1-1-1966

Polarographic and potentiometric studies of alpha substituted cystines.

Robert J. Walton
University of Windsor

POLAROGRAPHIC AND POTENTIOMETRIC STUDIES OF ALPHA SUBSTITUTED CYSTINES

BY

ROBERT J. WALTON

A Thesis
Submitted to the Faculty of Graduate Studies through the
Department of Chemistry in Partial Fulfillment
of the Requirements for the Degree of
Master of Science at the
University of Windsor

Windsor, Ontario

1966

ABN4296

Approved

Roger J. Thibert

Alex Gryff

W. J. Hall

147098

ABSTRACT

Because of the recent syntheses of some alpha substituted cystines, it became necessary to have a method of determining these compounds. The polarographic determination of these compounds in 0.1 N hydrochloric acid, using thymol as a maximum suppressor, was investigated. A linear relationship of the diffusion current to the concentration of the compound was observed. The influence of substituent, thymol concentration, and pH on the apparent half-wave potential and shape of the polarogram was determined. The half-wave potentials were determined in 0.1 N hydrochloric acid using 1.2×10^{-4} M thymol as a maximum suppressor. The systems were not reversible.

The titration curves obtained for these alpha substituted cystines were affected by the substituent in the alpha position.

ACKNOWLEDGEMENTS

The author wishes to acknowledge with gratitude the direction of Dr. R.J. Thibert without whose patience and guidance this work could not have been done.

He also wishes to acknowledge support from the Province of Ontario in the form of a Province of Ontario Graduate Fellowship and from the National Research Council of Canada. Finally, thanks to Mr. J.F.G. Diederich for his assistance in these studies.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER I

INTRODUCTION

The polarographic studies of cystine by Kolthoff and Barnum (1) and of α -methyl-DL-cystine in this laboratory (2) have prompted further investigation into the effect of other substituent groups in the alpha position of cystine. The object of this study is to investigate the effect of these substituent groups on the half-wave potential, the diffusion current, and the shape of the polarograms.

In order to carry out this investigation the following compounds were synthesized: α -ethyl-, α -n-propyl-, α -isopropyl-, α -n-butyl-, and α -phenyl-DL-cystine. It should be noted that the above compounds represent various degrees of polar, resonance, and steric effects which should then be manifested by changes in the half-wave potential, the diffusion current, and the shape of the polarograms.

Cystine and cysteine constitute a typical oxidation-reduction system:

$$2 \text{ cysteine} \rightleftharpoons \text{cystine} + 2H^{+} + 2e$$

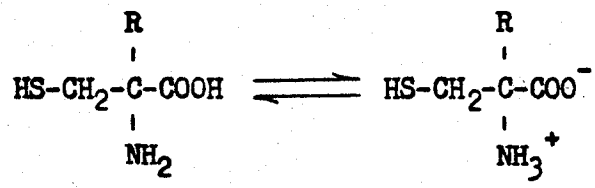
The oxidation of cysteine can be effected by atmospheric oxygen:

$$2 \text{ R-SH} \xrightarrow{(O_2)} \text{R-S-S-R}$$

However, this is a slow process and it can be enhanced by such metals as iron (3), nickel and manganese (4). Cystine is usually reduced to

cysteine by the action of tin metal on hydrochloric acid solutions of cystine (5) or by hydrogen with a catalyst.

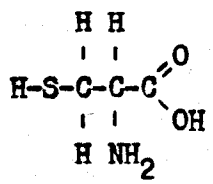
The presence of both acidic and basic groups in the amino acid molecule leads to partial internal neutralization with the formation of a dipolar ion (6):



Zwitterion

The dipolar ion produces a saltlike character and gives amino acids a high melting point, Many decompose before they melt. Alpha-amino acids, like alpha-hydroxy acids, form cyclic derivatives. The form of these cyclic diamides interferes with a simple synthesis of polyamides (proteins) (7).

Cysteine has three functional groups: the sulfhydryl group (-SH), the carboxyl group (-COOH), and the amine group ($-NH_2$) as shown by the following structure:



Structure of Cysteine

Thus if substitution could be effected in the alpha position it should have an influence on the reactivity of these functional groups.

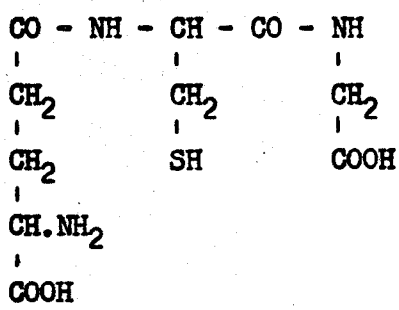
Amino acids readily form chelates with copper salts if a primary or secondary amino group is close to the carboxyl group (8). In

general, cysteine has the fundamental requirements of a chelating agent with the acid group (-COOH) and the basic amine group ($-NH_2$) both situated so as to form a six-membered, strain-free ring.

Amino mercaptans also react with aqueous solutions of heavy metal salts to form highly insoluble mercaptides. Kolthoff and Stricks (9) obtained the copper mercaptide of cystine, RSCu, by reacting cystine with cuprous chloride in near neutral solution. Lead, zinc, and mercury also readily form mercaptides (10). Substituent groups affect the acid-base strength as well as the solubility of these complex ions. The addition of hydrocarbon substituents generally decreases the solubility of the precipitate and so increases the effectiveness of the agent (11). It would be of interest to study the influence of various hydrocarbon groups in the alpha position of cystine on the solubility of the chelates. It would also be interesting to study the effect of these substituent groups on the chelating strength of the substituted cysteines.

Both cystine and cysteine undergo alkaline hydrolysis with the formation of pyruvic acid, ammonia, hydrogen sulfide, and sulfur (12).

In biochemistry, cysteine and glutathione are often referred to together because they undergo similar reactions and have similar physiological effects. Glutathione is a tripeptide which is biosynthesized from the component amino acids L-glutamic, L-cysteine, and glycine (13):



Structure of Reduced Glutathione (14)

Cysteine and glutathione form an oxidation-reduction equilibrium which is common with thiol-disulfide systems (15):

$$2GSH + CSSC \rightleftharpoons GSSG + 2CSH$$

Rapkine (16) and Dickens (17) found that cysteine and glutathione combine with halogen acids giving the thio ethers of cysteine and glutathione. According to Rapkine, the reaction with cysteine is faster than with glutathione. Furthermore, the rate of reaction with cysteine was dependent on the pH value of the solution. Not all halogen acids react with thiols; cysteine was found to react with iodoacetate, bromoacetate, and chloroacetate, but not with fluoroacetate:

$$R\text{-}SH + Hal\text{-}CH_2\text{-}COO^- \longrightarrow RSCH_2\text{-}COO^- + H\text{-}Hal$$

The rates were those of bimolecular reactions. It would be of interest to see if substituent groups in the alpha position of cysteine would have any effect on these reaction rates. At least the steric effect of these groups on the rate of saponification of the ethyl esters of the alpha substituted cystine should be significant.

Studies on the hydrolysis of para-nitrophenyl acetate as catalyzed by various thiols have also appeared in the literature recently (18). These studies were made prior to undertaking a study of the mechanism by

which certain enzymes operate. Therefore, it would be of interest to study the possible effects of various sterically important alkyl groups on the rate of catalysis.

Most of the sulfur proteins in the body are represented by the methionine and cystine present, though small amounts of cysteine can be found. Cystine is produced in the metabolic breakdown of methionine, which is an essential amino acid (19). Therefore, it is possible to replace cystine with methionine; however, the presence of cystine in the diet decreases the demand for the formation of cystine from methionine.

Cysteine participates in a novel form of detoxication in the formation of mercapturic acids (20). Aromatic hydrocarbons and many of their monohalogenated derivatives are conjugated with cysteine in the body, the cysteine moiety is then acetylated, and the resulting mercapturic acid excreted in the urine. These include benzene, polycyclic hydrocarbons (e.g. naphthalene), and ring halogenated hydrocarbons (e.g. bromobenzene).

The sulfhydryl and disulfide groups present in amino acids, peptides, and enzymes are known to play important biological and physiological roles (21). Cysteine has been found to be in intact protein molecules, but it is only obtained in the products of protein hydrolysis if precautions are taken against oxidation, since the sulfhydryl group of cysteine is readily converted to the disulfide of cystine.

The sulfhydryl groups of protein have been found to be essential in the reactivity of a great number of enzymes and as binding sites between protein and some prosthetic groups. Many enzymes contain sulfhydryl groups derived from the side-chains of cysteine residues. Blockage through chemical transformation of these groups results in inhibition of

the enzymes (22). An example is the conversion of sulfhydryl groups to disulfides with oxidants such as ferricyanide:

$$2\ \text{R-SH} \xrightarrow[-2\text{H}]{\text{Ferricyanide}} \text{R-S-S-R}$$

The enzyme combines with the substrate to form the "Michaelis-Menten enzyme-substrate complex". This complex then breaks down to yield the enzyme plus the product. For example, D-glyceraldehyde-3-phosphate dehydrogenase converts 3-phosphoglyceraldehyde to 1,3-diphosphoglyceric acid:

$$\text{Enzyme} + \text{Substrate} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \text{ES}^* \xrightarrow{k_3} \text{Enzyme} + \text{Product}$$

* "Michaelis-Menten Complex"

The formation and dissociation of the Michaelis-Menten complex, as well as the rate constants (k_1, k_2, and k_3) are influenced by the side chains of the protein molecule which contain sulfhydryl, amine, amide, carboxyl, and phenol groups. Therefore it would be conceivable to think that a substituent group in the alpha position to one of these groups would have some influence as well.

It has been found that sulfhydryl content increases steadily with cell growth and reaches a maximum just before cell division. Upon division the sulfhydryl content at first is low and increases again until the second division occurs. To illustrate this, Rapkine (23) added small amounts of mercuric chloride to fertilized sea urchin eggs until mitosis was arrested completely. Mitosis was again initiated when cysteine was added.

It has also been found that there is a greater amount of sulfhydryl protein present in tumorous tissue. The mechanism of sulfhydryl protein in the growth and division of cells is unknown. If, however, an unreactive sulfhydryl protein could be substituted for a reactive one then the rapid growth and division of cells in a tumor could be arrested. That is, if an unreactive amino acid, such as an alpha substituted cysteine, could be substituted for a reactive amino acid and inhibit the enzyme, the reaction will go only as far as the Michaelis-Menten complex and would not break down into the desired product, thus slowing down the over-all process. In other words it would act as a metabolic antagonist:

$$\text{Enzyme} + \text{Substrate} \rightleftharpoons \underset{\text{(unreactive)}}{ES^*} \longrightarrow \text{No Product}$$

The metabolic behaviour of various cystines and their corresponding cysteines has been shown to be of interest. Umbreit (24) found that α-methyl-DL-glutamic acid is inert to dehydrogenation and transamination, inhibits glutamine breakdown, decarboxylation, and glutamotransferase; thus similarly substituted cystines should also act as metabolic antagonists. It has also been shown that alpha-methyl substituted, aliphatic amino acids (such as alanine, valine, and serine) are inert to metabolic reactions (24).

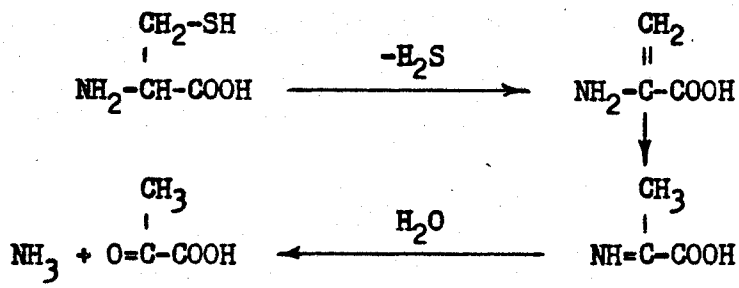
The effects of ionizing radiation on the sulfhydryl and disulfide groups are of primary concern in radiation biochemistry, and have recently been reviewed by Barron (25). Cysteine and cystine have long been the object of radiochemical investigations because the information from such studies is of help in understanding the radiation chemistry of more complex sulfhydryl and disulfide compounds.

Ionizing radiation oxidizes the sulfhydryl group (-SH) to the disulfide (-S-S-). Thus radiation inhibits sulfhydryl enzymes and lowers free sulfhydryl groups essential in cell growth. Sulfhydryl compounds such as glutathione and cysteine are more susceptible to oxidation by radiation than most sulfhydryl enzymes. Patt et al. (26) and Chapman et al. (27) found it possible experimentally to protect rats against lethal doses of radiation after injecting them with cysteine or glutathione. Therefore, derivatives of cysteine may be a step closer to the problem of radiation sickness. There is also evidence that cystines may afford such chemical protection as it has been shown that cystamine, a disulfide, possesses such properties (28). It has been shown that the maximum protection is obtained if the amine group and the thiol group are separated by two carbon atoms, as is the case in these compounds (29).

Cysteine and related compounds readily form chelates with copper salts. The instability constants of these resulting compounds, determined polarographically, have been shown to vary inversely with the ability of the sulfur compound to afford some protection against the effects of ionizing radiation on the cell (30). The instability constant should vary with the alpha substituted groups present. The ability of cysteine and related compounds such as cysteamine and glutathione to lower the effects of ionizing radiation has been shown (31,32). Substituted cystines would probably act in a similar manner.

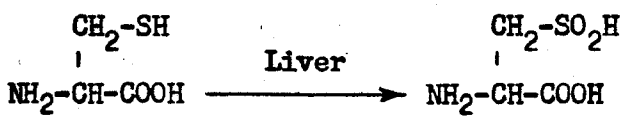
Various flavoproteins usually catalyze the oxidative deamination of amino acids. However, deamination of some amino acids is brought about by means of a non-oxidative reaction with enzymes. Deamination of cysteine can be effected by cysteine desulfhydrase which catalyses the

removal of the elements of hydrogen sulfide from cysteine (33):



It can be seen from the above mechanism that if the alpha hydrogen were substituted that, unless this substituent group were removed by cysteine desulfhydrase, the reaction would be inhibited and the substituted cysteine would act as an anti-metabolite.

L-Cysteine is converted to L-cysteine sulfinic acid in the liver which is then further metabolized (34):



L-Cysteine L-Cysteine sulfinic acid

Here again it would be interesting to see if steric effects have any influence on the reaction.

Cystine can be determined colorimetrically by reduction to cysteine and by using reagents which form colors in the presence of free sulfhydryl groups (35,36). Cystine can also be determined polarographically, and studies of this type have been reported by Kolthoff and Barnum (1).

The oxidation potential of the biologically important cysteine-cystine system has been the subject of many investigations (37). However, the reduction of cystine at the dropping mercury electrode has received very little attention from investigators in the field, until

the work of Kolthoff and Barnum (1) in 1941 and Kalousek, Grubner, and Tochstein (38) in 1953.

Brdicka (39) found that when cystine was electrolyzed, in a buffer solution of 0.1 N ammonium chloride and 0.1 N ammonia, a polarographic wave was obtained, the height of which was observed to be proportional to the concentration of cystine. A somewhat more detailed study was made by Roncato (37). He studied the polarographic reduction of cystine in unbuffered neutral, acid, and alkaline solutions of lithium chloride. However, the waves cannot be subjected to an exact analysis as the solutions were unbuffered (40) and the reduction potential depends on the pH:

$$RSSR + 2H^+ + 2e \longrightarrow 2\ RSH$$

Kolthoff and Barnum (1) found that cystine waves had peculiar characteristics and their interpretation is quite involved. They found that the addition of capillary-active substances such as phenol, thymol, gelatin or camphor suppressed the maximum. However, varying results were obtained and from a practical viewpoint thymol was found to be the most suitable for the elimination of the cystine maximum. Therefore, it was decided to adopt it for use in this study. They found that the analysis of the current-voltage curves shows that the reduction of cystine at the dropping mercury electrode does not occur reversibly and that the pH has a marked effect on the reduction potential. They also found that cystine may be determined polarographically at a pH of 1 using thymol as the maximum suppressor. The diffusion current was found to be proportional to the concentration.

Borsook et al. (41) determined the ionization constants of cystine.

A knowledge of the ionization constants of the alpha substituted cystines would aid in the interpretation of the shift in half-wave potential due to these substituents in the alpha position.

The half-wave potentials of various alkyl disulfides were determined by Hall (42). The half-wave potentials of these compounds could be useful in interpreting the results obtained for alpha substituted cystines.

Obolentsev et al. (43) have studied various disulfides employing tetramethylammonium iodide in N,N-dimethyl-formamide as supporting electrolyte.

The polarography of diphenyl, dibenzyl, di-n-butyl, and di-tert-butyl disulfide has been carried out by Karchmer and Walker (44).

Zuman (45) has shown how the shifts of half-wave potentials in reaction series consisting of compounds of the type X-A-R, containing the polarographically active group R, characteristic for the reaction series given and bearing different substituents X, are explained in terms of polar, resonance, and steric effects. A similar approach might be employed in the study of alpha substituted cystines.

CHAPTER II

EXPERIMENTAL

A. Polarographic Investigation of Some Alpha Substituted Cystines

Materials and Methods

The Sargent (E.H. Sargent and Co.) Model XXI Polarograph was employed for this study. An H-cell with saturated calomel reference electrode (S.C.E.) was used during this work. The characteristics of the capillary used were: $m = 2.126$ mg.sec.$^{-1}$, $t = 4.04$ sec., $m^{2/3}t^{1/6} = 2.09$ mg.$^{2/3}$sec.$^{-1/2}$. The height of the mercury column was 52.5 cm. All pH measurements were made with a Beckman Model G pH meter. Buffer solutions employed were prepared according to Clark and Lubs (46). A conductivity bridge was used to measure the cell resistance. A Fluke Model 825A Differential Voltmeter was used to measure the potential against the S.C.E.

The alpha substituted cystines were synthesized by Mr. J.F.G. Diederich (47). A stock solution of thymol in 0.1 N hydrochloric acid was prepared for use as a maximum suppressor.

No damping was used in recording any of the polarograms obtained for this study.

Procedure

The solutions used for analysis were prepared in a 50-ml. volumetric flask by adding the appropriate amount of alpha substituted cystine

and thymol and diluting to volume with 0.1 N hydrochloric acid or buffer.

The solutions were transferred to the polarographic cell and nitrogen (purified by passing through a gas washing bottle containing copper turnings and a 1:1 ammonium hydroxide solution saturated with ammonium chloride) was bubbled through for 5 minutes. All polarograms were recorded at 25 ± 0.1°C.

B. Titration Curves of Some Alpha Substituted Cystines

Materials and Methods

Titration curves were obtained with a Radiometer Type TTT1c automatic titrator equipped with glass and calomel electrodes. The temperature of the titration vessel was maintained at 25 ± 0.1°C and an atmosphere of nitrogen was maintained over the solutions during the titrations. The pH meter was calibrated with buffers of pH 1.10 (0.1 M hydrochloric acid), 4.01 (0.05 M potassium acid phthalate), and 7.02 (Beckman buffer solution 3581).

Procedure

A sufficient quantity of amino acid (2.5×10^{-5} moles) to give a final concentration of 5×10^{-3} M was dissolved in 1 ml. of carbonate-free sodium hydroxide and 4 ml. of 0.1 M potassium chloride. Nitrogen was bubbled through the solution and an atmosphere of nitrogen was maintained over the solution during the titration. The amino acids were titrated with 0.1000 N hydrochloric acid.

CHAPTER III

RESULTS

A. Polarographic Investigation of Some Alpha Substituted Cystines

Effect of Concentration on Diffusion Current

A linear relationship of the diffusion current to the concentration of alpha substituted cystine (using thymol as the maximum suppressor) was observed (Table I). The diffusion currents and half-wave potentials are based on the prewave plus the top wave wherever a prewave occurs. This type of prewave also occurs with cystine and has been studied by Kalousek, Grubner, and Tochstein (38).

The diffusion current was calculated by measuring the height, h, of the polarographic wave in millimeters (Figure 1) and substituting into the following relationship:

$$i_D = \text{Sens.} \times h$$

Where i_D = the diffusion current

Sens. = the sensitivity

h = the wave height

e.g. Sens. = 0.030 μamp. per mm.

h = 203 mm.

Therefore, $i_D = 0.030 \times 203$

$= 6.09$ μamp.

The apparent half-wave potentials were calculated by measuring the

TABLE I

EFFECT OF CONCENTRATION ON DIFFUSION CURRENT

Amino Acid Concentration (Mole/Liter)	Diffusion Current (μamp.) for α-R-DL-cystine*				
	R				
	Ethyl	n-Propyl	Isopropyl	n-Butyl	Phenyl
2.0×10^{-3}	8.69	12.27	12.06	11.18	
1.5×10^{-3}	6.49	9.07	9.12	7.92	
1.0×10^{-3}	4.32	5.97	5.65	5.35	
7.5×10^{-4}	3.87+	4.55	4.42	4.14	
5.0×10^{-4}	1.92	2.94	2.88	2.60	2.89
3.75×10^{-4}					2.09
2.5×10^{-4}					1.32
1.88×10^{-4}					0.98
1.25×10^{-4}					0.61

* Thymol concentration used was 1.2×10^{-4} M except for α-n-butyl-DL-cystine which was 4.8×10^{-4} M.

\+ The concentration of α-ethyl-DL-cystine was 8.7×10^{-4} M.

FIGURE 1

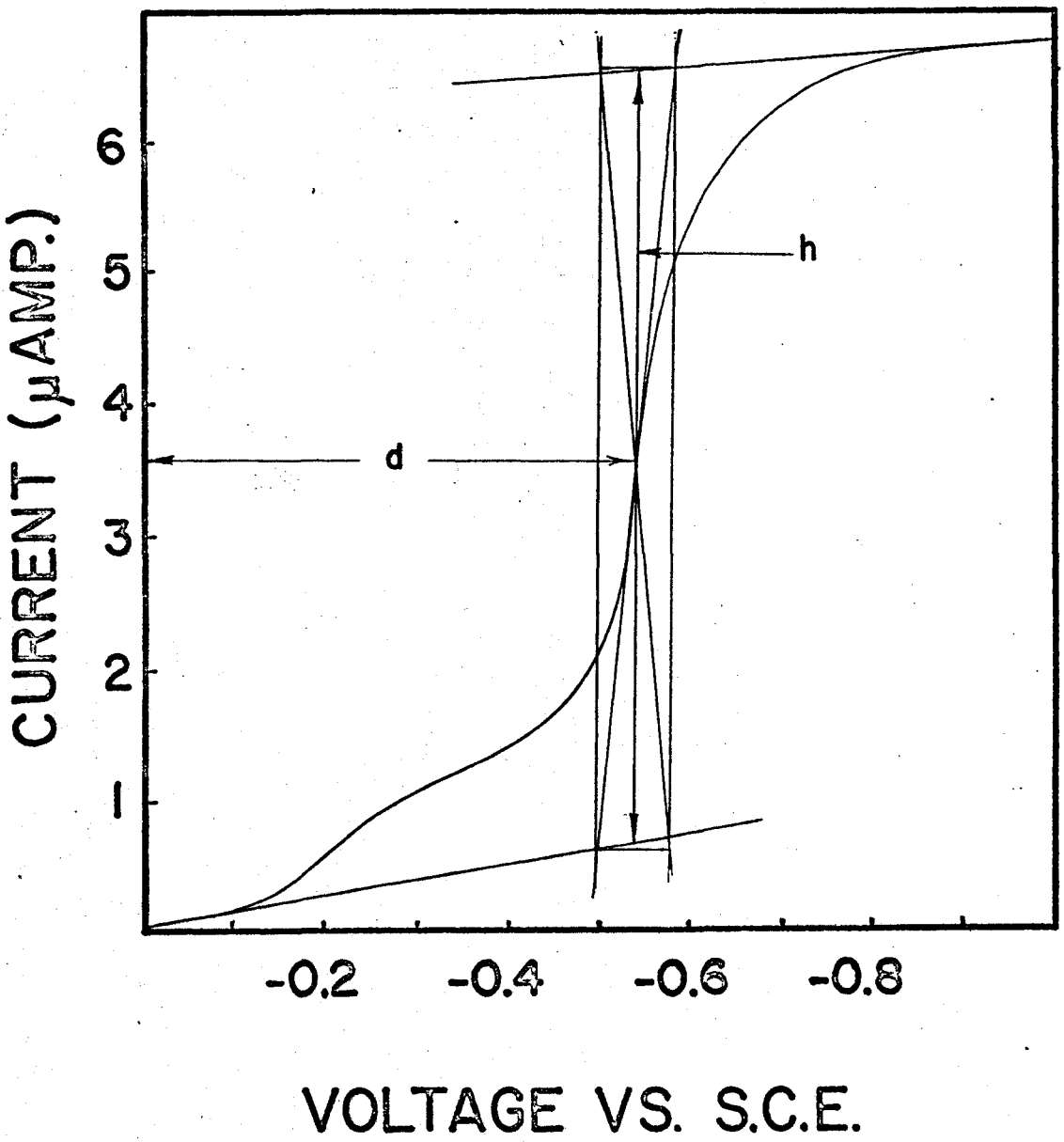
TYPICAL POLAROGRAM

Legend

This is a typical example of a polarogram of an alpha substituted cystine. The determination of the height of the diffusion current (h), and the distance for the half-wave potential (d) are shown.

FIGURE 1

TYPICAL POLAROGRAM



distance in inches from the beginning of the polarogram to the middle of diffusion curve (48), as shown in Figure 1.

This information was then substituted into the following:

$$E_{\frac{1}{2}app.} = (-1/13.5) \times d$$

Where $E_{\frac{1}{2}app.}$ = the apparent half-wave potential

$-1/13.5$ = a constant for the polarograph (a polarogram is 13.5 inches long for every span; since the span used is 1.00 volt, then 13.5 inches represents 1.00 volt on the polarogram, and the voltage at any time is 1/13.5 of the distance in inches from the beginning of the span - the negative sign is due to the fact that the voltage increases negatively)

d = the distance in inches from the beginning of the polarogram to the half-wave potential

e.g. Assuming the polarogram was begun at 0.00 volt:

d = 8.25 inches

Therefore $E_{\frac{1}{2}app.} = (-1/13.5) \times 8.25$

$= -0.611$ volt

Effect of pH on Apparent Half-Wave Potential

Solutions of α-n-propyl-, α-isopropyl-, and α-n-butyl-DL-cystine (1.0×10^{-3} M) were prepared using media of various pH levels (46) with thymol (4.8×10^{-4} M) as the maximum suppressor. Stock solutions of 0.2 M potassium chloride, 0.2 M hydrochloric acid, and 0.2 M potassium acid phthalate were prepared. The apparent half-wave potentials were determined (Table II).

TABLE II

EFFECT OF pH ON APPARENT HALF-WAVE POTENTIAL

Legend

* Values represent final solution to be analyzed. Buffers were prepared according to Clark and Lubs (46).

The amino acid concentration employed was 1.0×10^{-3} M.

TABLE II

EFFECT OF pH ON APPARENT HALF-WAVE POTENTIAL

Amino Acid	Thymol Concentration (Mole/Liter)	pH*	Apparent Half-Wave Potential (Volt)
α-n-Propyl-	4.8×10^{-4}	0.17	Two waves observed
DL-cystine		1.32	-0.492
		2.11	-0.482
		3.07	-0.452
		3.70	-0.494
α-Isopropyl-	4.8×10^{-4}	0.16	-0.512
DL-cystine		1.42	-0.550
		1.92	-0.572
		2.84	-0.580
		3.40	-0.622
α-n-Butyl-	4.8×10^{-4}	0.12	-0.162
DL-cystine		1.34	-0.312
		1.95	Maximum observed
		3.08	Maximum observed
		3.67	Maximum observed

The polarograms had different shapes (Figures 2-4) and the apparent half-wave potentials were observed to increase negatively with increase in pH with the exception of α-n-propyl-DL-cystine in which case the half-wave potential decreased up to pH 3.07 and then began to increase at pH 3.70.

Effect of Thymol on Apparent Half-Wave Potential

The effect of thymol concentration on the shape of the polarographic wave was studied. Solutions of 1.0×10^{-3} M α-n-propyl-, α-isopropyl-, and α-n-butyl-DL-cystine in 0.1 N hydrochloric acid were analyzed at thymol concentrations of 2.4×10^{-5}, 1.2×10^{-4}, 2.4×10^{-4}, and 4.8×10^{-4} M. The value of the apparent half-wave potential was determined (Table III). There were changes in the shapes of the polarographic waves (Figures 5-7).

Effect of Digestion on Polarographic Waves of Alpha Substituted Cystines

Samples of alpha substituted cystines were digested according to the method of Koch and McMeekin (49). A distilled water blank was treated similarly.

The alpha substituted cystine (5×10^{-5} moles) was transferred to a pyrex test tube, 5 ml. of distilled water, and 1 ml. of 1:1 sulfuric acid were added along with a glass bead. This was heated over a microburner to evaporate the water. When charring began and white fumes appeared in the tube, the size of the flame was reduced. Heating was continued until no further blackening occurred. The tube was cooled for approximately 1 minute, and then 1 drop of 30% hydrogen peroxide was added. The tube was heated to boiling. The above procedure was repeated

FIGURE 2

EFFECT OF pH ON APPARENT HALF-WAVE POTENTIAL OF α-n-PROPYL-DL-CYSTINE

Legend

The concentration of α-n-propyl-DL-cystine employed was 1.0×10^{-3} M. pH values represent final solutions to be analyzed.

1. pH 0.17
2. pH 1.32
3. pH 2.11
4. 3.07
5. 3.70

Buffers were prepared according to Clark and Lubs (46).

pH 0.17 (1 N HCl)

pH 1.32 (HCl,KCl)

pH 2.11 (HCl, KCl)

pH 3.07 (Potassium Acid Phthalate, HCl)

pH 3.70 (Potassium Acid Phthalate, HCl)

The thymol concentration employed was 4.8×10^{-4} M.

FIGURE 2

EFFECT OF pH ON APPARENT HALF-WAVE POTENTIAL OF α -n-PROPYL-DL-CYSTINE

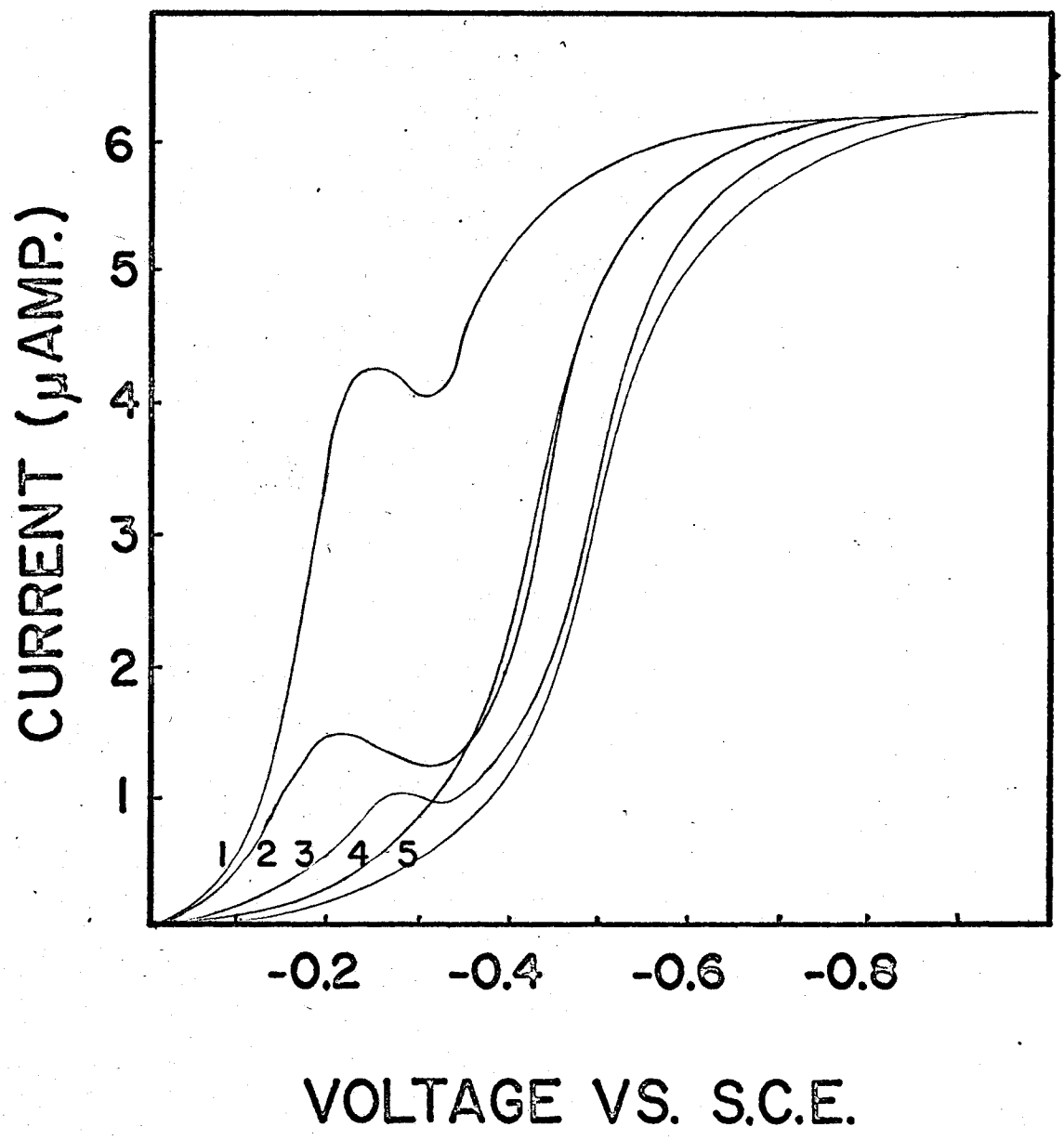


FIGURE 3

EFFECT OF pH ON APPARENT HALF-WAVE POTENTIAL OF α-ISOPROPYL-DL-CYSTINE

Legend

The concentration of α-isopropyl-DL-cystine employed was 1.0×10^{-3} M. pH values represent final solutions to be analyzed.

1. pH 0.16
2. pH 1.42
3. pH 1.92
4. pH 2.84
5. pH 3.40

Buffers were prepared according to Clark and Lubs (46).

pH 0.16 (1 N HCl)

pH 1.42 (HCl, KCl)

pH 1.92 (HCl, KCl)

pH 2.84 (Potassium Acid Phthalate, HCl)

pH 3.40 (Potassium Acid Phthalate, HCl)

The thymol concentration employed was 4.8×10^{-4} M.

FIGURE 3

EFFECT OF pH ON APPARENT HALF-WAVE POTENTIAL OF α-ISOPROPYL-DL-CYSTINE

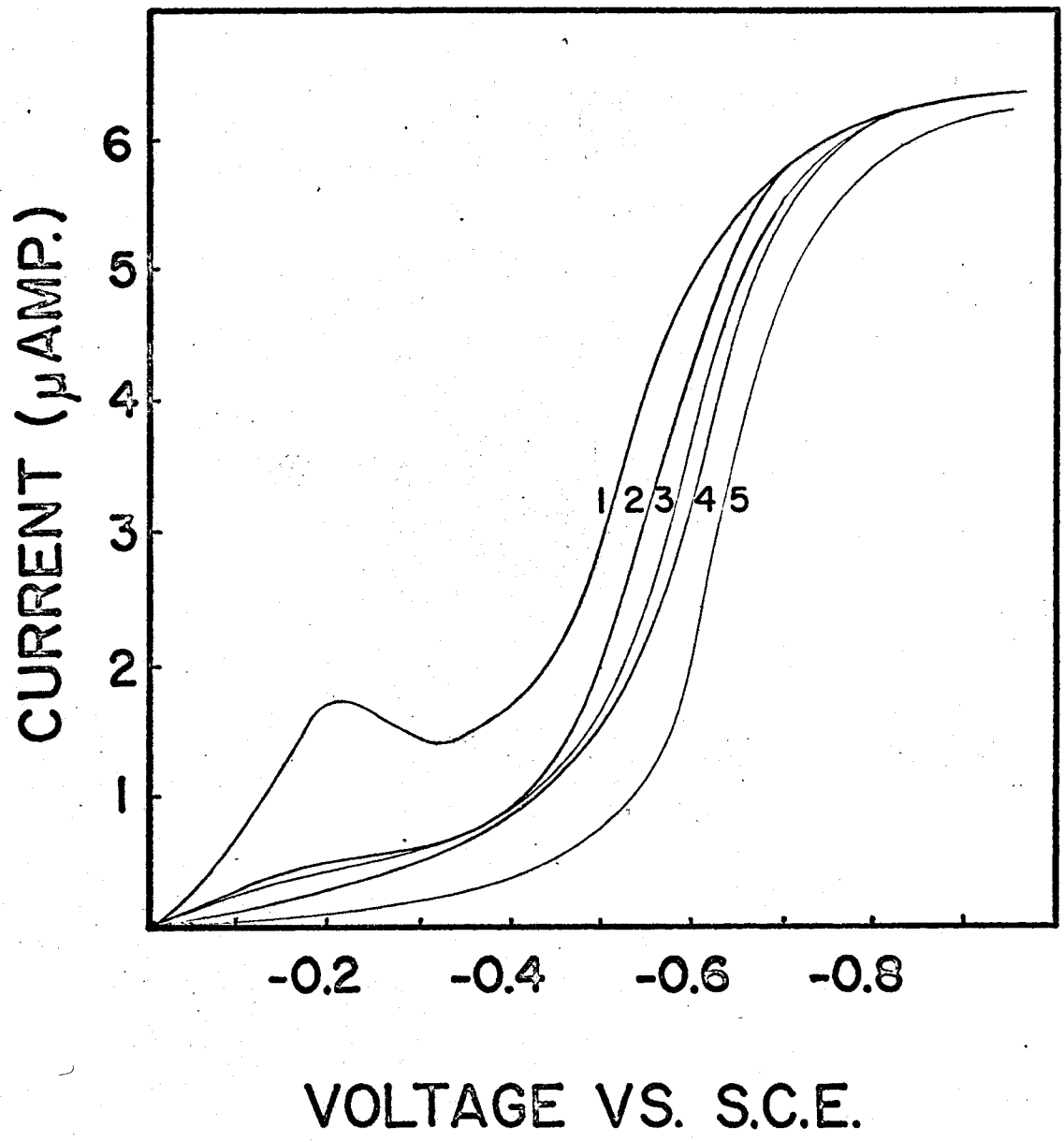


FIGURE 4

EFFECT OF pH ON APPARENT HALF-WAVE POTENTIAL OF α -n-BUTYL-DL-CYSTINE

Legend

The concentration of α -n-butyl-DL-cystine employed was 1.0×10^{-3} M. pH values represent final solutions to be analyzed.

1. pH 0.12
2. pH 1.34

Buffers were prepared according to Clark and Lubs (46).

pH 0.12 (1 N HCl)

pH 1.34 (HCl, KCl)

The thymol concentration employed was 4.8×10^{-4} M.

FIGURE 4

EFFECT OF pH ON APPARENT HALF-WAVE POTENTIAL OF α-n-BUTYL-DL-CYSTINE

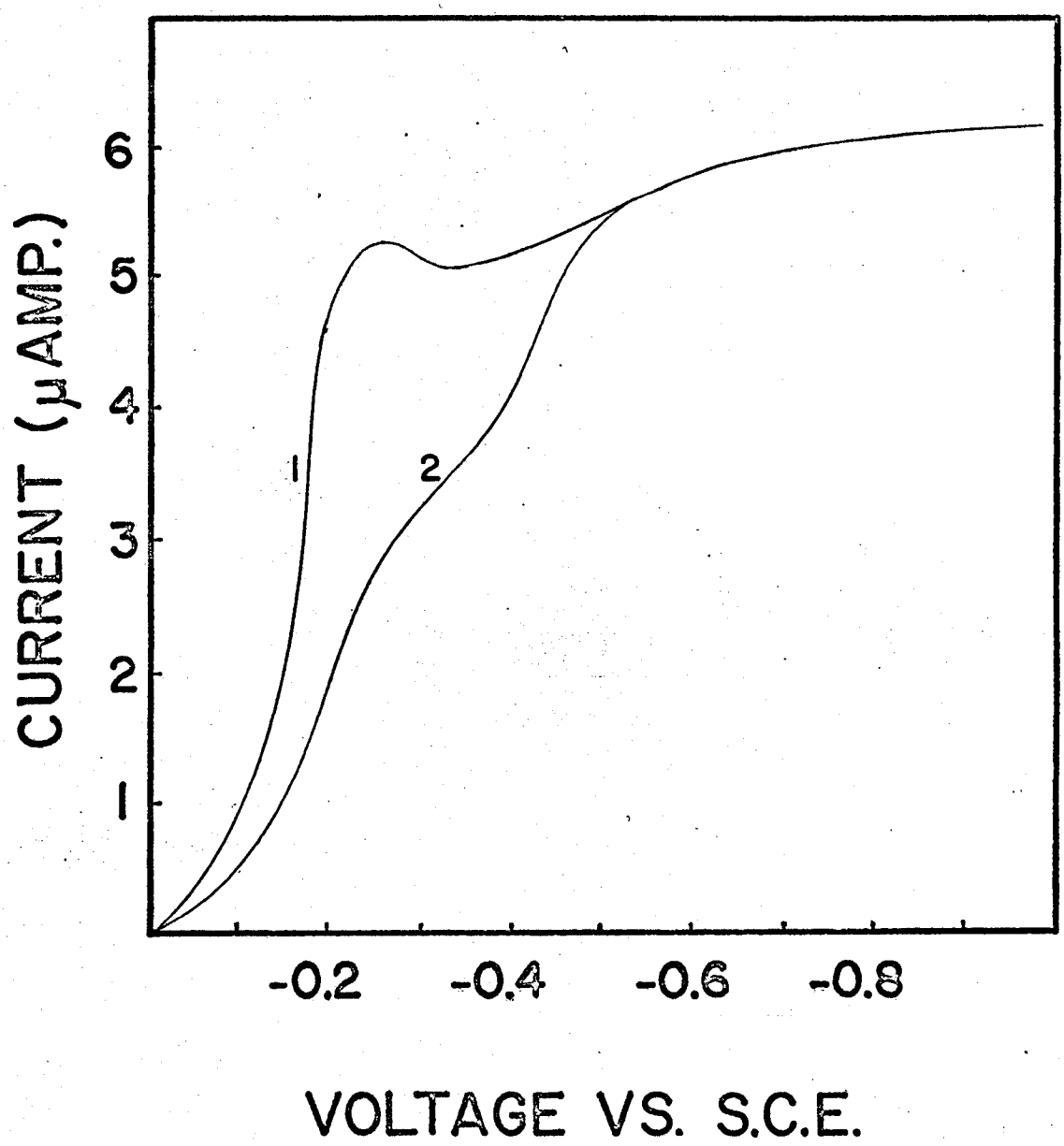


TABLE III

EFFECT OF THYMOL CONCENTRATION ON APPARENT HALF-WAVE POTENTIAL

Amino Acid*	Thymol Concentration (Mole/Liter)	Apparent Half-Wave Potential (Volt)
α-n-Propyl-	2.4×10^{-5}	-0.192
DL-cystine	1.2×10^{-4}	-0.194
	2.4×10^{-4}	-0.198
	4.8×10^{-4}	-0.478
α-Isopropyl-	2.4×10^{-5}	-0.258
DL-cystine	1.2×10^{-4}	-0.354
	2.4×10^{-4}	-0.450
	4.8×10^{-4}	-0.544
α-n-Butyl-	2.4×10^{-5}	Maximum observed
DL-cystine	1.2×10^{-4}	-0.196
	2.4×10^{-4}	-0.205
	4.8×10^{-4}	-0.314

* The amino acid concentration employed was 1.0×10^{-3} M.

FIGURE 5

EFFECT OF THYMOL CONCENTRATION ON SHAPE OF POLAROGRAM FOR ∝-n-PROPYL-DL-CYSTINE

Legend

A concentration of 1.0×10^{-3} M ∝-n-propyl-DL-cystine was employed.

1. 2.4×10^{-5} M
2. 1.2×10^{-4} M
3. 2.4×10^{-4} M
4. 4.8×10^{-4} M

The amino acid was dissolved in 0.1 N hydrochloric acid.

FIGURE 5

EFFECT OF THYMOL CONCENTRATION ON SHAPE OF POLAROGRAM FOR α -n-PROPYL-DL-CYSTINE

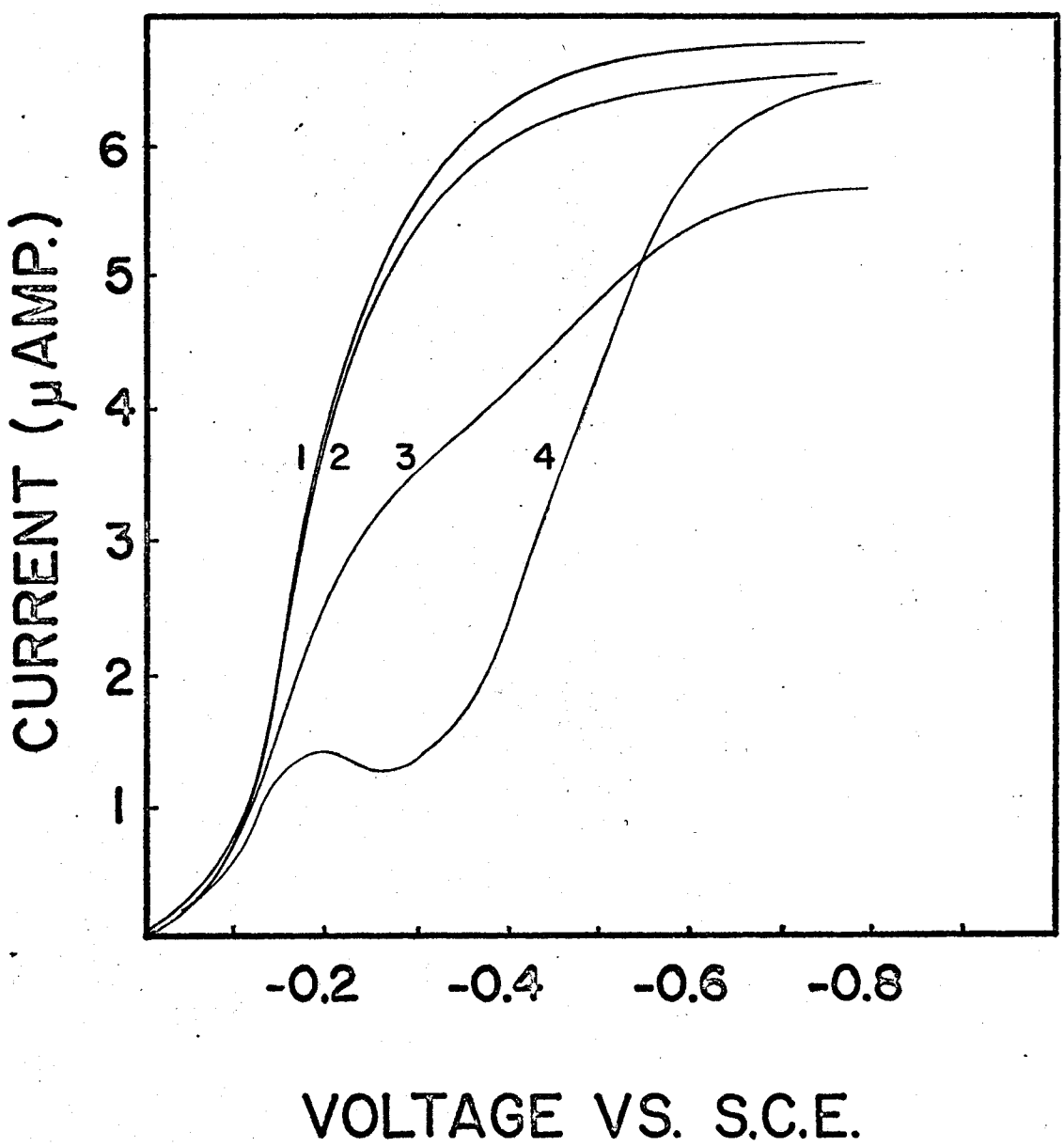


FIGURE 6

EFFECT OF THYMOL CONCENTRATION ON SHAPE OF POLAROGRAM FOR α -ISOPROPYL-DL-CYSTINE

Legend

A concentration of 1.0×10^{-3} M α -isopropyl-DL-cystine was employed.

1. 2.4×10^{-5} M
2. 1.2×10^{-4} M
3. 2.4×10^{-4} M
4. 4.8×10^{-4} M

The amino acid was dissolved in 0.1 N hydrochloric acid.

FIGURE 6

EFFECT OF THYMOL CONCENTRATION ON SHAPE OF POLAROGRAM FOR α-ISOPROPYL-DL-CYSTINE

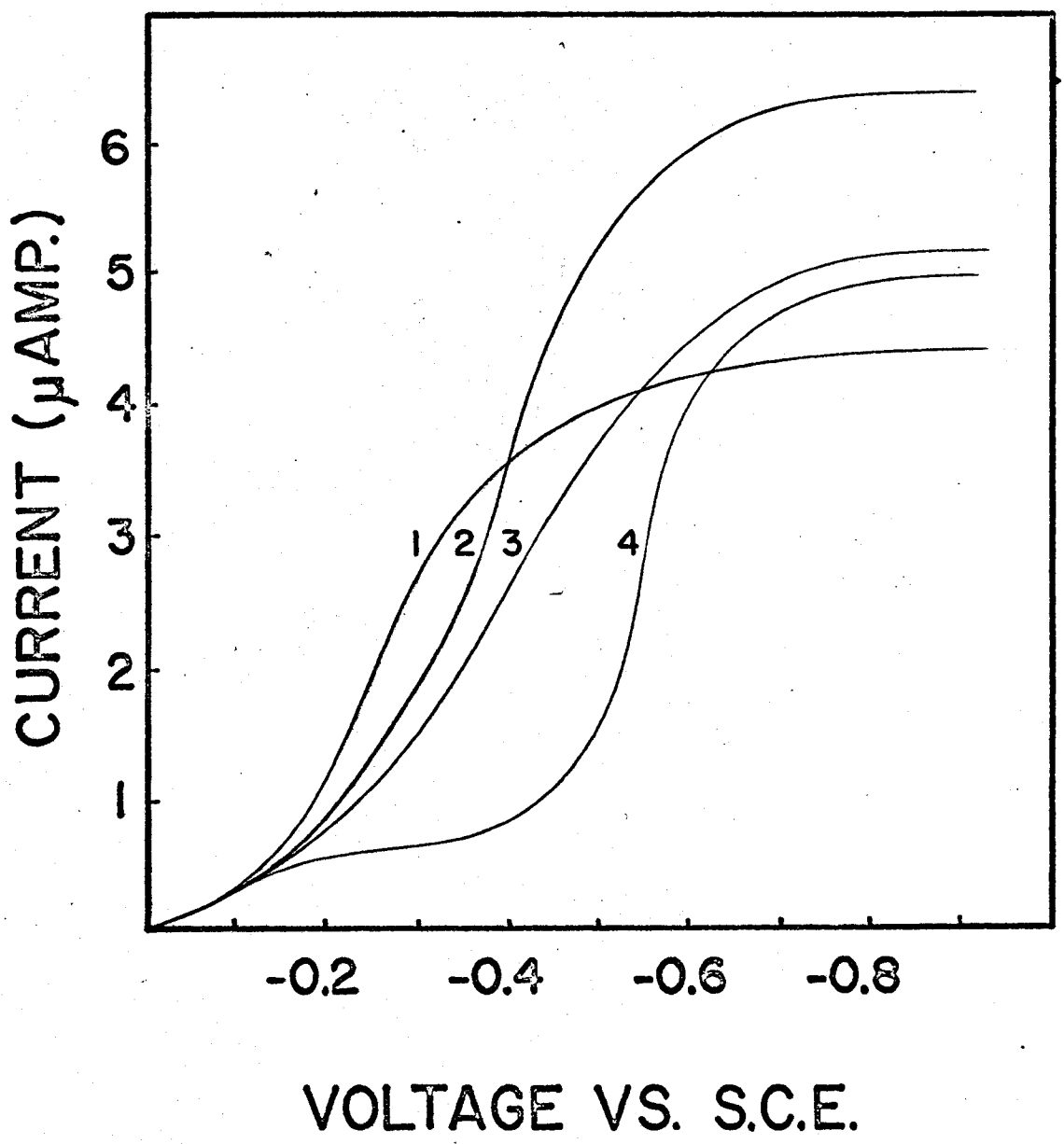


FIGURE 7

EFFECT OF THYMOL CONCENTRATION ON SHAPE OF POLAROGRAM FOR α -n-BUTYL-DL-CYSTINE

Legend

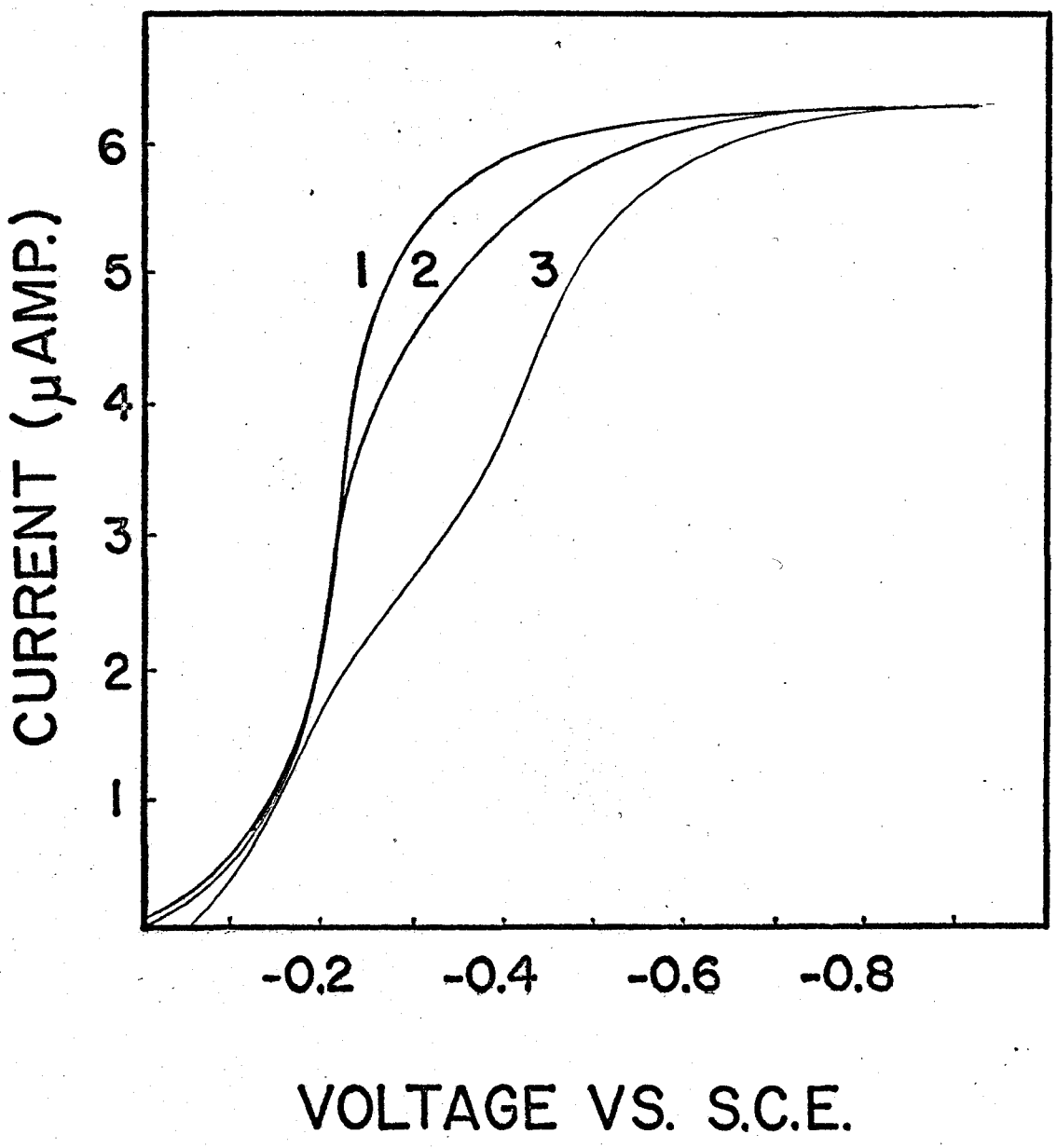
A concentration of 1.0×10^{-3} M α -n-butyl-DL-cystine was employed.

1. 1.2×10^{-4} M
2. 2.4×10^{-4} M
3. 4.8×10^{-4} M

The amino acid was dissolved in 0.1 N hydrochloric acid.

FIGURE 7

EFFECT OF THYMOL CONCENTRATION ON SHAPE OF POLAROGRAM FOR α-n-BUTYL-DL-CYSTINE



until the solution was decolorized. Finally the mixture was boiled gently for 5 minutes. It was cooled and transferred to a 50-ml. volumetric flask, 5 ml. of 1.2×10^{-3} M thymol were added and diluted to the mark with 0.1 N hydrochloric acid.

Polarographic analysis of the digests in 0.1 N hydrochloric acid using 1.2×10^{-4} M thymol revealed that the characteristic waves were absent in both blanks and samples. This was taken as evidence that no substance present in the reagents was responsible for the waves usually obtained with the alpha substituted cystines.

Diffusion Coefficient and Reversibility of the Reaction

By substitution in the Ilkovic equation, the diffusion coefficients for L-cystine, α -ethyl-, α -n-propyl-, α -isopropyl-, and α -n-butyl-DL-cystine were found using 1.2×10^{-4} M thymol as a maximum suppressor and 0.1 N hydrochloric acid as supporting electrolyte (Table IV).

$$i_D = 607n\, D^{1/2}\, C\, m^{2/3} t^{1/6}$$

Where i_D = diffusion current in microamperes

n = number of faradays of electricity required per mole of the electrode reaction (n = 2 for cystines)

D = diffusion coefficient of the reducible or oxidizable substance in the units $cm.^2 sec.^{-1}$

C = concentration in millimoles per liter

m = rate of flow of mercury from the dropping electrode capillary in the units $mg.sec.^{-1}$

t = drop time in seconds

The reversibility of the reactions was tested by plotting E versus

TABLE IV

DIFFUSION COEFFICIENT

Amino Acid	Diffusion Current (μamp.)	Diffusion Coefficient ($cm.^2 sec.^{-1} \times 10^6$)
L-Cystine	6.06	5.70
α -Ethyl-DL-cystine	3.99	3.27
α -n-Propyl-DL-cystine	5.60	4.87
α -Isopropyl-DL-cystine	5.06	3.98
α -n-Butyl-DL-cystine	4.80	3.58

The amino acid concentration was 1.0×10^{-3} M except for α -ethyl-DL-cystine which was 8.7×10^{-4} M.

$\log (i_D - i)/i^2$ for L-cystine, α-ethyl-, α-n-propyl-, α-isopropyl-, α-n-butyl-, and α-phenyl-DL-cystine using 1.2×10^{-4} M thymol and 0.1 N hydrochloric acid (Table V). Straight lines were obtained except for α-n-butyl-DL-cystine (Figure 8). The slopes of these plots are given in Table VI.

Half-Wave Potential

The half-wave potentials ($E_{\frac{1}{2}}$) of L-cystine, α-methyl-, α-ethyl-, α-n-propyl-, α-isopropyl-, α-n-butyl-, and α-phenyl-DL-cystine were determined in 0.1 N hydrochloric acid using 1.2×10^{-4} M thymol (Table VII).

Corrections were made for residual current and voltage drop across the cell. The half-wave potentials were determined from a plot of E versus $\log (i_D - i)/i$ (50).

B. Titration Curves of Some Alpha Substituted Cystines

Titration curves were obtained for L-cystine, α-methyl-, α-ethyl-, α-n-propyl-, α-isopropyl-, α-n-butyl-, and α-phenyl-DL-cystine. A typical titration curve for an alpha substituted cystine is given in Figure 9. Values for the pH at the two inflection points in the alkaline region of the titration curves are given in Table VIII.

TABLE V

VALUES OF E AND $\log (i_D - i)/i^2$

Amino Acid	E (Volt)	$\log (i_D - i)/i^2$
L-Cystine	-0.500	0.113
(1.0×10^{-3} M)	-0.520	-0.216
	-0.540	-0.529
	-0.560	-0.827
	-0.580	-1.113
	-0.600	-1.402
α -Ethyl-DL-cystine	-0.160	0.800
(8.7×10^{-4} M)	-0.180	0.407
	-0.196	0.169
	-0.220	-0.080
	-0.240	-0.217
	-0.260	-0.355
	-0.280	-0.458
	-0.296	-0.527
	-0.320	-0.623
	-0.340	-0.713
	-0.360	-0.800

TABLE V (Continued)

VALUES OF E AND $\log (i_D - i)/i^2$

Amino Acid	E (Volt)	$\log (i_D - i)/i^2$
α -n-Propyl-DL-cystine	-0.140	0.796
(1.0×10^{-3} M)	-0.160	0.214
	-0.170	-0.046
	-0.180	-0.285
	-0.190	-0.521
	-0.200	-0.739
	-0.210	-0.937
α -Isopropyl-DL-cystine	-0.280	0.371
(1.0×10^{-3} M)	-0.320	0.015
	-0.340	-0.145
	-0.360	-0.315
	-0.380	-0.475
	-0.400	-0.635

TABLE V (Continued)

VALUES OF E AND $\log (i_D - i)/i^2$

Amino Acid	E (Volt)	$\log (i_D - i)/i^2$
α -n-Butyl-DL-cystine	-0.140	1.165
(1.0×10^{-3} M)	-0.160	0.561
	-0.180	0.057
	-0.200	-0.326
	-0.220	-0.667
	-0.240	-0.911
	-0.260	-1.127
α -Phenyl-DL-cystine	-0.140	1.256
(Saturated solution)	-0.160	0.579
	-0.180	0.017
	-0.196	-0.446
	-0.220	-1.067
	-0.240	-1.471

FIGURE 8

PLOT OF $\log (i_D - i)/i^2$ VS. E

Legend

Data for these plots were taken from current-voltage curves corrected for residual current. Amino acid concentrations were 1.0×10^{-3} M except for α-ethyl-, and α-phenyl-DL-cystine which were 8.7×10^{-4} M and saturated, respectively. Plots are given for: L-cystine (●), α-ethyl- (△), α-n-propyl- (□), α-isopropyl- (■), α-n-butyl- (○), and α-phenyl-DL-cystine (●).

The amino acids were dissolved in 0.1 N hydrochloric acid and 1.2×10^{-4} M thymol was used.

FIGURE 8

PLOT OF $\log (i_D - i)/i^2$ VS. E

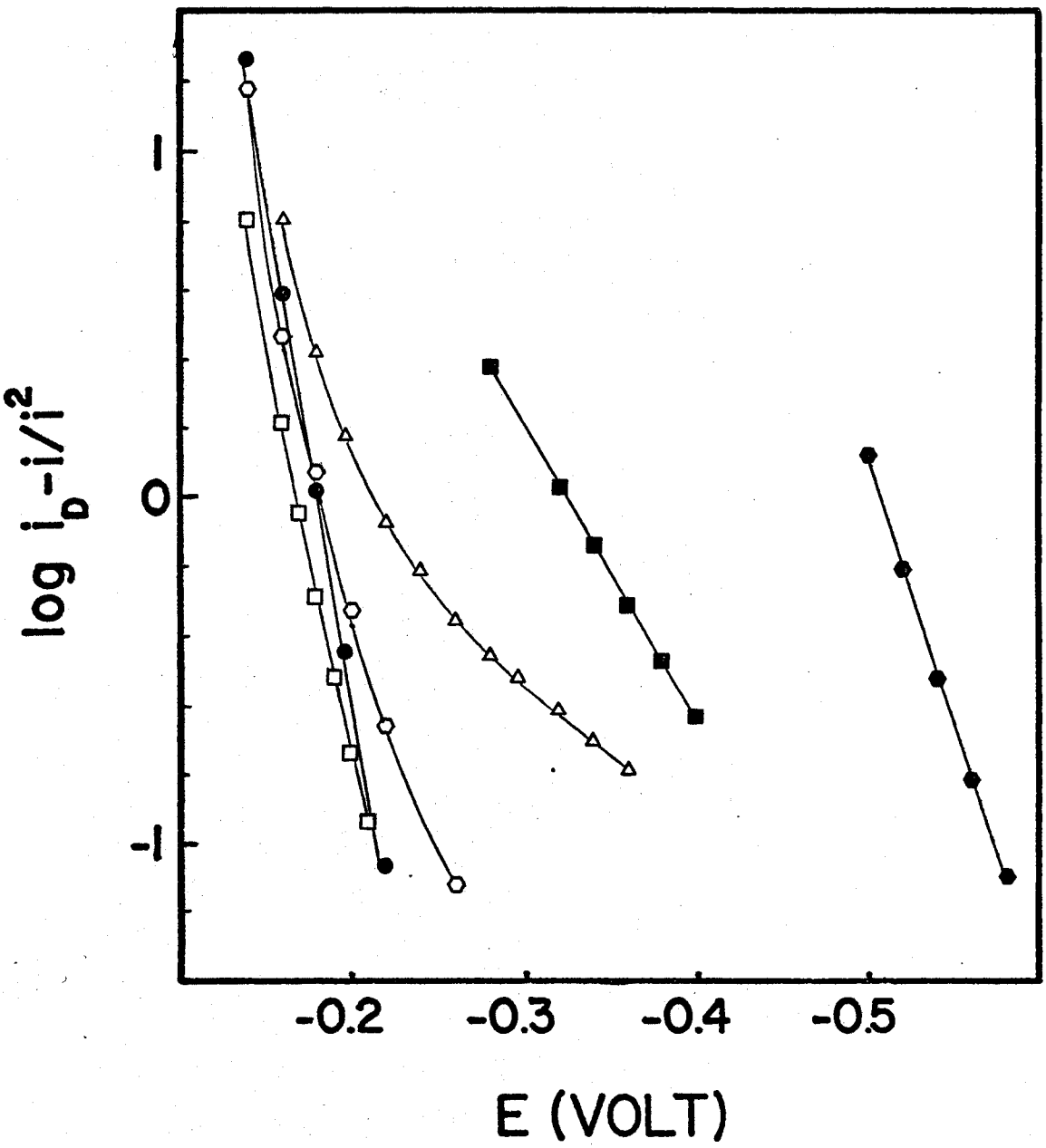


TABLE VI

SLOPE OF E VS. log $(i_D - i)/i^2$ PLOT

Amino Acid	Slope
L-Cystine	0.067
α-Ethyl-DL-cystine	0.236
α-n-Propyl-DL-cystine	0.044
α-Isopropyl-DL-cystine	0.125
α-Phenyl-DL-cystine	0.035

TABLE VII

HALF-WAVE POTENTIAL

Amino Acid*	Half-Wave Potential (Volt)
L-Cystine	-0.5361
α-Methyl-DL-cystine	-0.5142
α-Ethyl-DL-cystine[a]	-0.2492
α-n-Propyl-DL-cystine	-0.1865
α-Isopropyl-DL-cystine	-0.3703
α-n-Butyl-DL-cystine	-0.2024
α-Phenyl-DL-cystine[b]	-0.1841

* The amino acid concentration employed was 1.0×10^{-3} M unless otherwise specified.

[a] 8.7×10^{-4} M.

[b] Saturated solution.

FIGURE 9

TYPICAL TITRATION CURVE

Legend

Samples (2.5×10^{-5} moles) of amino acid were dissolved in 1 ml. of 0.1 N carbonate-free sodium hydroxide and 4 ml. of 0.1 M potassium chloride and titrated with 0.1000 N hydrochloric acid.

FIGURE 9

TYPICAL TITRATION CURVE

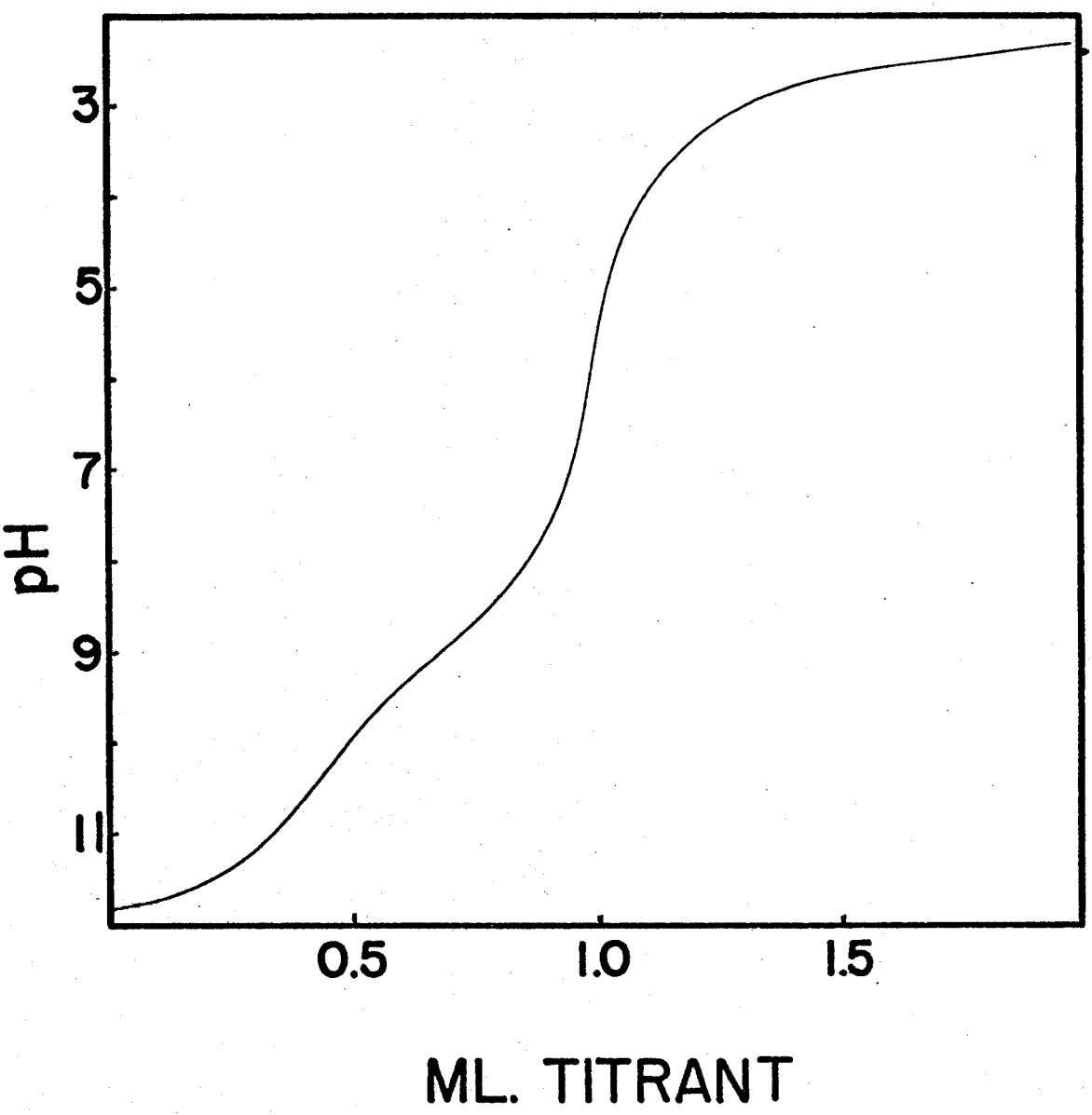


TABLE VIII

pH VALUES AT INFLECTION POINTS

Amino Acid	pH at First Inflection Point	pH at Second Inflection Point
L-Cystine	10.25	8.68
α -Methyl-DL-cystine	10.44	9.08
α -Ethyl-DL-cystine	10.33	8.62
α -n-Propyl-DL-cystine	10.32	8.63
α -Isopropyl-DL-cystine	10.38	8.67
α -n-Butyl-DL-cystine	10.48	8.78
α -Phenyl-DL-cystine	9.89	8.19

CHAPTER IV

DISCUSSION

A. Polarographic Investigation of Some Alpha Substituted Cystines

The recent syntheses of a number of alpha substituted cystines in this laboratory (47) have made it desirable to obtain a method for their quantitative determination. A linear relationship of the diffusion current to the concentration of alpha substituted cystine was observed for all the cystines investigated using thymol as the maximum suppressor and 0.1 N hydrochloric acid as solvent and supporting electrolyte. Thus a simple and rapid polarographic method is feasible for their determination.

Since the electrode reaction is pH dependent, it was important to study the effect of pH on the half-wave potential and the shape of the polarographic wave. It was observed that as the pH was increased the more nearly S-shaped the polarograms became (Figures 2-4). The polarograms had different shapes (Figures 2-4), and the apparent half-wave potential was observed to increase with increase in pH with the exception of α-n-propyl-DL-cystine. This increase in apparent half-wave potential with increase in pH is to be expected since as the pH increases, the amino acid molecule assumes an increasing negative charge. Thus the reduction at the dropping mercury electrode should become increasingly difficult as the degree of negative charge on the amino acid molecule is increased. It was also noted that the magnitude of the prewave

decreased as the pH increased.

The value of the apparent half-wave potential increased as the thymol concentration increased (Figures 5-7). In order to explain the shift of the cystine waves to more negative potentials Kolthoff and Barnum (1) postulated that cystine has to be oriented (adsorbed) in a favourable position at the surface of the dropping mercury electrode to be reduced. Apparently capillary-active substances counteract or prevent the orientation (adsorption) of cystine at the interface and thus displace the waves to more negative potentials. These same postulates should be applicable to the case of the alpha substituted cystines. Thymol concentration also affected the shape of the polarograms. In general, as the thymol concentration increased, the magnitude of the prewave also increased. Thus the appearance of a prewave seems to be associated with the thymol concentration. Also, an increase in thymol concentration decreased the wave height in the case of α-n-propyl- and α-isopropyl-DL-cystine but had very little effect on that of α-n-butyl-DL-cystine.

Polarographic analyses of solutions of supporting electrolyte (0.1 N hydrochloric acid and thymol) as well as those of the digests revealed that no substance present in the reagents was responsible for the waves usually obtained with the alpha substituted cystines.

In order to determine whether the reduction of the alpha substituted cystines was reversible, plots of E vs. $\log (i_D - i)/i^2$ were made. With the exception of α-n-butyl-DL-cystine, straight lines were obtained with slopes ranging from 0.035 to 0.236 (Figure 8 and Table VI). These values differ from the theoretical slope (0.0296). In this respect the results are similar to those of Kolthoff and Barnum (1), and Kalousek,

Grubner, and Tochstein (38) who concluded that the cystine reduction was not reversible.

By substitution in the Ilkovic equation, the diffusion coefficients for the compounds were found (Table IV). The value of 5.70×10^{-6} $cm.^2 sec.^{-1}$ obtained for cystine was close to that obtained by Kolthoff and Barnum (1) (5.3×10^{-6} $cm.^2 sec.^{-1}$). The values for the substituted cystines were found to be less than that of cystine due in part to the greater bulk effect of the molecule.

The effect of substituents on the half-wave potential of alpha substituted cystines can be seen in Table VII. Zuman (45) has shown that there are stringent experimental conditions necessary for obtaining comparable data if a quantitative treatment is desired. These experimental conditions are summarized as follows:

a) The compared values of half-wave potentials are to be measured under exactly the same experimental conditions. For the interpretation of results described in the literature usually only the results obtained by one author are to be compared. In the future it would be desirable (in case polarographic measurements should be planned as an extension of a previous work) to choose the experimental conditions as close as possible to the previous ones, in order to compare the new and the old in one reaction series.

b) The mechanism should be similar for all systems under comparison. Because the reduction products and the mechanism are not usually determined for all members of the reaction series, the following conditions must be fulfilled:

(i) The height of all compared diffusion currents ought to correspond to the same number of electrons transferred.

(ii) The shape of all compared waves should be nearly the same.

(iii) The number of protons transferred during the electrode process must be the same. It is preferable to compare the half-wave potentials under conditions where they are independent of pH. If such a pH range is inaccessible experimentally, one must choose such a pH range where the slope $dE_{\frac{1}{2}}/dpH$ is approximately the same. The choice of an arbitrary pH value for the comparison (like pH 0 or pH 7) is unfounded and sometimes leads to erroneous conclusions about the influence of the substituent.

c) For the verification of some of the mentioned relations, compounds are to be chosen to cover the polar, steric. and resonance effects of substituents in the reaction series given in as wide a range as possible.

The presence of the carboxyl and amine groups further complicates the prediction of the shift in half-wave potential of the various alpha substituted cystines. Attempts to correlate the Lewis acid strength of compounds with the shift in half-wave potential have been reported. Karchmer and Walker (44) in studies on disulfides have shown that, in basic medium, the greater the Lewis acid strength of the disulfide, the lower the half-wave potential. This is also consistent with the thermal stabilities of the disulfides.

This complication could be removed if the dissociation constants of the amino acids were known. It would then be possible, by adjusting the pH, to obtain an amino acid molecule with a certain charge. This could then be done to all the amino acids. Then a species with the same charge would be presented to the dropping mercury electrode. In this case, only the polar and steric effects of the substituent groups should be operative. However, the removal of this complicating factor does not

solve the problem since it has already been shown that one must select a pH range such that $dE_{\frac{1}{2}}/dpH$ is approximately the same for all the compounds. Therefore, only if there is agreement between the pH chosen to obtain the amino acid in the desired form and the pH range chosen so that $dE_{\frac{1}{2}}/dpH$ is approximately the same, can the shift in half-wave potential be predicted.

Thus it is not possible at this time to explain the shift in half-wave potential in terms of the total polar substituent constant (σ) and the total steric substituent constant (E_s) as given by Zuman (45). However, on a qualitative basis it can be seen that in a system consisting of 0.1 N hydrochloric acid as supporting electrolyte and 1.2×10^{-4} M thymol as maximum suppressor, the half-wave potentials of all the substituted cystines were lower than that of L-cystine. In other words, the replacement of the hydrogen on the alpha carbon of cystine with various hydrocarbon groups always resulted in a lowering of the half-wave potential.

B. Titration Curves of Some Alpha Substituted Cystines

Since the substituent groups exert varying effects it was felt that these effects should be manifested by changes in the pK values of the amine and carboxylic acid groups of the alpha substituted cystines.

Titration curves were obtained for the amino acids by dissolving them in 1 ml. of 0.1 N hydrochloric acid and 4 ml. of 0.1 M potassium chloride and titrating with 0.1 N sodium hydroxide, but these curves did not exhibit usable inflection points due to the small amount of amino acid in solution which afforded little buffering. Thus it was decided to dissolve them in sodium hydroxide and titrate with hydro-

chloric acid. The amino acids proved to be much more soluble in base than in acid thereby making it possible to obtain suitable titration curves.

The four equilibrium expressions for an alpha substituted cystine are:

$$K_1 = \frac{\gamma_{\pm+}(R^{\pm+})(H^+)}{\gamma_{++}(R^{++})} \qquad K_2 = \frac{(R^{\pm\pm})(H^+)}{\gamma_{\pm+}(R^{\pm+})} \qquad K_3 = \frac{\gamma_{\pm-}(R^{\pm-})(H^+)}{(R^{\pm\pm})}$$

$$K_4 = \frac{\gamma_{=}(R^{=})(H^+)}{\gamma_{\pm-}(R^{\pm-})}$$

Where K = dissociation constant

γ = activity coefficient

(R) = amino acid concentration in moles per liter

(H^+) = hydrogen ion concentration in moles per liter

Cannan and Knight (51), Sano (52), Greenstein et al., and Borsook et al. (41) found that in the case of cystine, the two acid dissociation constants as well as the two basic ones were found to overlap. Van Slyke (53) has shown that the apparent dissociation constants (pK') of two buffer acids must be at least 2.5 units apart in order that the buffer effect of each may be less than 1 per cent that of the latter's maximum. Borsook et al. (41) have calculated the four pK values for cystine and their data is regarded as the most reliable. In order to calculate the pK values extensive solubility data is required. This data was not yet available for the substituted cystines; hence only relative values for the pH at the inflection points could be determined

from the titration curves. It was impossible to determine inflection points in the acid region of the titration curves since no points of inflection were apparent upon inspection. Two points of inflection were found in the alkaline region of the titration curve. As can be seen from Table VIII, the pH values at the inflection points for the substituted cystines do not differ greatly from that of L-cystine except for α-phenyl-DL-cystine.

CHAPTER V

SUMMARY AND CONCLUSIONS

The analyses of the current-voltage curves show that the reduction of α -ethyl-, α -n-propyl-, α -isopropyl-, α -n-butyl-, and α -phenyl-DL-cystine does not occur reversibly. The half-wave potentials and shapes of the waves of α -n-propyl-, α -isopropyl-, and α -n-butyl-DL-cystine were affected by pH and by thymol concentration. The diffusion current was found to be proportional to the concentration for all the compounds studied. All the compounds studied except α -phenyl-DL-cystine may be determined polarographically within the concentration range of 2×10^{-3} to 5×10^{-4} M using thymol as the maximum suppressor and 0.1 N hydrochloric acid as supporting electrolyte. Due to the limited solubility of α -phenyl-DL-cystine in 0.1 N hydrochloric acid the analytically useful concentration range was 5×10^{-4} to 1.25×10^{-4} M using 1.2×10^{-4} M thymol. The substituent groups in the alpha position affected the shape of the polarogram, the magnitude of the diffusion current, the diffusion coefficient, the behaviour of the compound under varying conditions of pH and thymol concentration as well as the half-wave potential.

The large difference in half-wave potential between some of the compounds studied should make possible a simultaneous determination.

The substituent groups also affect the titration curves of the alpha substituted cystines. However, an accurate determination of the

dissociation constants was not possible at this time.

BIBLIOGRAPHY

1. Kolthoff, I.M., and Barnum, D., J. Am. Chem. Soc., 63, 520 (1941).

2. Thibert, R.J., and Ottenbrite, R.M., Anal. Chem., 32, 106 (1960).

3. Krebs, H.A., Biochem. Z., 204, 322 (1929).

4. Rosenthal, S.M., and Voegtlin, C., U.S. Pub. Health Repts., 48, 347 (1933).

5. Baumann, E., and Preusse, C., Z. physiol. Chem., 5, 309 (1881).

6. Greenstein, J.P., and Winitz, M., Chemistry of the Amino Acids, Vol. 1, John Wiley and Sons, Inc., New York, 1961, p.441.

7. Bachmann, G.B., Organic Chemistry, McGraw-Hill Company, Toronto, 1949, p. 148.

8. Christensen, H., and Riggs, T., J. Biol. Chem., 22, 265 (1956).

9. Kolthoff, I.M., and Stricks, W., J. Am. Chem. Soc., 73, 1723 (1951).

10. Barron, E.S.G., Advances in Enzymology, 2, 201 (1951).

11. Martell, A., and Calvin, M., Chemistry of the Metal Chelate Compounds, Prentice-Hall Inc., New Jersey, 1956, p. 281.

12. Fruton, J.S., and Simmons, S., General Biochemistry, 2nd Edition, John Wiley and Sons, Inc., New York, 1960, p. 58.

13. Block, K., and Anker, H.S., J. Biol. Chem., 169, 765 (1947).

14. Harington, C.R., and Mead, H.T., Biochem. J., 29, 1602 (1935).

15. Dixon, M., and Webb, E.C., Enzymes, Academic Press Inc., New York, 1958, p. 408.

16. Rapkine, L., Compt. rend. soc. biol., 112, 790 (1933); J. chim. phys., 33, 493 (1936).

17. Dickens, F., Nature, 131, 130 (1933); Biochem. J., 27, 1141 (1933).

18. Ogilvie, J.W., Tildon, J.T., and Strauch, B.S., Biochem., 3, 754 (1964).

19. West, E.D., and Todd, W.R., Textbook of Biochemistry, 2nd Edition, Macmillan Co., New York, 1957, p. 1117-30.

20. Cantarow, A., and Schepartz, B., Biochemistry, Saunders Company, Philadelphia, 1954, p. 283.

21. Barron, E.S.G., Advances in Enzymology, 11, 201 (1951).

22. Sumner, J.B., and Myrbach, K., The Enzymes, Vol. 1, Part 1, Academic Press Inc., New York, 1951, p. 327.

23. Rapkine, L., J. chim. phys., 34, 416 (1937).

24. Umbreit, W.W., Symposium on Amino Acid Metabolism, Rahway, N.J., 1955, p. 48-62.

25. Barron, E.S.G., Ann. N.Y. Acad. Sci., 59, 574 (1955).

26. Patt, H.M., Tyree, E.B., Stranbe, R.L., and Smith, D.E., Science, 110, 213 (1949).

27. Chapman, W.H., et al., Project NM 006012.08.25, Naval Medical Institute, Bethesda, Md., Sept. 1949.

28. Eldjarn, L., Bremer, J., and Borresem, H.C., Biochem. J., 82, 192 (1962).

29. Doherty, D.G., Burnett, W.T., and Shapiro, R., Radiation Res., 7, 13 (1957).

30. Jones, M.M., Nature, 185, 96 (1959).

31. Kalkwarf, D.R., Nucleonics, 18, No.5, 76 (1960).

32. Kalkwarf, D.R., Nucleonics, 18, No 5, 130 (1960).

33. Smythe, C.V., J. Biol. Chem., 142, 387 (1942).

34. Lin, K.H., Li, L., and Huang, Y.T., Chinese Chem. Soc., 15, 31 (1947).

35. Chinard, F.P., and Hellerman, L., Methods of Biochem. Anal., 1, 1 (1954).

36. Snell, F.D., and Snell, C.T., Colorimetric Methods of Analysis, 3rd ed., Vol. III, Van Nostrand, New York, 1953, p. 478.

37. Roncato, A., Arch. sci. biol. (Italy), 20, 146 (1934).

38. Kalousek, M., Grubner, O., and Tochstein, A., Chem. listy, 47, 1143 (1953)

39. Brdicka, R., Collection Czech. Chem. Comm., 5, 238 (1933).

40. Muller, O.H., Chem. Revs., 24, 95 (1939).

41. Borsook, H., Ellis, E.L., and Huffman, H.M., J. Biol. Chem., 117, 281 (1937).

42. Hall, M.E., Anal. Chem., 25, 556 (1953).

43. Obolentsev, R.D., Gavrilova, L.D., and Bulatova, B.T., C.A., 63, 1627g (1965).

44. Karchmer, J.H., and Walker, M.T., Anal. Chem., 26, 271 (1954).

45. Zuman, P., Ricerca sci., 30, Suppl. No. 5, 229 (1960).

46. Hawk, P.B., Oser, B.L., and Summerson, W.H., Practical Physiological Chemistry, 13th ed., McGraw-Hill, New York, 1954, p. 35.

47. Thibert, R.J., Diederich, J.F.G., and Rutherford, K.G., Can. J. Chem., 43, 206 (1965).

48. Willard, H.H., Merritt, L.L., and Dean, J.A., Instrumental Methods of Analysis, 3rd ed., D. Van Nostrand Co. (Canada), Ltd., Toronto, 1958, p. 544.

49. Hawk, P.B., Oser, B.L., and Summerson, W.H., Practical Physiological Chemistry, 13th ed., McGraw-Hill, New York, 1954, p. 547.

50. Taylor, J.K., and Smith, S.W., J. Res. Nat. Bur. Std., 56, 143 (1956).

51. Cannan, R.K., and Knight, B.C.J.G., Biochem. J., 21, 1384 (1927).

52. Sano, K., Biochem. Z., 168, 14 (1926).

53. Van Slyke, D.D., J. Biol. Chem., 52, 525 (1922).

VITA AUCTORIS

Born:

August 13, 1940; Kitchener, Ontario, Canada.
Son of Mr. and Mrs. K.F. Walton.

Primary School:

St. Theresa, Windsor, Ontario, Canada.

Secondary School:

Port Arthur Collegiate Institute, Port Arthur, Ontario, Canada.

University:

University of Windsor, Windsor, Ontario, Canada.
Degree: B. Sc. in Chemistry - 1962.

Awards:

Province of Ontario Graduate Fellowship

Professional Societies:

Chemical Institute of Canada