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Alpha-substituted cystines as substrates for cystine reductase, and L-amino acid oxidase.

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ALPHA-SUBSTITUTED CYSTINES AS SUBSTRATES FOR CYSTINE REDUCTASE, AND L-AMINO ACID OXIDASE

BY

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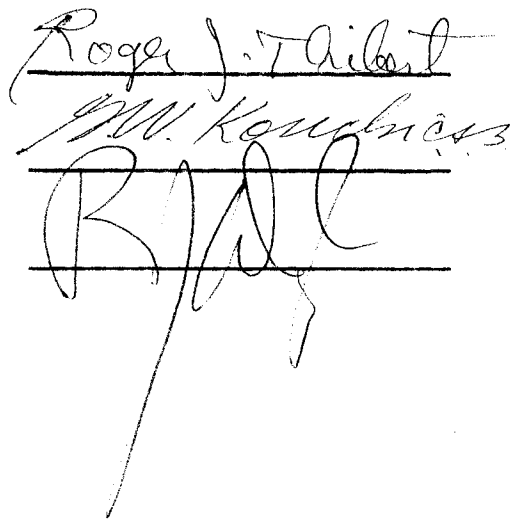

ABSTRACT

The ability of L-cystine, DL-cystine, ∝-methyl-, ∝-ethyl-, ∝-n-propyl-, ∝-isopropyl-, ∝-n-butyl-, and ∝-phenyl-DL-cystine to act as substrates for cystine reductase and L-amino acid oxidase has been studied. A new method for the simultaneous determination of cysteine and cystine is described along with its application to the enzymatic system, cystine reductase.

All of the alpha-substituted cystines acted as substrates for a crude extract of cystine reductase from bakers' yeast. Lineweaver-Burk plots were constructed and the K_m and V_{max} determined for L-cystine, DL-cystine, and four of the six alpha-substituted-DL-cystines. None of the analogues of cystine acted as substrates for L-amino acid oxidase from Crotalus adamanteus venom, nor did they inhibit the oxidative deamination of L-cystine by the enzyme.

ACKNOWLEDGMENTS

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ABBREVIATIONS

aa	amino acid
a_o	initial concentration of substrate
a_o-x	amount of substrate remaining after a specific time interval
α	alpha
E	equivalent weight
FAD	flavin adenine dinucleotide
g	gram
μg	microgram
ia	imino acid
I	concentration of inhibitor
k	apparent first order rate constant
ka	keto acid
K_m	substrate concentration at which the reaction develops half the maximum velocity
M	molar
μM	micromole
ml	millilitre
mg	milligram
min	minute
NADH	reduced nicotinamide adenine dinucleotide
NADPH	reduced nicotinamide adenine dinucleotide phosphate
N	normal

NBS	N-bromosuccinimide
r.p.m.	revolutions per minute
S	substrate concentration
t	time
V	volume
v_o	initial velocity
V_{max}	maximum velocity due to net catalysis, obtained when the enzyme is saturated with substrate
v/v	volume to volume
w/v	weight to volume

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CHAPTER I

INTRODUCTION

The purpose of this study is to see if the alpha-hydrogen atom of cystine is necessary for this compound to act as a substrate for the enzymes cystine reductase (EC 1.6.4.1), and L-amino acid oxidase (EC 1.4.3.2). In order to carry out this study, six alpha-substituted cystines were synthesized in this laboratory (1,2). In place of the alpha-hydrogen, a methyl, ethyl, n-propyl, isopropyl, n-butyl, or a phenyl group was substituted.

In general, the 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, alpha-methyl-DL-glutamic acid was inert in dehydrogenation of glutamic acid (3). When alpha-methylalanine and alpha-methyl-alpha-amino-n-butyric acid were administered to dogs, the amino acids were largely excreted in the urine (4,5). In another study, alpha-methyl-DL-serine did not undergo oxidative deamination in the presence of L- or D-amino acid oxidase (6). In a study by Bender and Krebs (7) seven alpha-substituted amino acids were found to be inert to oxidation by L- or D-amino acid oxidase. There are, however, examples in the literature of enzymatic reactions undergone by alpha-substituted amino acids. Snell and co-workers (8,9) observed that alpha-methylserine was attacked by cell-

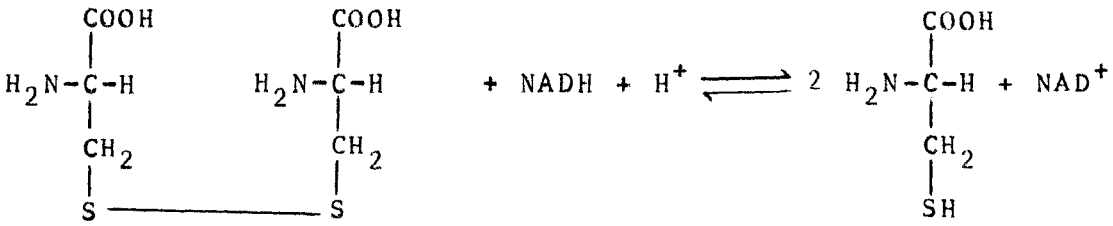
free extracts of a bacterium of the genus Pseudomonas producing D-alanine; alpha-hydroxymethylserine and alpha-ethylserine also acted as substrates.

In this laboratory, it has been established that alpha-substituted cystines do not act as substrates for cysteine desulfhydrase or, as it is referred to by its systematic name, "L-cysteine hydrogen-sulfide lyase (deaminating) (EC 4.4.1.1)" (2). It was shown by Flavin and Cavallini (10, 11, 12) however, that cystine, which is always present in small amounts and is spontaneously regenerated, is the actual substrate for this enzyme in a cyclic reaction. It was also shown that the alpha-substituted cystines competitively inhibit the enzyme homoserine deaminase or homoserine dehydratase [L-homoserine hydro-lyase (deaminating) (EC 4.2.1.15)] when L-homoserine is the substrate (2).

The property of reducing disulfides to sulfhydryl compounds has long been attributed to plant and animal tissues. Tunnicliffe (13) in 1925) reported the existence of a thermostable substance in tissue residues capable of reducing disulfides. Abderhalden and Wertheimer (14) found that frog muscle reduced cystine. This reducing capacity was lost when the muscle was washed but could be restored by the addition of heated extracts of yeast, muscle, or liver. This suggested the participation of a thermostable coenzyme.

Romano and Nickerson (15, 16) reported the existence of an enzyme in pea seeds and yeasts, catalyzing the reduction of cystine to cysteine by reduced coenzyme I (NADH). The

authors were the first to call the enzyme "cystine reductase". The systematic name for the enzyme is NADH$_2$: L-cystine oxidoreductase (EC 1.6.4.1). The overall reaction may be illustrated as



The presence of NADH is essential for the reduction of cystine (15, 16, 17). The enzyme was found to lose its activity after four hours of dialysis (16). The activity was restored by the addition of a portion of an undialyzed enzyme preparation that had been boiled and filtered. When NADH was substituted for the boiled and filtered preparation, activity was restored. NADPH was inactive in the cystine reductase system (16).

Proskuryakov and Buachidze (17) established that reduction of cystine by extracts of pressed yeast and pea seeds is most rapid under anaerobic conditions, although it also occurs in the presence of air. The reduction was activated by additions of malic acid and glucose -1-phosphate which acted as hydrogen donors. A partially-purified cystine reductase preparation obtained by precipitation of a phosphate extract of pea seeds by eighty per cent acetone was able to reduce cystine in a model system containing cystine, NAD, and malic acid (17).

In 1959 Powning and Irzykiewicz (18) reported a

NADPH-linked cystine reductase in the clothes moth, Tineola bisselliella. Some activation was caused by NADH but considerably more by NADPH. Cystine reductase activity has also been determined in wheat flour (19) and in twelve strains of Aspergillus niger (20).

The purpose of using the alpha-substituted cystines in the cystine reductase system was to determine if the alpha-hydrogen atom is necessary for reduction. Also, the rates of reduction could be compared to the half-wave potentials previously determined polarographically for each of these compounds (21), if the alpha-substituted compounds act as substrates.

A new titrimetric method was developed during the course of this work which allowed the simultaneous determination of cysteine and cystine using N-bromosuccinimide (22). The method was applied to the determination of cysteine produced from the reduction of cystine by the enzyme, cystine reductase. Previous methods used to detect cysteine in this enzymatic system have centered mainly around the use of iodine (15, 16) or iodate (17, 19, 20). These methods determine only the amount of cysteine present at any specific time, but the remaining cystine was not determined by an independent method. The use of N-bromosuccinimide gives reliable results whereas values determined by potassium iodate were shown to be high (22).

The method was shown to be adaptable to the determination of the alpha-substituted cystines with some revisions in the calculations. It is accurate for the determination of the alpha-substituted cysteines without revision in the calculations.

Enzymes that catalyze the complete reaction illustrated below are termed amino acid oxidases:

$$RCHNH_2COOH + O_2 + H_2O \longrightarrow RCOCOOH + NH_3 + H_2O_2$$

In the presence of catalase, the hydrogen peroxide formed in this reaction is decomposed to oxygen and water. In its absence, the hydrogen peroxide reacts with the keto acid product nonenzymatically in an oxidative decarboxylation reaction (23).

$$RCOCOOH + H_2O_2 \longrightarrow RCOOH + H_2O + CO_2$$

The amino acid oxidases fall into two broad categories: those that catalyze the oxidative deamination of L-amino acids and those that perform a similar function employing the D-isomers as substrates. Enzymes of both classes are reasonably widely distributed in living material. L-amino acid oxidases have been detected in the venom of most poisonous snakes, in avian liver, in molds, and in bacteria (24-31).

Although the deamination of amino acids was implied shortly after the turn of the century by experiments that showed interconversion of alpha-keto and alpha-amino acids, careful and detailed studies concerning the enzymes responsible for these reactions were not initiated until 1935. At that time, in a classical paper, Krebs (32) reported the oxidation of L- and D-amino acids by homogenates of mammalian kidney and liver. This work stimulated a general study of the enzymes involved in oxidative deamination of amino acids. To date, the best understood enzymes are the L-amino acid oxidases from the venom of the cottonmouth, moccasin, and the diamondback rattlesnake, and the D-amino acid oxidase from mammalian kidney.

The L-amino acid oxidases of snake venoms have been obtained in homogenous form and that of rattlesnake, Crotalus adamanteus, has been crystallized (31). These enzymes exhibit molecular weights near 150,000 and contain two moles of FAD per mole of enzyme (31).

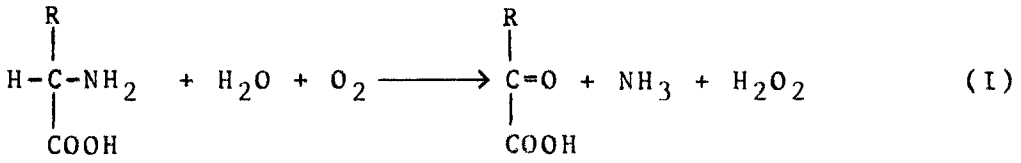
A considerable amount of work has been done on the specificity of L-amino acid oxidase from snake venom (36-55). Zeller (40) concluded that besides a free carboxyl group and an unsubstituted alpha-amino group in the L-configuration, a free alpha-hydrogen was necessary. A hydroxy or methoxy group in the alpha position nearly abolished the substrate suitability of an L-alpha-amino acid, while the replacement of a β-hydrogen by chlorine or sulfur increased the oxidation rate (40). On the basis of this information and from experiments carried out with a deuterium-containing amino acid, it was concluded that the first step in the degradation of the L-amino acid consists of an alpha-beta dehydrogenation. However, Meister (56) showed that phenylglycine and its derivatives were oxidized by L-amino acid oxidase supporting the hypothesis that the reaction proceeds via the alpha-amino acid rather than through an alpha-beta unsaturated compound.

Zeller (48) concluded that although the carboxylic and amino groups do not contribute significantly to the binding energy of the L-amino acid to the enzyme, they are essential for the preliminary orientation of the substrate molecule to the binding site. Recent studies, however, have shown that L-3,4-dehydroproline is a substrate for L-amino acid oxidase

which contradicts the belief that this enzyme requires the presence of a free primary amino group for activity (45).

It does seem imperative that the amino acid have an alpha-hydrogen atom present for it to act as a substrate. Frieden (43) found that alpha-methylserine was inert to oxidative deamination by L-amino acid oxidase. It has been shown that alpha-methylvaline and alpha-methylphenylalanine do not act as substrates for D-amino acid oxidase (3). Also supporting this hypothesis are the results of Triggle and Moran (49); DL-Tyrosine and DL-phenylglycine substituted with deuterium in the alpha position were found to be oxidized slower than the unlabeled derivative by L-amino acid oxidase. The kH/kD ratio was 1.41 for both compounds. It was concluded that the removal of the alpha-hydrogen was the rate-limiting step in the reaction.

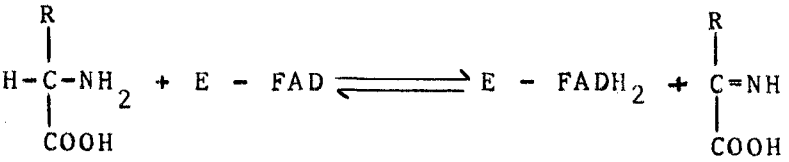
Wellner and Meister (33) have proposed a mechanism of action of L-amino acid oxidase from Crotalus adamanteus venom. The enzyme catalyzes the oxidative deamination of a number of L-amino acids according to the following equation:



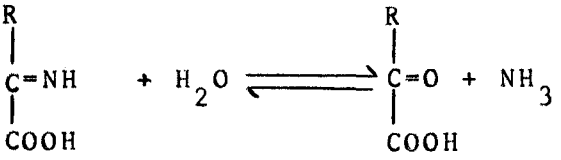
(I)

The reaction takes place in at least two steps, reduction of the enzyme by amino acid, and reoxidation of the enzyme by molecular oxygen, the first of these steps being reversible (34).

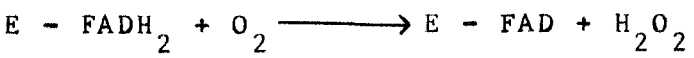
The reaction may now be written as:



(II)



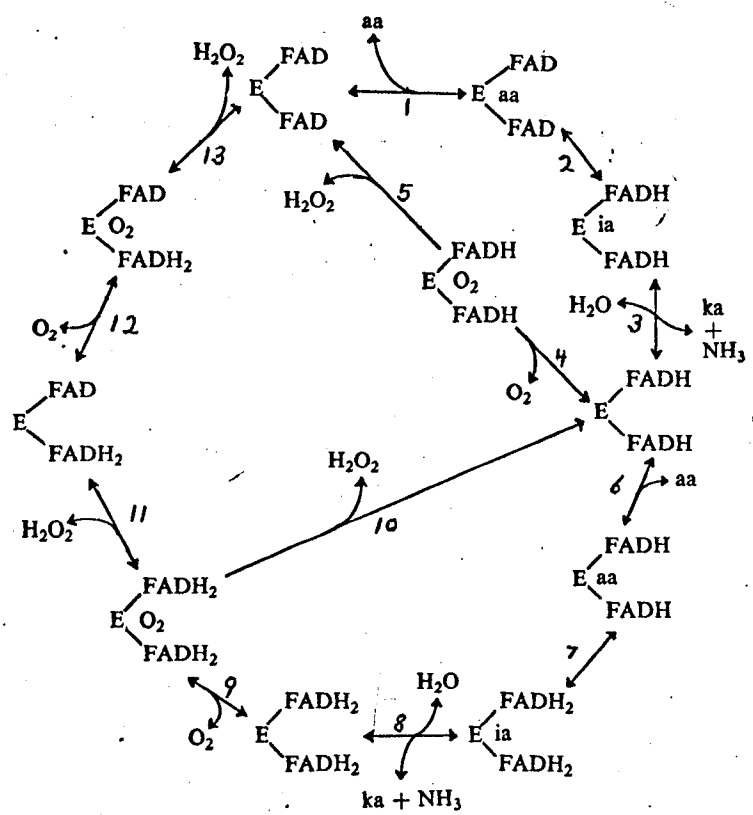
(III)



(IV)

The enzyme exhibits marked substrate inhibition and this is accounted for by the fact that reaction (II) takes place in two steps, the first of which leads to the formation of a half-reduced enzyme capable of being further reduced by substrate or of being reoxidized by molecular oxygen more rapidly than the completely reduced enzyme (35).

Wellner and Meister (33) have proposed the following overall mechanism based on kinetic and spectrophotometric data.



Tentative Scheme for the Mechanism of L-Amino Acid Oxidase. aa: Amino acid; ka: keto acid; ia: imino acid.—*D. Wellner and A. Meister, J. Biol. Chem., 236: 2357 (1961).*

The amino acid forms a complex with the enzyme and donates two hydrogen atoms, one to each FAD, yielding a dihydroenzyme, and an imino acid which is hydrolyzed to ammonia and the corresponding keto acid (steps 1, 2, and 3). This half-reduced enzyme is rapidly reoxidized by oxygen (steps 4 and 5). In the absence of oxygen, or in the presence of high concentrations of amino acid, a second amino acid molecule reacts with the half-reduced enzyme to yield a fully-reduced enzyme (steps 6, 7, and 8), which reacts slowly with oxygen (steps 9 and 10). It is also possible that oxygen may react with the fully-reduced enzyme by removing two hydrogen atoms from the same FAD (step 11) to yield an enzyme containing one reduced and one oxidized FAD. This complex can react slowly with oxygen (steps 12 and 13) to yield the fully-oxidized enzyme.

CHAPTER II

EXPERIMENTAL

MATERIALS AND METHODS

Materials

Bakers' yeast was obtained from the Fleischmann Company; L-amino acid oxidase, Type I and IV and L-cysteine from Sigma Chemical Co.; selenium from Hengar Co.; mossy zinc from Matheson, Coleman and Bell; DL-cystine (B grade) from Calbiochem; soluble starch from Merck and Co. Ltd.; mercury (triple distilled) from Engelhard Industries of Canada Ltd.; H_2SO_4, HCl, and Bordeaux Red from Allied Chemical; KH_2PO_4, K_2HPO_4, Na_2SO_4, NaOH, L-cystine, N-bromosuccinimide, KIO_3, KI, Na metal, and sodium pyrophosphate from Fisher Scientific Co. Ltd.; H_3BO_3, bromo-phenol blue, phospho-24-tungstic acid, paraffin oil, and toluene from the British Drug Houses Ltd.

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

Methods Used in the Cystine Reductase System

Cystine reductase was prepared from bakers' yeast obtained from the Fleischmann Company in the form of pressed cakes. An acetone powder was made according to the procedure as given by Umbreit, Burris, and Stauffer (57). A 20 per cent

(w/v) suspension of the acetone powder in 0.025 M phosphate buffer (pH 6.2) was stirred for 30 minutes on a magnetic stirrer at 5°C and used as the enzyme suspension. Protein content was determined by the Kjeldahl procedure as given by Vogel (58).

In studying the kinetics of the cystine reductase system, the reaction vessel contained 2.5 ml of enzyme suspension (171 mg protein), 0.025 M phosphate buffer (pH 6.3), and concentrations of L-cystine, DL-cystine, or alpha-DL-substituted cystines ranging from 4×10^{-4} M to 1×10^{-3} M. The cystines were dissolved in 0.5 N HCl and an equal volume of 0.5N NaOH was added to neutralize the acid. The final volume of the reaction mixture was made up to 10 ml with deionized water. The controls contained 0.025 M phosphate buffer (pH 6.2) in place of the cystine. The enzyme was added last, the flasks tightly stoppered, and placed in a Dubnoff metabolic shaking incubator at 37 $\pm$ 0.5°C. Flasks were removed at 0, 5, 10, 15, 20, 30, 60, and 90 minutes. The reaction was stopped by the addition of 0.5 ml 10 per cent (w/v) phospho-24-tungstic acid in 5 per cent (v/v) HCl. The mixtures were centrifuged at 10,000 r.p.m. for 10 minutes and the clear, yellow supernatant used for titration with NBS. All determinations were done in triplicate. The values for the control flasks were subtracted from the experimental values and the amount of cysteine produced from cystine determined.

It was found that there was sufficient endogenous NADH present in the crude, undialyzed enzyme preparation and therefore none was added in these experiments. When NADH was added in a final concentration of 3.5×10^{-5} M there was no significant increase in the amount of cystine reduced to cysteine when

compared to the controls containing no NADH.

Methods used for the Determination of Cysteine and Cystine Using NBS.

In the comparative study between potassium iodate and NBS as titrimetric methods for the determination of cysteine, eight 125 ml Erlenmeyer flasks each contained 0.025 M phosphate buffer (ph 6.2), 2 x 10^{-3} M cystine, and 20 ml of enzyme suspension. The final volume of the reaction mixture was 80 ml. Eight flasks containing no cystine served as controls. The enzyme was added last, the flasks tightly stoppered and placed in a Dubnoff metabolic shaking incubator at 37 $\pm$ 0.5° for the appropriate time interval. The reaction was stopped and the proteins precipitated by the addition of 4 ml of 10 per cent (w/v) phospho-24-tungstic acid in 5 per cent (v/v) HCl. The mixtures were centrifuged at 10,000 r.p.m. for 10 minutes and the clear, yellow supernatant used for titration. Aliquots (5 ml) were taken from both the controls and the experimental flasks and titrated for cysteine using NBS and also with potassium iodate. Values of the controls were subtracted from the experimental values before calculation of cysteine.

The method for the determination of cysteine using NBS consisted of adding 30 ml of 0.05 M phosphate buffer (pH 7.0) to the 5-ml aliquot, 10 ml of a 10 per cent (w/v) solution of potassium iodide, one ml of a one per cent (w/v) starch solution, and titrating with 0.001 N NBS to a blue endpoint which persists for 30 seconds. The NBS used in these investigations had been previously recrystallized.

The method used for the determination of cysteine

with potassium iodate was essentially the same as that of Woodward and Fry (59). No buffer was added in the case of potassium iodate since iodine is only liberated in an acid medium. One millilitre of 5 per cent (v/v) HCl was added to lower the pH to 2.0.

On a third aliquot, cystine was determined using NBS with Bordeaux Red as the indicator (22). To a one-ml aliquot was added 20 ml of deionized water and two drops of Bordeaux Red. Buffer was not necessary as the method is independent of pH. The solution was then titrated with standard NBS until the last drop just caused the red colour to turn yellow. The controls were titrated in the same manner and the values subtracted from the experimental titers before calculations of cystine.

In order to see if the alpha-substituted cystines could be determined by the NBS method, a solution containing approximately one mg per ml of each of the alpha-substituted compounds was prepared. One ml of the stock solution was added to a 125 ml Erlenmeyer flask and 20 ml of deionized water was added. Two drops of Bordeaux Red were added and the solution titrated with 0.005N NBS as mentioned above.

In the case of the alpha-substituted cysteines, the alpha-cystines were first quantitatively reduced to alpha-substituted cysteines with a one per cent sodium-mercury amalgam (60). The amalgam was prepared by cutting one gram of sodium metal into small pieces (5 mm square) and transferring quickly to a small casserole dish containing paraffin oil to

avoid air oxidation. This was heated over a small Bunsen burner until the sodium melted. While still hot, 100 grams of purified mercury was added very slowly with stirring. The amalgam was stirred until it was as homogenous as possible, cooled, and the liquid paraffin decanted off. The amalgam is washed with toluene and a layer of toluene kept over the amalgam prior to use. When the reduction is to be carried out, the toluene is removed by washing with distilled water. To the washed amalgam is added 5 ml of distilled water in a 60 ml seperatory funnel. The solid alpha-substituted cystine is added and the reduction allowed to proceed for one hour at room temperature. The amalgam is then separated from the mixture by use of the separatory funnel and the solution of alpha-substituted cysteine neutralized with 1N HCl and made up to a known volume.

One millilitre of the alpha-substituted cysteine solution (2 mg per ml) was added to a 125 ml Erlenmeyer flask with 20 ml of 0.05 M phosphate buffer (pH 7.0). Ten millilitres of a 10 per cent (w/v) solution of potassium iodide and one ml of a one per cent starch solution was added and the solution titrated with 0.005 N NBS to a blue end-point which persisted for at least 30 seconds.

Methods Used in the L-Amino Acid Oxidase System

Oxygen consumption by L-amino acid oxidase [(L-amino acid:O_2 oxidoreductase)(deaminating), (EC 1.4.3.2)] was followed using an oxygen electrode produced by the Yellow Springs

Instrument Company in conjunction with a Sargent SR recorder. The reaction vessel was kept at 25 ± 0.2°C by a Haake circulating water pump. The electrode was standardized according to the method of Dixon and Kleppe (62). The experiments were carried out with solutions initially saturated with air. The electrode was always standardized with distilled water saturated with air at 25°C. It was found convenient to adjust the reading arbitrarily to 90 on the chart by means of the sensitivity control. At standard barometric pressure, this reading corresponds to an O_2 content of 0.257 μM per ml of water (62) or 1.028 μM O_2 in the total volume of 4 ml used in the reaction vessel. Thus the readings could be converted into μM O_2 in the vessel by multiplying by the constant 1.028/90 = 0.0114. However, the presence of dissolved substances can change this constant and allowances for this were made (61). The new constant with all components present except enzyme was found to be 0.0103 μM per division on the chart paper.

For kinetic studies the reaction vessel contained 0.05 M pyrophosphate buffer (pH 8.0), 0.06 units of L-amino acid oxidase and concentrations of L- cystine or DL-cystine ranging from 1×10^{-4} M to 2×10^{-3} M. A unit of L-amino acid oxidase is defined as that amount which will oxidatively deaminate one μM of L-leucine per minute at pH 7.8 and 37°C. The total volume of the reaction mixture was 4 ml. The initial rate was found to be linear for at least the first two minutes.

All the solutions were air saturated at 25°C. The enzyme was added last, the electrode lowered and all air bubbles excluded. No gas phase was present during the course of the reaction.

Experiments with alpha-substituted cystines were carried out in exactly the same manner. Enzyme concentrations of 0.06 and 0.288 units were used. The concentrations of the alpha-substituted-DL-cystines were 1×10^{-3}M and 5×10^{-3}M. Stock solutions of the alpha-substituted cystines were dissolved in 0.05 M pyrophosphate buffer (pH 8.0) by gentle heating.

The inhibition experiments were carried out with 1×10^{-4}M L-cystine, 0.05 M pyrophosphate buffer (pH 8.0), 0.288 units of L-amino acid oxidase and varying concentrations of each of the alpha-substituted-DL-cystines. These concentrations of inhibitor were selected to give $[I/S]$ values of 0, 5, 10, 20, 30, and 50 thus covering a wide range of ratios.

CALCULATIONS

Cysteine Titration Employing the NBS Displacement of Iodine

mg cysteine = N x V x E (I)

N = Normality of NBS

V = Volume (ml) of NBS

E = Equivalent weight of cysteine (mol wt/1)

Cysteine Titration Employing the Potassium Iodate Method

mg cysteine = N x V x E (II)

N = Normality of potassium iodate

V = Volume (ml) of potassium iodate

E = Equivalent weight of cysteine (mol wt/1)

Cystine Titration Employing NBS with Bordeaux Red as an Indicator

Mg cystine = N x V x E (III)

N = Normality of NBS

V = Volume (ml) of NBS

E = Equivalent weight of cystine (mol wt/10)

Alpha-Substituted Cysteine Titration Employing the NBS Displacement of Iodine

mg α-substituted cysteine = N x V x E (IV)

N = Normality of NBS

V = Volume (ml) of NBS

E = Equivalent weight of alpha-substituted cysteine (mol wt/1)

Alpha-Substituted Cystine Titration Employing NBS with Bordeaux Red as the Indicator

mg α-substituted cystine = N x V x E (V)

N = Normality of NBS

V = Volume of NBS

E = Equivalent weight of alpha-substituted cystine (mol wt/14)

Apparent First Order Rate Constants and the Determination of Initial Velocities

The overall reaction can be illustrated as:

$$RSSR \xrightarrow{k} 2RSH$$

where RSSR represents cystine and RSH represents cysteine.

The rate of disappearance of cystine is given by:

$$-d\frac{[RSSR]}{dt} = k[RSSR]$$

The initial velocity is given by:

$$v_o = k[a_o]$$

k = first order rate constant

$[a_o]$ = initial concentration of cystine

v_o = initial velocity

The first order rate constants were calculated from the first order rate equation in the following manner.

$$k = \frac{2.303}{t} \log \frac{[a_o]}{[a_o - x]}$$

k = first order rate constant

$[a_o]$ = initial concentration of cystine

$[a_o - x]$ = the amount of cystine remaining at any time t

$\log \frac{[a_o]}{[a_o - x]}$ was plotted against time, the slope of the straight line obtained being equal to k/2.303.

Reciprical velocities, V_{max} and K_m values were determined using an IBM 1620 computer by the method of least squares analysis. The computer program is shown in the Appendix.

CHAPTER III

RESULTS

Determination of Cysteine and Cystine Using NBS

The results of the comparative study of determining cysteine by the NBS titrimetric method versus the potassium iodate method for the enzymatic system, cystine reductase, are given in Table I and Figure 1. Table I shows the amount of cystine remaining in the reaction vessel after various time intervals as well as the amount of cysteine present. The cysteine was titrated with NBS as well as potassium iodate using starch as an indicator. The cystine was determined with NBS using Bordeaux Red as an indicator as described under Methods. It can be seen that the values determined with potassium iodate are considerably higher than those obtained for NBS. Figure 1 shows that the theoretical amount of cysteine possible is greatly exceeded using the potassium iodate method while the NBS titrimetric method does not exceed this limit to any significant extent.

Table II and III show the results of the determination of the alpha-substituted cystines and cysteines in pure solutions using NBS.

Table I. The simultaneous determination of cysteine and cystine in the cystine reductase system. Each value shown is the average of five determinations. Aliquots (5 ml) were taken from both the controls and the experimental flasks and titrated for cysteine using both NBS and potassium iodate. Cystine was determined by titrating a one ml aliquot from the experimental and control flasks with NBS using Bordeaux Red as an indicator.

TABLE I

The Simultaneous Determination of Cysteine and Cystine in the Cystine Reductase System.

Time min	Cystine taken µg/ml	Cysteine found µg/ml NBS	Cysteine found µg/ml KIO_3	Cystine found µg/ml NBS
0	457	5	12	436
5	457	24	65	422
10	457	57	119	414
15	457	98	169	354
20	457	144	192	308
30	457	214	265	229
60	457	392	478	48
90	457	463	588	2

Figure 1. The determination of cysteine from cystine by the enzyme, cystine reductase. The experimental flasks contained 0.025M phosphate buffer (pH 6.2), 20 ml of enzyme suspension (1.37 g of protein), and 2 x 10^{-3}M cystine. The total volume was 80 ml. The controls contained no cystine. The reaction was stopped with 4 ml of 10 per cent (w/v) phospho-24-tungstic acid in 5 per cent (v/v) HCl. Aliquots (5 ml) were used for titration with both the NBS (●) assay and the potassium iodate (▲) assay for cysteine. Values obtained for the controls were subtracted from the experimental values. Each value is the average of 5 determinations. The dashed (---) line represents the theoretical amount of cysteine possible.

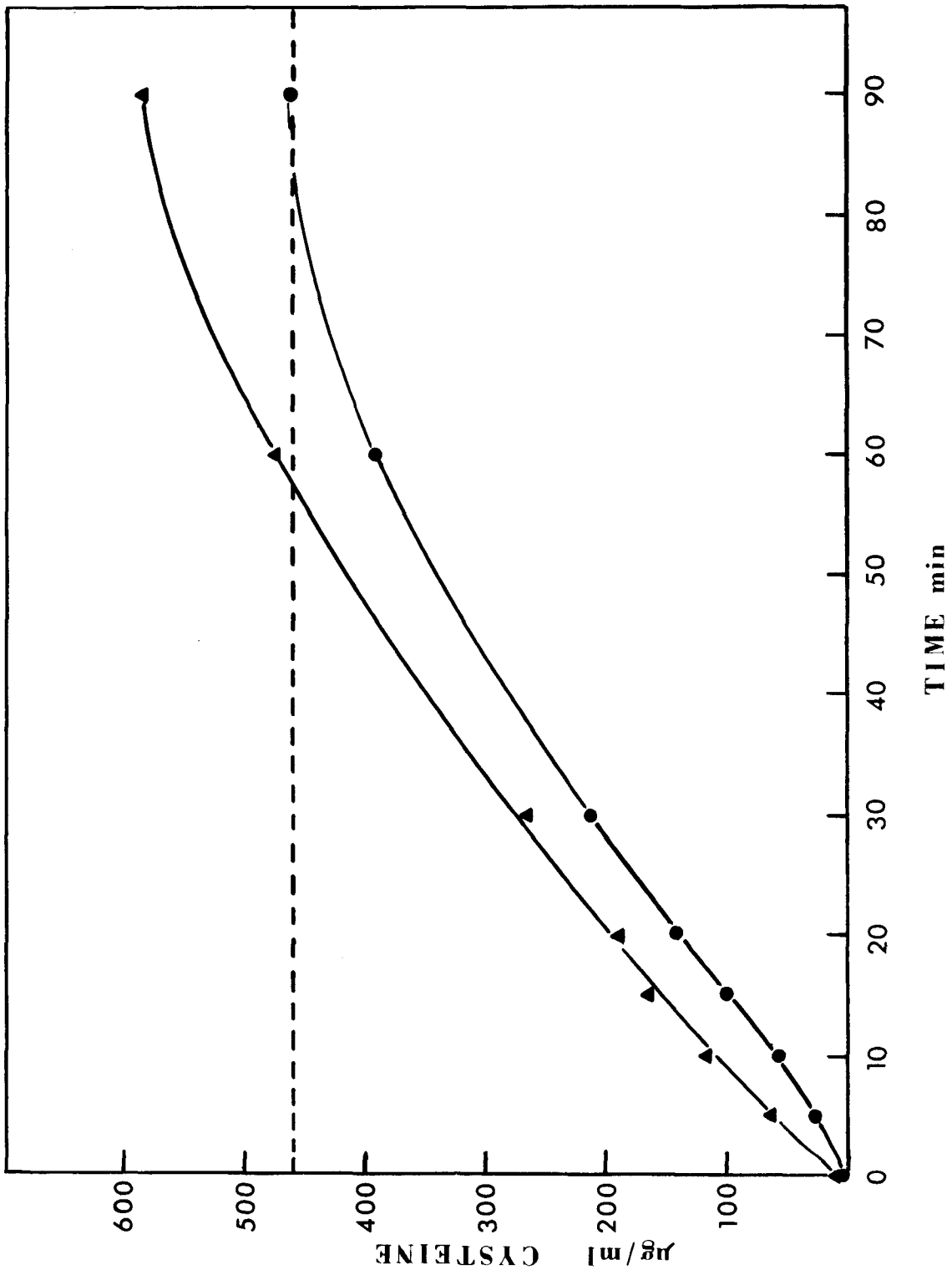


FIGURE 1

Table II. The determination of alpha-substituted cystines with NBS. The titrations were carried out in pure solutions as opposed to a protein free filtrate. A one ml aliquot of a stock solution containing approximately one mg per ml was added to a 125 ml Erlenmeyer flask along with 20 ml of deionized water and 2 drops of Bordeaux Red. The solution was titrated with 0.005 N NBS to a yellow end-point. Each value is the average of five determinations.

TABLE II

The Determination of Alpha-Substituted Cystines with NBS

Cystine used	Sample mg	Found mg	Deviation per cent
L-Cystine	1.03	1.06	+2.92
α-Methyl-DL-	1.06	1.08	+1.88
α-n-Propyl-DL-	1.02	1.04	+1.96
α-Phenyl-DL-	1.09	1.12	+2.75

Table III. The determination of alpha-substituted cysteines with NBS. The alpha-substituted cystines were first reduced to the corresponding alpha-substituted cysteine by a one per cent sodium-mercury amalgam. A one ml aliquot of a stock solution containing approximately 2 mg per ml was added to a 125 ml Erlenmeyer flask along with 20 ml of 0.05 M phosphate buffer (pH 7.0) and 10 ml of a 10 per cent (w/v) solution of potassium iodide. One ml of a one per cent starch solution was added and the solution titrated with 0.005 N NBS to a blue end point. Each value is the average of 5 determinations.

TABLE III

The Determination of Alpha-Substituted Cysteines with NBS

Cysteine used	Sample mg	Found mg	Deviation per cent
L-Cysteine	2.06	2.09	+1.46
α-Methyl-DL-	2.00	2.04	+2.00
α-n-Propyl-DL-	2.08	2.11	+1.44
α-Phenyl-DL-	2.03	2.07	+1.94

Substrate Behaviour of the Alpha-Substituted Cystines in the Cystine Reductase System

Figure 2 shows Lineweaver-Burk plots of L-cystine, DL-cystine, ∝-methyl-, ∝-n-propyl-, ∝-isopropyl-, and ∝-phenyl-DL-cystine. Alpha-ethyl- and ∝-n-butyl-DL-cystine were omitted due to lack of compound. The reaction was shown to obey first order kinetics and the initial velocities were determined by use of the first order rate constants obtained and the initial concentration of substrate used as described under Calculations. All determinations were done in triplicate. The points were programmed and the best straight line obtained by the method of least squares analysis using an IBM 1620 computer.

Table IV lists the V_{max} and the K_m values for L-cystine, DL-cystine, ∝-methyl-, ∝-n-propyl-, ∝-isopropyl-, and ∝-phenyl-DL-cystine.

Table V lists the first order rate constants and the initial velocities obtained for a 1×10^{-3} M substrate concentration in the cystine reductase system. The polarographic half-wave potentials previously obtained for these compounds (22) are also shown. No correlation can be seen between the case of reduction of these compounds enzymatically as compared to polarographically.

Figure 2. Substrate behaviour of the alpha-substituted cystines in the cystine reductase system. The NBS assay was used. Each flask contained 2.5 ml of enzyme suspension (171 mg of protein), 0.025 M phosphate buffer (pH 6.2), and concentrations of substrate ranging from 4×10^{-4}M to 1×10^{-3}M. The total volume of the reaction mixture was 10 ml. The reaction was stopped by adding 0.5 ml of 10 per cent (w/v) phospho-24-tungstic acid in 5 per cent (v/v) HCl. All determinations were done in triplicate. Values of cysteine for the controls were subtracted from the values for the experimental flasks. The substrates were as follows: (●) L-cystine; (□) DL-cystine and α-methyl-DL-cystine; (■) α-n-propyl-DL-cystine; (○) α-phenyl-DL-cystine; (▲) α-isopropyl-DL-cystine. Initial velocities were calculated as outlined in the Calculations. The lines represent the best fit relationship between the experimental points computed by an IBM 1620 computer by the method of least squares.

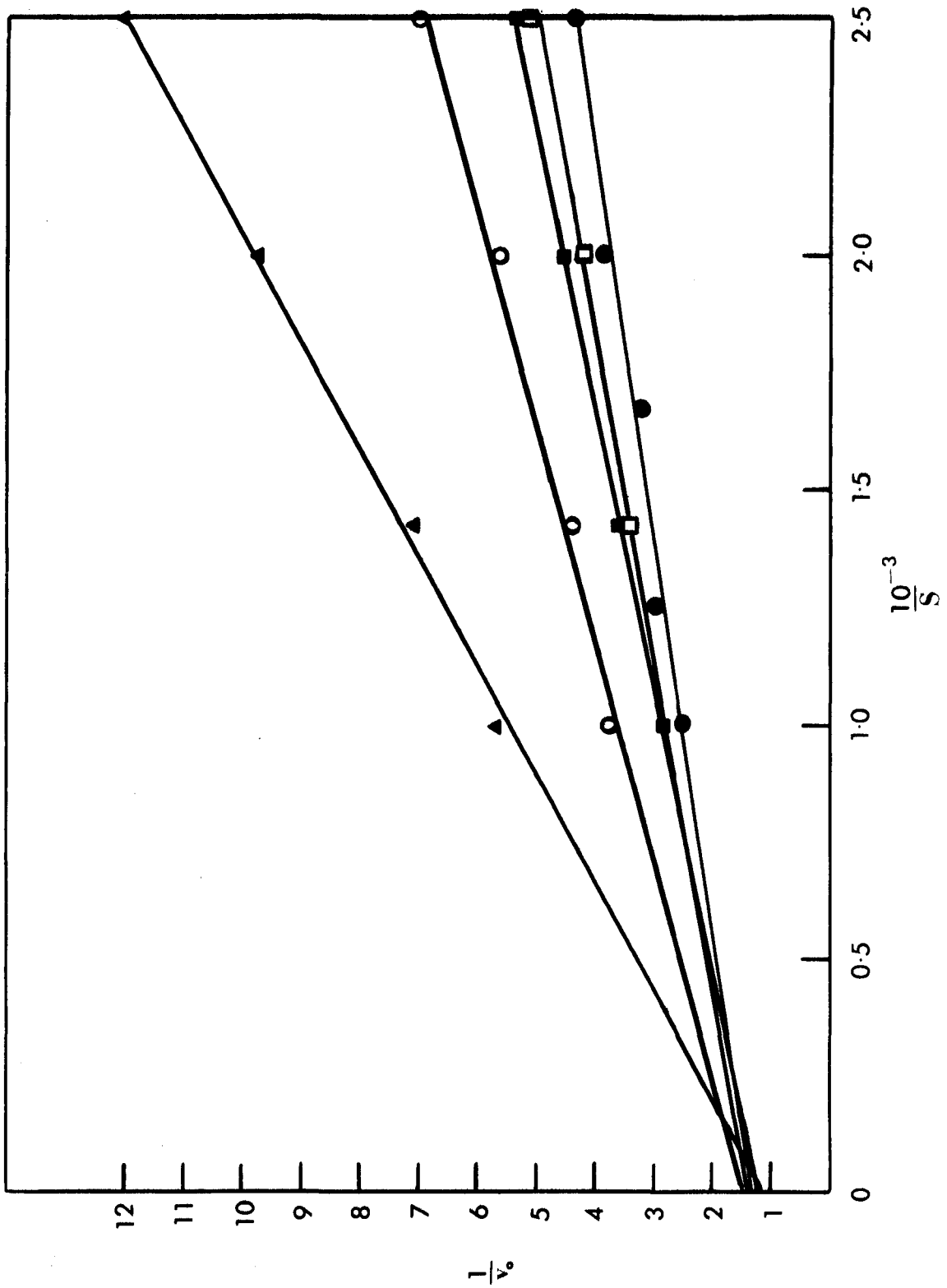


FIGURE 2

Table IV. K_m and V_{max} values for the cystine reductase system. All values were obtained by the method of Lineweaver and Burk (64). The determination of cysteine produced from cystine was followed by the NBS titrimetric method. Each flask contained 2.5 ml of enzyme suspension (171 mg of protein), 0.025 M phosphate buffer (pH 6.2), and concentrations of substrate ranging from 4×10^{-4}M to 1×10^{-3}M. The reaction was stopped by adding 0.5 ml of 10 per cent (w/v) phospho-24-tungstic acid in 5 per cent (v/v) HCl. All determinations were done in triplicate. Values obtained for the controls were subtracted from the values for the experimental flasks. The K_m and V_{max} values were calculated by the IBM 1620 computer by the method of least squares analysis.

TABLE IV

K_m and V_{max} Values for the Cystine Reductase System

Amino Acid	K_m ($\times 10^3$)	V_{max} ($\mu M\ min^{-1}$)
L-Cystine	0.901	0.741
DL-Cystine	1.056	0.718
α-Methyl-DL-	1.164	0.774
α-n-Propyl-DL-	1.396	0.832
α-Isopropyl-DL-	3.537	0.820
α-Phenyl-DL-	1.468	0.687

Table V. Enzymatic versus polarographic reduction of the alpha-substituted cystines. Initial velocities and first order rate constants were determined using 1 x 10^{-3}M L-cystine, DL-cystine, or alpha-substituted cystine, 0.025 M phosphate buffer (pH 6.2), and 2.5 ml of enzyme suspension (171 mg of protein) in a total volume of 10 ml. The reaction was stopped by adding 0.5 ml of 10 per cent (w/v) phospho-24-tungstic acid in 5 per cent (v/v) HCl. The cysteine produced was determined using the NBS assay. The values listed are the average of three determinations. The first order rate constants were calculated from the first order rate equation. The polarographic half-wave potentials were previously determined in this lab (22).

TABLE V

Enzymatic Versus Polarographic Reduction of the Alpha-Substituted Cystines

Amino acid	v_o ($\mu M\ min^{-1}$)	k (min^{-1})	Half-wave potential (volt)
L-Cystine	0.415	415	-0.536
α-Methyl	0.350	350	-0.514
DL-Cystine	0.346	346	--
α-n-Propyl	0.345	345	-0.186
α-n-Butyl	0.318	318	-0.202
α-Phenyl	0.264	264	-0.184
α-Ethyl	0.190	190	-0.249
α-Isopropyl	0.167	167	-0.370

L-amino Acid Oxidase

Figure 3 shows Lineweaver-Burk plots of L-cystine and DL-cystine obtained for the L-amino acid oxidase system. L-cystine gave a K_m of 5.5×10^{-4}M while DL-cystine a value of 2.7×10^{-3}M. V_{max} for L-cystine was 0.120 μM per minute, while for DL-cystine V_{max} was 0.200 μM per minute.

Table VI shows the inability of the alpha-substituted cystines to act as substrates for L-amino acid oxidase. Table VII illustrates the failure of the alpha-substituted cystines to inhibit the oxidative deamination of L-cystine by the enzyme, L-amino acid oxidase.

Figure 3. Lineweaver-Burk plots of L-amino acid oxidase with L-cystine and DL-cystine as substrates. The consumption of oxygen was measured using an oxygen electrode. The reaction vessel contained 0.05 M pyrophosphate buffer, 0.06 units of L-amino acid oxidase (Type I), and concentrations of substrate ranging from 1 x 10^{-4}M to 2 x 10^{-3}M. The total volume was 4 ml. All the solutions were air saturated at 25°C. Initial velocities were measured by taking the slope of the initial rates which were linear for at least the first 2 minutes. Individual points for L-cystine (▲) as well as for DL-cystine (●) are the average of 3 determinations.

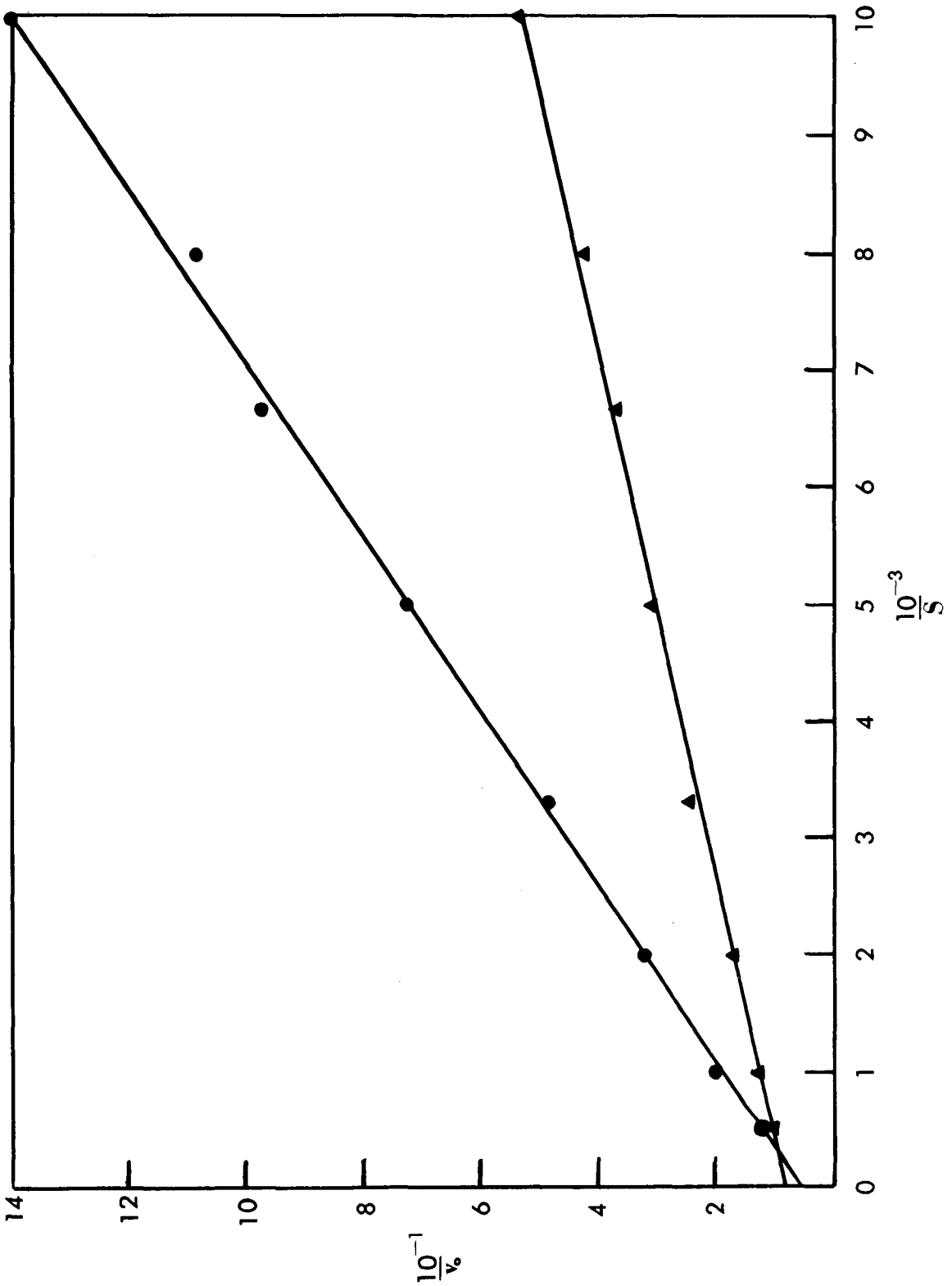


FIGURE 3

Table VI. Alpha-substituted cystines as substrates for L-amino acid oxidase. Oxygen consumption was measured using an oxygen electrode. When L-cystine and DL-cystine were used as substrates, the reaction vessel contained 0.05M pyrophosphate buffer, 0.06 units of L-amino acid oxidase (Type I), and 1 x 10^{-4}M, 5 x 10^{-4}M, or 2 x 10^{-3}M concentrations of substrate. When the alpha-substituted cystines were used as substrate, the reaction vessel contained the same concentrations of components except for the enzyme. Two concentrations of L-amino acid oxidase were used, 0.06 units and 0.288 units. The total volume of the reaction mixture was 4 ml.

TABLE VI

Alpha-Substituted Cystines as Substrates for L-Amino Acid Oxidase

Amino Acid	Concentration	v_o ($\mu M\ min^{-1}$)
L-Cystine	1×10^{-4} M	0.021
	5×10^{-4} M	0.062
	2×10^{-3} M	0.108
DL-Cystine	1×10^{-4} M	0.007
	5×10^{-4} M	0.031
	2×10^{-3} M	0.085
α-Methyl-	1×10^{-4} M	0.000
	5×10^{-4} M	0.000
	2×10^{-3} M	0.000
α-Ethyl-	1×10^{-4} M	0.000
	5×10^{-4} M	0.000
	2×10^{-3} M	0.000
α-n-Propyl-	1×10^{-4} M	0.000
	5×10^{-4} M	0.000
	2×10^{-3} M	0.000
α-Isopropyl-	1×10^{-4} M	0.000
	5×10^{-4} M	0.000
	2×10^{-3} M	0.000
α-n-Butyl-	1×10^{-4} M	0.000
	5×10^{-4} M	0.000
	2×10^{-3} M	0.000
α-Phenyl-	1×10^{-4} M	0.000
	5×10^{-4} M	0.000
	2×10^{-3} M	0.000

Table VII. Alpha-substituted cystines as inhibitors of L-amino acid oxidase. Oxygen consumption was measured using an oxygen electrode. The reaction vessel contained 0.05 M pyrophosphate buffer, 0.288 units of L-amino acid oxidase, 1×10^{-4}M L-cystine, and 5×10^{-4}M, 1×10^{-3}M, 2×10^{-3}M, 3×10^{-3}M, and 5×10^{-3}M concentrations of alpha-substituted cystine. The reaction was allowed to proceed for 5 minutes and the initial rate measured by taking the initial slope. Triplicate runs were made at each concentration of inhibitor.

TABLE VII

Alpha-Substituted Cystines as Inhibitors of L-Amino Acid Oxidase

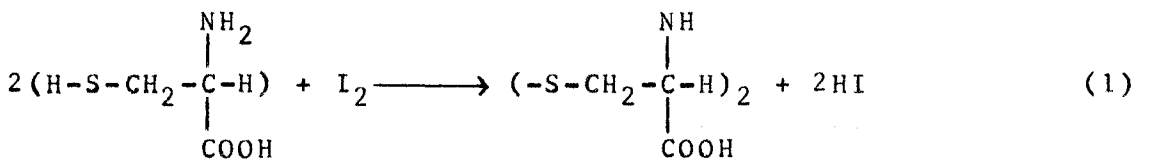
Amino acid	I/S	v_o (μMmin^{-1})	Inhibition per cent
α-Methyl-	0	0.066	0
	5	0.066	0
	10	0.066	0
	20	0.066	0
	30	0.066	0
	50	0.066	0
α-Ethyl-	0	0.066	0
	5	0.066	0
	10	0.066	0
	20	0.066	0
	30	0.066	0
	50	0.066	0
α-n-Propyl-	0	0.066	0
	5	0.066	0
	10	0.066	0
	20	0.066	0
	30	0.066	0
	50	0.066	0
α-Isopropyl-	0	0.066	0
	5	0.066	0
	10	0.066	0
	20	0.066	0
	30	0.066	0
	50	0.066	0
α-n-Butyl-	0	0.066	0
	5	0.066	0
	10	0.066	0
	20	0.066	0
	30	0.066	0
	50	0.066	0
α-Phenyl-	0	0.066	0
	5	0.066	0
	10	0.066	0
	20	0.066	0
	30	0.066	0
	50	0.066	0

CHAPTER IV

DISCUSSION

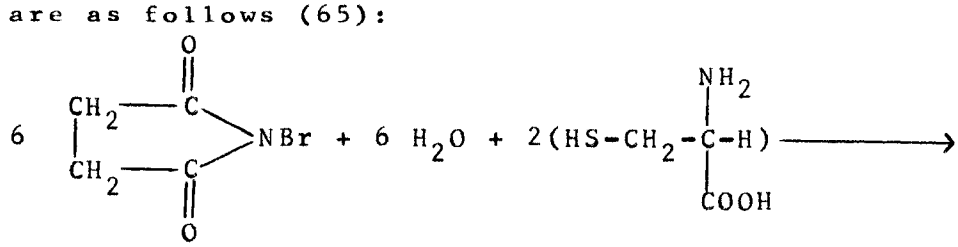
Determination of Cysteine and Cystine Using NBS

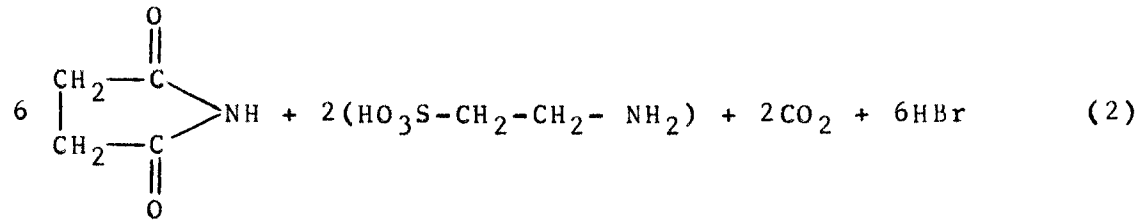
In Aqueous solution, N-bromosuccinimide hydrolyzes to form succinimide and hypobromous acid (64) which is thought to be responsible for the oxidizing properties of this compound. The reactions using N-bromosuccinimide in the determination of sulfur amino acids are quantitative (65). When titrated with N-bromosuccinimide, iodine is liberated preferentially from potassium iodide and then reacts with cysteine. The reaction is as shown below:



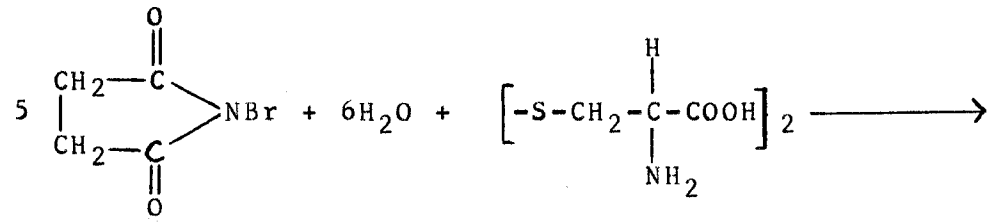
(1)

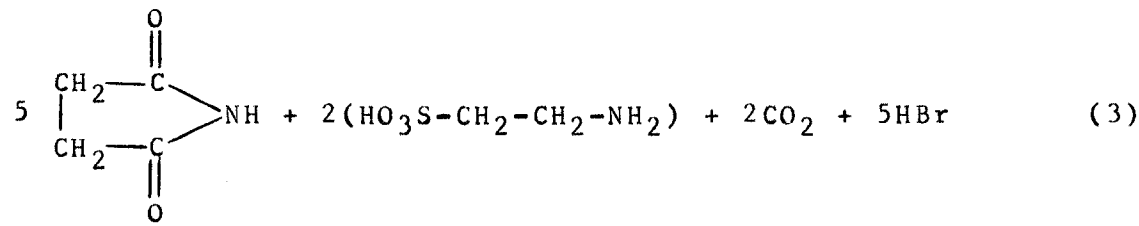
It has been found that at pH 7 the oxidation of cysteine (R-SH) does not go beyond cystine (R-S-S-R). N-Bromosuccinimide, used as a primary standard, can serve to liberate iodine from potassium iodide quantitatively at pH 7. Potassium iodate cannot be used at pH 7, and if used at low pH values, oxidizes The RSH beyond the R-S-S-R stage as shown in Table I and Figure 1. The reactions of N-bromosuccinimide with cysteine and cystine are as follows (65):





(2)





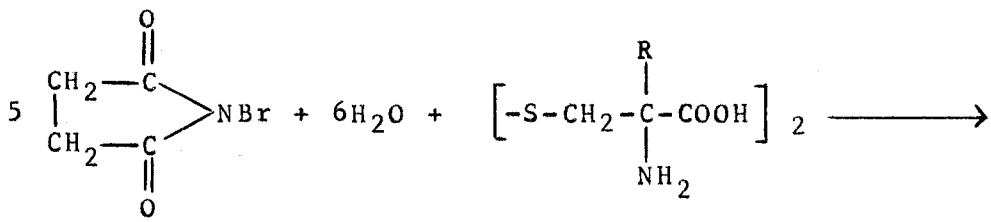
(3)

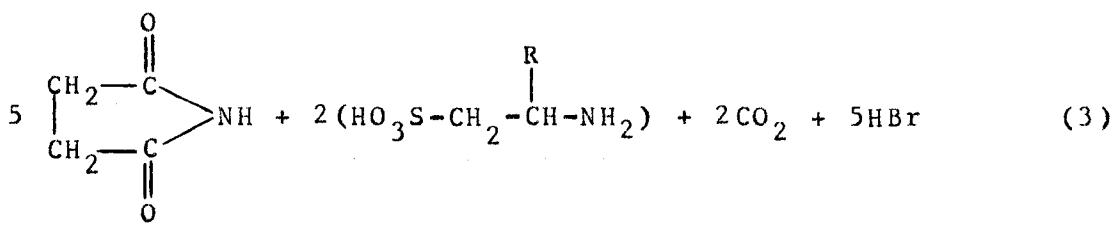
From equation (1), it can be seen that cysteine sulfur goes from an oxidation state of -2 to -1 with the loss of one electron. In equation (2), the sulfur changes its oxidation state from -2 to +4 with a loss of 6 electrons. In cystine there are two sulfur atoms which change their oxidation state from -1 to +4 with a loss of 10 electrons which is shown in equation (3). In N-bromosuccinimide, the oxidation state of bromine goes from +1 to -1 with the gain of 2 electrons. The equivalent weights of the respective substances were calculated taking these changes into consideration.

The Determination of Alpha-Substituted Cysteines and Cystines with N-Bromosuccinimide.

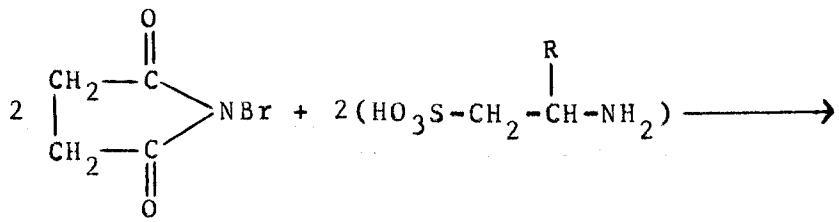
The same principles discussed above for the determination of cysteine with N-bromosuccinimide hold for the alpha-

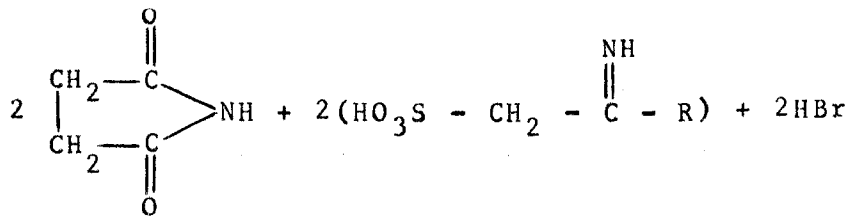
substituted cysteines. Table III shows that the alpha-substituted cysteines can be determined within +2 per cent error on a 2 mg sample in pure solutions. Using starch as the indicator provides for a very sharp end-point which is easily determined. However, when the alpha-substituted cystines were titrated with N-bromosuccinimide with Bordeaux Red as an indicator, an error of +40 per cent was consistently obtained. It was concluded that the cystine was going beyond the end product (taurine) as shown in equation (3). The following mechanism was proposed:





(3)





(4)

$$2(HO_3S\text{-}CH_2\text{-}\overset{\overset{NH}{\|}}{C}\text{-}R) + 2H_2O \longrightarrow 2(HO_3S\text{-}CH_2\text{-}\overset{\overset{O}{\|}}{C}\text{-}R) + 2NH_3 \qquad (5)$$

It can be seen that instead of the loss of 10 electrons, there are now 14 electrons lost. Thus the equivalent weight of the alpha-substituted cysteine is equal to the molecular weight divided by 14 instead of by 10. This scheme accounts for the +40 per cent error in the titrimetric determinations. The results are shown in Table II.

To further substantiate the proposed reaction mechanism, the presence of ammonia was determined using Nessler's reagent (67). The reagent went from clear to cloudy when ∝-methyl-DL-Cystine was mixed with an excess of NBS and the released ammonia aerated over into a tube of Nessler's reagent. When NBS was treated alone in the same manner, the Nessler's reagent did not turn cloudy to any extent indicating no significant release of ammonia. A further piece of supporting evidence was the successful detection of a methyl-ketone. According to the proposed mechanism, when ∝-methyl-DL-cystine is titrated with NBS, a methyl-ketone should be obtained. This was found to be the case as the iodoform test (68) was positive yielding yellow crystals with a distinct odour of iodoform.

Substrate Behaviour of the Alpha-Substituted Cystines in the Cystine Reductase System

From Figure 2 and Table IV it is seen that all of the alpha-substituted cystines act as substrates for cystine reductase. The K_m values range from 0.901×10^{-3}M to

3.537×10^{-3}M while the V_{max} values from 0.678 to 0.832 μM per minute. The results indicate that the alpha-hydrogen atom of cystine is not necessary for binding of the substrate to the enzyme and that it does not play a role in the enzymatic reduction of cystine to cysteine. On the basis of the K_m values obtained, an order of affinities of the substrates for the enzyme can be written as: L-cystine > DL-Cystine > α-methyl > α-n-propyl > α-phenyl > α-isopropyl-DL-cystine. On the basis of the initial velocities and the first order rate constants obtained in Table V for a 1×10^{-3}M concentration of substrate, the following order of ease of reduction by the enzyme can be written: L-cystine > α-methyl > DL-cystine > α-n-propyl > α-n-butyl > α-phenyl > α-ethyl > α-isopropyl-DL-cystine. No correlation between the ease of reduction of these compounds enzymatically as compared to polarographically is seen by comparing the half-wave potentials and the initial velocities or the apparent first order rate constants for these compounds illustrated in Table V. It might therefore be postulated that the enzyme is specific for the disulfide bond and non-specific for the rest of the molecule. Black et al. (66) have reported a nonspecific enzyme in yeast which will reduce disulfides to thiols. This enzyme was found to reduce hydroxyethyl disulfide, L- or D-cystine, homocystine, DL-Lipoic acid, oxidized glutathione, oxytocin, and the three disulfide bonds of insulin. It seems more likely that upon purification the reduction of these compounds could be attributed to a series of different, specific enzymes. The fact that cystine reductase has never

been purified and only crude homogenates of yeast, pea seeds, wheat flour, or bacteria have been used in previous studies (15-20) throws some suspicion on the validity of the existence of a specific cystine reductase.

L-Amino Acid Oxidase

The results shown in Table VI are in agreement with previous investigations with alpha-substituted amino acids as substrates for L-amino acid oxidase (6,7). The compounds are inert to oxidative deamination indicating that the alpha-hydrogen atom is essential either in binding of the substrate to the enzyme or in the enzymatic reaction mechanism involved. The fact that no inhibition of the enzyme was found when L-cystine was the substrate and the alpha-substituted cystines added as inhibitors, indicates that the alpha-hydrogen atom of cystine is necessary for binding of the substrate to the active site of the enzyme. These results tend to agree with those of Umbreit (3) for D-amino acid oxidase. Alpha-methylvaline was found to slightly inhibit methionine and valine but did not inhibit the oxidative deamination of phenylalanine. Alpha-methylphenylalanine slightly inhibited methionine oxidation while valine and phenylalanine were not inhibited. The substitution of deuterium in place of the alpha-hydrogen of tyrosine and phenylglycine resulted in an isotopic effect with kH/kD equal to 1.41, indicating that the alpha-hydrogen atom is important in the reaction mechanism (49). This piece of evidence, as well as the fact that none of the alpha-substituted amino acids tested to date act as substrate, supports the imino acid

intermediate proposed by Wellner and Meister (33,56).

CHAPTER V

SUMMARY

A new titrimetric method for the determination of cysteine and cystine (65) was tested and found suitable for the quantitative determination of cysteine from cystine in the enzyme system, cystine reductase. The method was shown to be applicable to the quantitative determination of alpha-substituted cysteines and cystines with a small change in the calculations.

Alpha-methyl-, α-ethyl-, α-n-propyl-, α-isopropyl-, α-n-butyl-, and α-phenyl-DL-cystine were found to be reduced by the enzyme, cystine reductase. K_m and V_{max} values were determined for all but α-n-butyl- and α-ethyl-DL-cystine. No correlations could be seen between the ease of reduction of these compounds by cystine reductase when compared to the polarographic half-wave potentials.

The above mentioned alpha-substituted cystines were inert to oxidative deamination by the enzyme L-amino acid oxidase. These compounds did not inhibit the oxidative deamination of L-cystine to any extent. The results indicate that the alpha-hydrogen atom is necessary for the binding of the amino acid to the active site of L-amino acid oxidase.

APPENDIX

Computer Program for the Determination of K_m and V_{max}.

```
340003200701360003200702490240251196361130010200
ZZJOB 5
ZZFORX5
      DIMENSION Y(50),X(50)
C     LEAST SQUARES EVALUATION OF KINETIC DATA
    1 READ 2,R,N
    2 FORMAT (A3,I4)
    3 READ 4,(Y(I),X(I),I=1,N)
    4 FORMAT(6(F6.3,F5.0))
C     LEAST SQUARES EVALUATION
      SX=0.
      SY=0.
      SXY=0.
      SXSQ=0.
      DO 5 I=1,N
      SX=SX+X(I)
      SY=SY+Y(I)
      SXY=SXY+X(I)*Y(I)
      SXSQ=SXSQ+X(I)*X(I)
    5 CONTINUE
      XN=N
C     CALCULATION OF SLOPE,XINT,YINT
      SLOPE=(SX*SY-XN*SXY)/(SX*SX-XN*SXSQ)
      YINT=(SX*SXY-SXSQ*SY)/(SX*SX-XN*SXSQ)
    6 XINT=-YINT/SLOPE
      VMAX=1./YINT
      AKM=-1./XINT
      PUNCH 100,R,VMAX,AKM
  100 FORMAT(A3,5X,5HVMAX=,E10.3,5X,3HKM=,E10.3)
   11 GO TO 1
   10 CALL EXIT
      END
```

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