</td>
<td>0.10</td>
<td>0.08</td>
<td>0.06</td>
<td>0.04</td>
</tr>
<tr>
<td>L-Homocystine</td>
<td>0.12</td>
<td>0.10</td>
<td>0.08</td>
<td>0.06</td>
<td>0.04</td>
</tr>
<tr>
<td>L-Djenkolic Acid</td>
<td>0.12</td>
<td>0.10</td>
<td>0.08</td>
<td>0.06</td>
<td>0.04</td>
</tr>
<tr>
<td>S-Ribosyl-L-Homo-cysteine</td>
<td>0.12</td>
<td>0.10</td>
<td>0.08</td>
<td>0.06</td>
<td>0.04</td>
</tr>
<tr>
<td>S-Adenosyl-L-Homo-cysteine</td>
<td>0.12</td>
<td>0.10</td>
<td>0.08</td>
<td>0.06</td>
<td>0.04</td>
</tr>
<tr>
<td>S-Adenosyl-L-Methionine</td>
<td>0.12</td>
<td>0.10</td>
<td>0.08</td>
<td>0.06</td>
<td>0.04</td>
</tr>
</tbody>
</table>
Fig. 6. -- The effect of pH on the rate of oxidation of sulfur amino acids by snake venom L-amino acid oxidase. Oxygen consumption and keto acids were measured as outlined in Fig. 5. Vessels contained 5 μmoles substrate, 100 μmoles phosphate buffer, pH 7.5, 100 μmoles KCl, 100 units catalase and 2 μg enzyme protein per ml.

a Measured by keto acid formation.
values for the other substrates tend to be lower with the snake venom enzyme. In general the enzyme from both sources had a greater affinity for the free sulfhydryls and thioethers, particularly methionine. A marked decrease in affinity was observed with the addition of a side chain, i.e., ribose or adenosine, with a further decrease when the thioether was oxidized to the sulfoxide.
Fig. 7. -- Lineweaver-Burk double reciprocal plot of initial reaction velocities of bacterial L-amino acid oxidase against S-adenosyl-L-homocysteine concentrations measured as a function of rate of oxygen consumption (---) and keto acid formation (X--X). The oxygen consumption was measured at 30°C using the Gilson oxygraph. The reaction vessel contained varying concentrations of substrate, 150 μmoles phosphate buffer, pH 7.5, 0.18 mg enzyme protein in the final volume of 1.5 ml. Keto acids were measured by 2,4-dinitrophenylhydrazine assay. Duplicate sets of tubes contained varying concentrations of substrate, 150 μmole phosphate buffer, pH 7.5, and 1.5 mg enzyme protein in final volume of 1.5 ml. One set of tubes was incubated at 30°C for 15 minutes and the other for 30 minutes. The initial reaction velocities were determined by plotting the rate of keto acid formation against time and extrapolating to zero time.
TABLE 5

<table>
<thead>
<tr>
<th></th>
<th>Bacteria</th>
<th>Snake Venom</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$K_m$ (mM)</td>
<td>$V_{max}$ c</td>
</tr>
<tr>
<td>L-Homocysteine</td>
<td>1.3</td>
<td>0.03</td>
</tr>
<tr>
<td>L-Cysteine</td>
<td>5.3</td>
<td>0.01</td>
</tr>
<tr>
<td>L-Methionine</td>
<td>9.6</td>
<td>0.42</td>
</tr>
<tr>
<td>L-Homocystine</td>
<td>12.5</td>
<td>1.43</td>
</tr>
<tr>
<td>S-Ribosyl-L-homocysteine</td>
<td>13.0</td>
<td>0.12</td>
</tr>
<tr>
<td>S-Adenosyl-L-homocysteine</td>
<td>14.0</td>
<td>0.26</td>
</tr>
<tr>
<td>S-Adenosyl-L-methionine</td>
<td>14.0</td>
<td>0.24</td>
</tr>
<tr>
<td>Methionine sulfoxide</td>
<td>45.0</td>
<td>0.03</td>
</tr>
<tr>
<td>Djenkolic acid</td>
<td>83.0</td>
<td>0.16</td>
</tr>
<tr>
<td>S-Adenosyl-L-homocysteine sulfoxide</td>
<td>100.0</td>
<td>0.06</td>
</tr>
<tr>
<td>L-Cystine</td>
<td>---b</td>
<td>5.0</td>
</tr>
</tbody>
</table>

a The values were determined from Lineweaver-Burk double reciprocal plots of initial reaction velocities as a function of keto acid formation. The rates of keto acid formation were determined as outlined under Fig. 1 and 2.

b Activity was below assayable level or too low at maximum substrate concentration for $K_m$ determinations.

c $V_{max}$ is expressed as μmoles keto acid formed/min/mg protein.
DISCUSSION

L-Amino acid oxidases have been isolated from several sources including microorganisms (11,12,14), snake venoms (7,8), rat kidney (3,23), bird livers (5,6) and invertebrates (9,10). To date activity of the enzyme toward sulfur amino acids has been demonstrated only for the following three sources: *Neurospora crassa* on cysteine, cystine, cystathionine (13); *Mytilus edulis* on methionine, cystathionine, djenkolic acid, cystine, and homocystine (9) and *Crotalus adamanteus* on ethionine, methionine, homocystine and cystine (14). The L-amino acid oxidase from *Proteus vulgaris* had no activity on either cysteine or cystine (14).

In the present study, the L-amino acid oxidases from both *Proteus rettgeri* and *Crotalus terrificus* venom were quite active toward several sulfur amino acids. The two enzymes differed in substrate specificity and rates of activity. Neither enzyme was active toward homocysteic acid, cysteic acid, lanthionine or cystathionine. The bacterial enzyme catalyzed the oxidation of the sulfoxides of S-adenosyl-L-homocysteine and methionine slowly, whereas there was no activity with either of these substrates with snake venom enzyme.

The relative rates of reaction with the bacterial enzyme at pH 7.5 in a descending order were L-homocystine, L-methionine, S-adenosyl-L-homocysteine, S-adenosyl-L-methionine, L-djenkolic acid,
S-ribosyl-L-homocysteine, S-adenosyl-L-homocysteine sulfoxide, L-homocysteine, methionine sulfoxide, cysteine and cystine, whereas for the snake venom enzyme at pH 7.5 it was L-cystine, L-methionine, L-djenkolic acid, S-ribosyl-L-homocysteine, S-adenosyl-L-homocysteine, L-homocysteine, L-homocysteine and L-cysteine.

The $K_m$ values for cysteine and homocysteine were nearly the same for both enzymes, whereas the $K_m$ values for the other substrates appeared to be lower with the snake venom enzyme, i.e. the affinities of both L-amino acid oxidases for cysteine and homocysteine were similar and the snake venom enzyme had higher affinities for other substrates. In general, the enzyme from both sources had a greater affinity for sulfhydryl and thioether containing compounds, particularly methionine, with a marked decrease by the addition of a side chain, i.e. ribose or adenine, with the lowest affinity and activity when the thioether oxidized to the sulfoxide.

The bacterial enzyme was quite active with the sulfonium compound, S-adenosyl-L-methionine with the structure shown in Scheme II. This compound is relatively resistant to attack by most enzymes, e.g. rat kidney L-amino acid oxidase (20), and snake venom L-amino acid oxidase from Crotalus terrificus terrificus in this study. The possibility arose that under the alkaline pH suitable for the oxidation to occur, the substrate may decompose chemically or enzymatically as shown in Scheme II. The results from thin layer chromatography of the reaction mixture showed that decomposition did occur with resultant formation of methylthioadenosine and homoserine. However, no measurable activity was found with homoserine.
SCHEME II

\[
\begin{align*}
&\text{S-ADENOSYL-L-METHIONINE} \\
&\rightarrow \text{DECOMPOSED CHEMICALLY} \\
&\text{SPONTANEOUSLY} \\
&\text{HOMOSERINE}
\end{align*}
\]
Both the snake venom and bacterial L-amino acid oxidases catalyzed the following overall reaction: (as shown in HISTORY, reaction (4)).

\[ \text{RCH(NH}_2\text{)COOH} + \text{O}_2 + \text{H}_2\text{O} \rightarrow \text{RCOOCOOH} + \text{NH}_3 + \text{H}_2\text{O}_2 \] (12)

In the presence of catalase the peroxide was converted to water and molecular oxygen.

The mechanism for the liberation of hydrogen sulfide from the free sulfhydryl substrates, cysteine and homocysteine, is not completely understood. Non-oxidative deamination of serine, threonine, cysteine, homocysteine and dicarboxylic amino acids has been shown to be catalyzed by a group of pyridoxal phosphate dependent enzymes. Several pyridoxal phosphate dependent enzymes including cysteine desulfhydrase \(^{(41,45)}\), cystathionase \(^{(45)}\) and tryptophanase \(^{(46)}\) catalyze the conversion of cysteine to pyruvate, ammonia and hydrogen sulfide. The resultant products are the same as those catalyzed by the bacterial and snake venom L-amino acid oxidases in this study. It is possible that both bacterial and the snake venom L-amino acid oxidases contained one of these enzymes. However, no activity was observed under anaerobic conditions, nor did pyridoxal phosphate stimulate the reaction. Hence, these enzymes can be ruled out. Similarly they could be contaminated with a \(\omega\)-mercapto-\(\alpha\)-keto transferase, whereby the sulfur liberated is reduced to hydrogen sulfide by a mercaptan or glutathionine \(^{(45,47)}\). However, both mercaptoethanol and glutathione (5 mM) inhibited the production of hydrogen sulfide. Another possibility is that the sulfur liberated might be reduced by substrate according to Scheme III.
SCHEME III

\[
\begin{align*}
\text{H-H-NH}_2 & \quad \text{L-AMINO ACID OXIDASE} \quad \text{H-H-O} \\
\text{H-S-C-C-C-C-COOH} & \quad \longrightarrow \quad \text{H-S-C-C-C-C-COOH} \\
\text{H-H-H} & \\
\text{HOMOCYSTEINE} & \quad \gamma\text{-MERCAPTO-\(\alpha\)-KETOBUTYRATE} \\
\end{align*}
\]

\[
\begin{align*}
\text{(?)} & \quad \text{CHEMICAL or} \\
\downarrow & \quad \text{TRANS-SULFURASE} \\
\text{H-C-C-C-C-COOH} & \\
\text{H-H-O} & \\
\text{S} & + \\
\text{HOMOCYSTEINE} & \quad \alpha\text{-KETOBUTYRATE} \\
\text{H}_2\text{S} + \text{R-S-S-R} & \\
\end{align*}
\]
L-Homocystine was found to be an excellent substrate for both the snake venom and the bacterial L-amino acid oxidases. Results from chromatographic analysis of the resultant keto acids could suggest that a monoketo-monoamine intermediate is formed during the course of the reaction with the ultimate product being 4,4'-dithio-bis(2-ketobutyric acid) (Scheme IV).

In the mammalian system (48) and in microorganisms (49,50), the utilization of methionine involved enzymatic activation of methionine with ATP resulting in the formation of S-adenosyl-L-methionine (active methionine). As an important methyl donor, S-adenosyl-L-methionine is involved in methylation reactions of several compounds including nucleic acid, nicotinamide, histamine, guadinoacetate (51,52). The demethylated product of S-adenosyl-L-methionine is reported to be S-adenosyl-L-homocysteine (53). The latter compound might further be metabolized by S-adenosylhomocysteine hydrolase EC 3.3.1.1. to adenosine and homocysteine in yeast (54,55) and mammals (56); or it might undergo nucleosidation by S-adenosyl-L-homocysteine nucleosidase, which was found in Escherichia coli, Aerobacter aerogenes and Salmonella typhimurium (57), to adenine and S-ribosyl-L-homocysteine. These metabolites were found to be good substrates for the L-amino acid oxidase from Proteus rettgeri. In the rat, the oxidation product of S-adenosyl-L-homocysteine---S-adenosyl-γ-thio-α-ketobutyrate---was eliminated in the urine (58), whereas in Proteus rettgeri, the fates of the resultant S-adenosyl-γ-methio-α-ketobutyrate, S-ribosyl-γ-thio-α-ketobutyrate, S-adenosyl-γ-thio-α-ketobutyrate are not yet known. It is assumed that these compounds might undergo further oxidation of
SCHEME IV

\[
\text{L-HOMOCYSTINE} \quad \xrightarrow{\text{INTERMEDIATE}} \quad \text{HOOC-C-C-C-S-S-C-C-C-COOH}
\]

\[
\text{4,4'-DITHIO-BIS (2-KETO BUTYRIC ACID)}
\]
the carbon side chain and/or nucleosidation releasing the purine moiety. The metabolic fate of these oxidation products needs further investigation.
SUMMARY

L-Amino acid oxidase from *Proteus rettgeri* which was formerly classified as an Achromobacter species was further characterized by its action on sulfur amino acids. Substrates specificity, pH optimum, $K_m$ and $V_{max}$ values for each substrate acted on by this bacterial enzyme are reported. The relative rates of oxidative deamination, in a descending order, are: homocysteine, methionine, S-adenosyl-L-homocysteine, S-adenosyl-L-methionine, djenkolic acid, S-ribosyl-L-homocysteine, S-adenosyl-L-methionine sulfoxide, homocysteine and cysteine. No activity was found with lanthionine, cystathionine, homocysteic acid, cysteic acid or S-ribosyl-L-homocysteine sulfoxide. With a sufficient amount of catalase present in the bacterial enzyme preparations, such preparations catalyzed the overall reaction:

$$R-\text{CH}(\text{NH}_2)\text{COOH} + \frac{1}{2} O_2 \rightarrow R\text{-CO-COOH} + \text{NH}_3$$

In this reaction, one atom of oxygen was consumed and one molecule of the corresponding keto acid and ammonia were formed. With the sulfhydryl substrates, homocysteine and cysteine, the oxygen consumption was disproportionate and hydrogen sulfide was produced in addition to ammonia and keto acids. No activity was found under anaerobic conditions, nor did pyridoxal phosphate affect the enzymic activity. The crystalline snake venom L-amino acid oxidase from *Crotalus terrificus terrificus* catalyzed the similar reaction. The characteristics of the snake venom L-amino acid oxidase were also studied for comparison.
APPENDICES
### Appendix 1

**Snell, Eakin, and Williams Medium (59)**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Amount per liter of water</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>20.0 g</td>
</tr>
<tr>
<td>Ammonium sulfate</td>
<td>3.0 g</td>
</tr>
<tr>
<td>DL-aspartic acid</td>
<td>0.2 g</td>
</tr>
<tr>
<td>Monopotassium phosphate</td>
<td>2.0 g</td>
</tr>
<tr>
<td>Calcium chloride</td>
<td>0.25 g</td>
</tr>
<tr>
<td>Magnesium sulfate $\cdot 7 \text{H}_2\text{O}$</td>
<td>0.25 g</td>
</tr>
<tr>
<td>Boric acid</td>
<td>1.0 mg</td>
</tr>
<tr>
<td>Zinc sulfate</td>
<td>1.0 mg</td>
</tr>
<tr>
<td>Manganese chloride</td>
<td>1.0 mg</td>
</tr>
<tr>
<td>Ferric chloride</td>
<td>0.5 mg</td>
</tr>
<tr>
<td>Copper sulfate $\cdot 5 \text{H}_2\text{O}$</td>
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</tr>
<tr>
<td>Potassium iodide</td>
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</tr>
<tr>
<td>Inositol</td>
<td>3.0 mg</td>
</tr>
<tr>
<td>Beta-alanine</td>
<td>1.0 mg</td>
</tr>
<tr>
<td>Nicotinic acid</td>
<td>0.1 mg</td>
</tr>
<tr>
<td>Pyridoxine</td>
<td>0.02 mg</td>
</tr>
<tr>
<td>Thiamine</td>
<td>0.02 mg</td>
</tr>
<tr>
<td>Para-aminobenzoic acid</td>
<td>0.02 mg</td>
</tr>
<tr>
<td>Biotin</td>
<td>0.01 mg</td>
</tr>
</tbody>
</table>

Adjust pH to 4.0 and sterilize at $121^\circ \text{C}$ for 12 minutes.
Appendix 2

**M-9 Medium (60)**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Amount per liter of water</th>
</tr>
</thead>
<tbody>
<tr>
<td>Potassium dihydrogen phosphate $(\text{KH}_2\text{PO}_4)$</td>
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</tr>
<tr>
<td>Dibasic potassium phosphate $(\text{K}_2\text{HPO}_4)$</td>
<td>6.0 g</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>3.0 g</td>
</tr>
<tr>
<td>Ammonium sulfate</td>
<td>2.0 g</td>
</tr>
<tr>
<td>Magnesium sulfate</td>
<td>0.1 g</td>
</tr>
<tr>
<td>Glucose</td>
<td>5.0 g</td>
</tr>
</tbody>
</table>

The following trace salts: 0.5 g ferric chloride, 1.0 g zinc sulfate, 1.0 g manganese chloride, 50 mg copper sulfate, 1.0 g calcium chloride, 50 mg potassium iodide were dissolved in 200 ml deionized water and 1.5 ml of this solution was added to each 15 liters M-9 media.
Appendix 3

Determination of Protein (Lowry's method)

Reagent A: 2.0% Na$_2$CO$_3$ in 0.1 N NaOH
Reagent B: 0.5% CuSO$_4$·5H$_2$O in water
Reagent C: 1.0% Na and K tartrate in water
Reagent D:

Reagent A 50 ml
Reagent B 0.5 ml
Reagent C 0.5 ml

Appendix 4

Determination of Ammonia nitrogen (Nessler's)

Nessler's Reagent:

KI 4 gm
H$_2$I$_2$ 4 gm
Gum ghatti 1.75 gm
H$_2$O 1000 ml

Dissolve KI and H$_2$I$_2$ in 25 ml of water. Select light colored gum ghatti, grind them in a mortar, drop the powder into 750 ml of boiling water and reflux until dissolved. Add KI and H$_2$I$_2$ solution to the gum ghatti solution. Dilute to one liter and filter. Replace filter paper periodically to speed filtration.
Appendix 5

Detection Reagents Used with Thin Layer Chromatography

Detection of Sulfur Compounds (61)

0.02 M Platinic chloride
1.0 M Potassium iodide
2.0 N Hydrochloric acid
Acetone

Spray the developed paper with the above reagent and dry in air at room temperature. Sulfur compounds will reduce the platinic iodide and result in white areas against a pink background.

Detection of Amino Acids (61)

A solution of 0.25% 1,2,3-triketohydrindene (ninhydrin) in acetone is sprayed on the developed paper. After the excess acetone has evaporated at room temperature, the paper is heated at 110° C for about 1 minute. Amino acids will yield blue-purple spots against a white background.

Detection of Carbohydrates (62)

Saturated aqueous solution of silver nitrate................. 0.1 ml
Acetone................................................................. 20.0 ml

Water (add dropwise with shaking until the suspended silver nitrate redissolves)

A) Spray the developed paper with the above reagent.
B) Spray the dry paper with 0.5 N sodium hydroxide in 95% ethanol.
c) Remove the excess silver oxide by immersion of the paper in 6 N ammonium hydroxide, wash for 1 hour with running water.

Reducing sugars will form black or dark brown spots against a white background.
REFERENCES


