36467

National Library of Canada | Bibliothèque nationale du Canada

CANADIAN THESES ON MICROFICHE | THÈSES CANADIENNES SUR MICROFICHE

NAME OF AUTHOR/NOM DE L'AUTEUR Ralf Sauter

TITLE OF THESIS/TITRE DE LA THÈSE Determination of Background-Active Surface-Containing Compounds by High Performance Liquid Chromatography with a Mercury-Based Electrochemical Detector

UNIVERSITY/UNIVERSITÉ University of Alberta

DEGREE FOR WHICH THESIS WAS PRESENTED/GRADE POUR LEQUEL CETTE THÈSE FUT PRÉSENTÉE Doctor of Philosophy

YEAR THIS DEGREE CONFERRED/ANNÉE D'OBTENTION DE CE GRADE 1978

NAME OF SUPERVISOR/NOM DU DIRECTEUR DE THÈSE Dr. Dallas L. Rabenstein

Permission is hereby granted to the NATIONAL LIBRARY OF CANADA to microfilm this thesis and to lend or sell copies of the film.

The author reserves other publication rights, and neither the thesis nor extensive extracts from it may be printed or otherwise reproduced without the author's written permission.

L'autorisation est, par la présente, accordée à la BIBLIOTHÈQUE NATIONALE DU CANADA de microfilmer cette thèse et de prêter ou de vendre des exemplaires du film.

L'auteur se réserve les autres droits de publication; ni la thèse ni de longs extraits de celle-ci ne doivent être imprimés ou autrement reproduits sans l'autorisation écrite de l'auteur.

DATED/DATÉ March 1st, 1978 SIGNED/SIGNÉ Ralf Sauter

PERMANENT ADDRESS/RÉSIDENCE FIXÉ #19, 11603 79 Ave, Edmonton, Alberta T6G 0P7

NL-91 (3-74)

NOTICE

The quality of this microfiche is heavily dependent upon the quality of the original thesis submitted for microfilming. Every effort has been made to ensure the highest quality of reproduction possible.

If pages are missing, contact the university which granted the degree.

Some pages may have indistinct print especially if the original pages were typed with a poor typewriter ribbon or if the university sent us a poor photocopy.

Previously copyrighted materials (journal articles, published tests, etc.) are not filmed.

Reproduction in full or in part of this film is governed by the Canadian Copyright Act, R.S.C. 1970, c. C-30. Please read the authorization forms which accompany this thesis.

THIS DISSERTATION
HAS BEEN MICROFILMED
EXACTLY AS RECEIVED

AVIS

La qualité de cette microfiche dépend grandement de la qualité de la thèse soumise au microfilmage. Nous avons tout fait pour assurer une qualité supérieure de reproduction.

S'il manque des pages, veuillez communiquer avec l'université qui a conféré le grade.

La qualité d'impression de certaines pages peut laisser à désirer, surtout si les pages originales ont été dactylographiées à l'aide d'un ruban usé ou si l'université nous a fait parvenir une photocopie de mauvaise qualité.

Les documents qui font déjà l'objet d'un droit d'auteur (articles de revue, examens publiés, etc.) ne sont pas microfilmés.

La reproduction, même partielle, de ce microfilm est soumise à la Loi canadienne sur le droit d'auteur, SRC 1970, c. C-30. Veuillez prendre connaissance des formules d'autorisation qui accompagnent cette thèse.

LA THÈSE A ÉTÉ
MICROFILMÉE TELLE QUE
NOUS L'AVONS REÇUE

THE UNIVERSITY OF ALBERTA

DETERMINATION OF BIOLOGICALLY ACTIVE SULFUR-CONTAINING COMPOUNDS BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY WITH A MERCURY-BASED ELECTROCHEMICAL DETECTOR

by

© ROLF SAETRE

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE

OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF CHEMISTRY

EDMONTON, ALBERTA

SPRING, 1978

THE UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled .....
DETERMINATION OF BIOLOGICALLY ACTIVE SULFUR-CONTAINING COMPOUNDS BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY WITH A MERCURY-BASED ELECTROCHEMICAL DETECTOR
submitted by ROLF SAETRE
in partial fulfilment of the requirements for the degree of DOCTOR OF PHILOSOPHY.

.....
D.L. Rabenstein,
(Supervisor).

.....
J.A. Plambeck.

.....
F.F. Cantwell.

.....
R.B. Jordan.

.....
J.C. Russell.

.....
F.W. Karasek,
(External Examiner).

Date: ....., 1978.

ABSTRACT

A mercury-based electrochemical detector for high performance liquid chromatography has been developed for the purpose of detecting biologically active sulfhydryl-containing molecules in eluates. The detector has an approximately 0.8-mm diameter Hg pool working electrode and is constructed from a machined block of Plexiglas. The characteristics of the detector and its response to sulfhydryl-containing amino acids and a variety of other compounds including inorganic sulfur compounds and some metal species have been studied. The detector has a potential range from about +0.2 V to about -1.2 V vs. SCE depending on pH.

Procedures have been developed for the determination of glutathione in whole blood, of reduced and total penicillamine (reduced plus oxidized penicillamine) in plasma, packed erythrocytes, whole blood, and urine, of reduced and total cysteine (reduced plus oxidized cysteine) in plasma and urine, of total homocysteine (reduced plus oxidized homocysteine) in plasma, and of cysteine and glutathione in fruit juices. The procedures are based on the separation of the thiols by high performance cation-exchange chromatography, followed by detection with the mercury-based electrochemical detector. The detector has a detection limit of approximately 10^{-6} M for a sample injection of 10 µL,

and at an electrode potential of +0.1 V vs. SCE, it is selective for sulfhydryl components in the biological fluids analyzed. Total thiol is determined by electrolytic reduction at a mercury pool electrode prior to the HPLC analysis.

The procedures have been used to determine glutathione in whole blood of normal adults, reduced and total penicillamine in blood and urine from patients on D-penicillamine therapy for rheumatoid arthritis, reduced and total cysteine in plasma and urine of normal adults, total homocysteine in plasma of normal adults, and cysteine and glutathione in juices from several fruits. For glutathione, the LC method has been checked by comparison with the colorimetric assay based on reaction with 5,5'-dithiobis-(2-nitrobenzoic acid). The LC results are consistently slightly lower, presumably because of the greater selectivity of the LC method. The results obtained for penicillamine suggest that the LC method with electrochemical detection may provide a useful technique for pharmacokinetic studies on D-penicillamine. For cysteine, results are presented which suggest that cysteine disulfide exchange reactions are a source of error in the iodoacetate method for the determination of cysteine in plasma.

ACKNOWLEDGEMENTS

My sincere thanks to Dr. D.L. Rabenstein for his guidance and patience throughout the course of this research.

I am also grateful to all my colleagues in the research group for their discussions and advice. Special thanks go to Dr. J.S. Percy, Dr. A.S. Russell, and Dr. P. Davis in the Rheumatic Disease Unit at the University of Alberta for providing access to patients treated with D-penicillamine for rheumatoid arthritis and to my wife, Angie, for obtaining the blood and urine samples.

I also want to thank Dr. F.F. Cantwell for numerous helpful discussions.

Financial support from the National Research Council of Canada, the Government of Alberta, and the University of Alberta is gratefully acknowledged.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

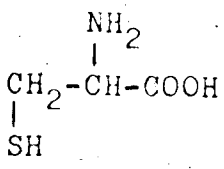
DETERMINATION OF BIOLOGICALLY ACTIVE SULFUR-CONTAINING COMPOUNDS BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY WITH A MERCURY-BASED ELECTROCHEMICAL DETECTOR

CHAPTER I

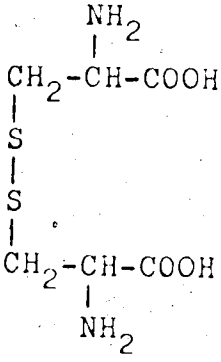
INTRODUCTION

A. Presence and Function of Some Biologically Active Disulfide and Sulfhydryl-Containing Molecules

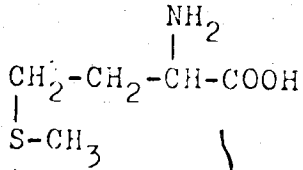
All living organisms require sulfur in some suitable form, and in higher animals this need is met by the amino acids L-cysteine (I), L-cystine (II), and L-methionine (III).



(I)



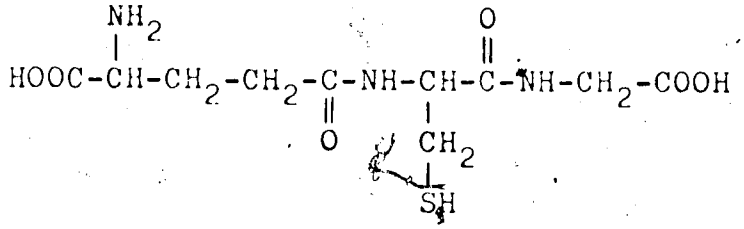
(II)



(III)

together with the heterocyclic compounds biotin and thiamine. The ingested sulfur appears in cells in only three principal chemical fractions which are to some extent interconvertible (1). One of these is the sulfide fraction, made up of methylated sulfhydryl groups from methionine residues of cellular proteins (1). Sulfur also appears as sulfate bound chiefly as ester or amide sulfate in various polysaccharides and steroids (1). Among naturally-occuring inorganic forms of sulfur is sulfite, which plays an intermediary role in sulfate metabolism and has been detected in seminal plasma (2). Mammalian urine generally contains thiosulfate and thiocyanate, and the latter is known to occur in secretions such as saliva (3).

The third fraction of sulfur is that present in sulfhydryl and disulfide forms. The disulfide-containing amino acid cystine was first discovered in 1810 by Wollaston (4) in stones of bladder, whence it obtained its name. Only years later was it found in proteins (5) and its structure established (6). In 1907 sulfhydryl groups in proteins were discovered (7), but it was the discovery of glutathione (γ-L-glutamyl-L-cysteinyl-glycine, IV)



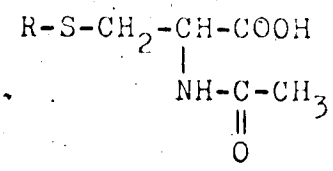
(IV)

and the study of its functions in the chemical dynamics of the cell (8) which excited wide interest in the investigation of sulfhydryl groups.

Glutathione (GSH) is the most widely distributed nonprotein thiol in living organisms. Its intracellular concentration is normally at least an order of magnitude higher than that of other nonprotein thiols (9). The extracellular glutathione concentration, however, is too low to be measured with available techniques. Despite its tendency to oxidize, the glutathione is maintained predominantly in the reduced form. Small amounts of the oxidized form, GSSG, are usually also detectable, but it is uncertain whether these are artifacts arising from the isolation procedure or whether they represent actual presence in tissues (10).

The high intracellular GSH concentration is thought to have several protective functions. In one of these, GSH is utilized as a reductant in several enzyme-catalyzed reductions, such as for example the reduction of H_2O_2 (11) and of several disulfides (12,13). GSH also protects intracellular protein against radiation (14).

When the GSH-GSSG status of the cell is disturbed, it is re-established by resynthesis of GSH and/or rapid reduction of GSSG. In the liver and kidney, glutathione is involved in the detoxication of a variety of potentially harmful electrophilic compounds (R) through enzyme-catalyzed conjugation (15). The end products of the reaction, mercapturic acids (V)



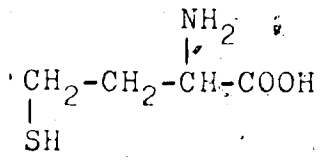
(V)

which are conjugates of N-acetyl-cysteine, can be detected in the urine.

Apart from its protective functions, glutathione has been shown to participate in the γ-glutamyl cycle, a series of six enzyme-catalyzed reactions which has been proposed (16) as one mechanism for the transport of amino acids across cell membranes. This cycle also accounts for the synthesis and degradation of glutathione. A number of enzymes also require GSH as coenzyme (17), such as for example glyoxalase in the hydration and rearrangement of methyl glyoxal to lactic acid.

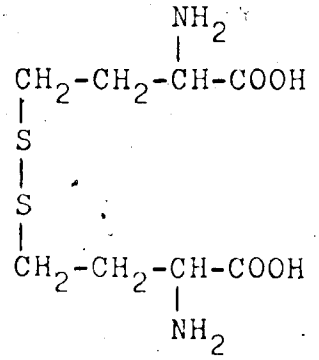
One of the components needed in the synthesis of glutathione is cysteine. Despite its abundance in bound forms, there is normally little free cysteine or cystine in cells and in extracellular fluids (18). Cysteine and

cystine are, however, both present in measurable quantities in the plasma and in the urine of normal persons. The level of cysteine, which is produced by reduction of cystine formed by hydrolysis of dietary protein and which appears as a metabolite in the degradative pathway of methionine to sulfate, is about 10% of the cystine level. Although cystine greatly predominates over cysteine in extracellular fluids, the reverse is true inside cells because absorbed cystine is rapidly reduced to cysteine. Since homocysteine (VI)



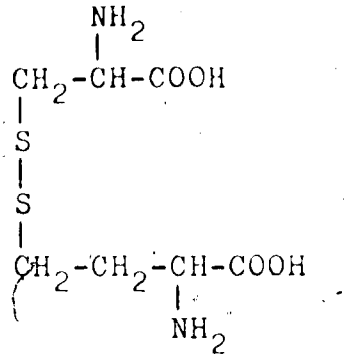
(VI)

appears as a metabolite linking methionine and cysteine (19), it would be expected to be present in normal plasma although in low concentration. So far, however, neither homocysteine nor homocystine (VII)



(VII)

have been detected in the free state. The cysteine-homocysteine mixed disulfide (VIII),



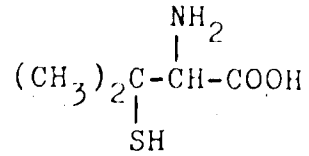
(VIII)

on the other hand, has been reported to be present in normal plasma (20).

In congenital metabolic defects such as cystinuria and homocystinuria, excessively large quantities of cystine and homocystine are excreted in the urine. Cystinuria arises from a defect in the reabsorption of cystine across the renal tubules (21) whereas homocystinuria usually is caused by a deficiency in the activity of the enzyme cystathionine synthetase (22). In the case of cystinuria, some of the cystine, due to its low solubility, crystallizes in the kidney as renal stones. In another human congenital abnormality, cystinosis, cystine accumulates in cells, probably due to a defect in the intracellular reductive mechanism of absorbed cystine (23).

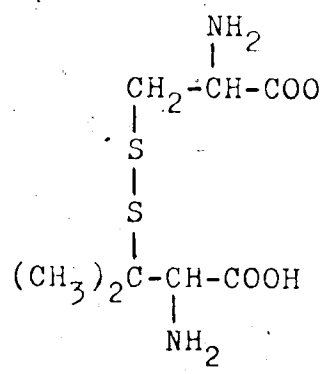
Several thiols and disulfides are also used as

drugs, which may be administered over prolonged periods. One of these is the sulfhydryl-containing amino acid D-penicillamine (IX),



(IX)

which has been extensively used since 1956 (24) for complexing copper ions in Wilson's disease and thus increasing their urinary excretion. The rate of elimination of other metals such as Pb, Hg, and As is also increased, but the effect on Hg and As is not comparable with that of BAL (2,3-dimercaptopropanol) (25), another sulfhydryl-containing compound. D-penicillamine is also used in the treatment of the above mentioned congenital disorders cystinuria and cystinosis where it functions by reacting with deposited insoluble cystine to form the soluble cysteine-penicillamine mixed disulfide (X),



(X).

which is readily excreted in the urine. D-penicillamine is also one of several drugs currently used for the treatment of rheumatoid arthritis (26). Its mechanism of action in this disease is still not understood.

B. Methods for Determination of Biologically Active Disulfide and Sulfhydryl-Containing Molecules

Because of the wide interest in the chemistry and biochemistry of disulfide and sulfhydryl-containing molecules, a multitude of methods has been devised for their determination. Most of the techniques, however, are rarely applied to biological samples because they have insufficient specificity or low sensitivity, or both. In addition, some techniques are too tedious to be of any practical value.

The nonprotein disulfides can be assayed either directly or indirectly. Direct assays include those based on fluorescent reagents such as o-phthalaldehyde which reacts with oxidized glutathione after derivatization of reduced glutathione with N-ethylmaleimide (27), specific enzyme-catalyzed reduction with $NADPH_2$ (10,28), and ion-exchange chromatography (29-35). In most indirect methods, the disulfides are first reduced in one of a variety of ways such as electrolytically (36), or with tin (37), zinc (38), sulfite (39), cyanide (40), thiols (41), potassium borohydride (42), or Thiolated Sephadex (43). The liberated thiol is then assayed by a suitable

technique for sulfhydryl groups. The most important of the methods for sulfhydryl groups can be classified as (a) titrations based on mercaptide formation, (b) redox titrations, (c) electrochemical methods, (d) spectroscopic methods, (e) enzymatic methods, and (f) chromatographic methods. Since glutathione, penicillamine, cysteine, and homocysteine have been of major interest in this thesis, references will be limited to the more important assays available for these molecules.

a) Titrations Based on Mercaptide Formation

In 1948 Benesch and Benesch (44) showed that glutathione and cysteine could successfully be titrated with silver ions in ammoniacal solution using an amperometric endpoint. They applied the technique to the determination of glutathione in rat blood and rat tissue (45). Since this initial work, silver ions have been extensively used for the determination of small sulfhydryl-containing molecules. With slight modifications of Benesch's conditions, Sluyterman (46,47) titrated glutathione and cysteine, and Grimes (48) determined glutathione routinely in erythrocytes. Aibara et al (49) and Shol'ts (50) reported that cysteine and penicillamine gave high results, whereas glutathione gave the theoretical titer. Glutathione, cysteine, and homocysteine have also been estimated potentiometrically with a silver electrode (51), and Ladenson and Purdy (52)

introduced coulometrically-generated silver for the titration of glutathione in whole blood.

Other metal ions used to titrate glutathione and cysteine include lead(II) with catechol violet as endpoint indicator (53) and mercury(II) in aqueous (54,55) and nonaqueous (56) solutions with potentiometric and amperometric endpoint detection. Because of the difficulty in controlling the stoichiometry of the reactions with silver(I), lead(II), and mercury(II), organo-mercury reagents containing only one available valency have replaced most of the other metal ions. Cysteine has been determined with p-chloromercurybenzoate (57) and phenylmercuric acetate (58), and Wronski (59) determined glutathione and cysteine in the presence of each other with o-hydroxymercurybenzoate using thiofluorescein as indicator.

b) Redox Titrations

Iodine is probably the most popular of the various oxidizing reagents used for thiols. As early as 1925, Tunncliffe (60) reported the direct titration of glutathione in various tissues with I_2 using nitroprusside as endpoint indicator. With minor modifications such as for example the introduction of starch as endpoint indicator or back titration of excess iodine, this procedure soon was widely applied to glutathione in blood and tissues (61-66). Cysteine has been determined with I_2 under similar conditions (67) and also with

coulometrically-generated I_2 (68).

Woodward and Fry (69) titrated glutathione with iodate after addition of iodide to the sample. Divin et al (70) stated that this method gave low results and recommended addition of iodide to the iodate titrant rather than to the sample. The iodate method has since been used for several glutathione determinations in biological materials (71-73) even in the presence of ascorbic acid (74). Other oxidizing agents which have been used as titrants, particularly for cysteine, include coulometrically-generated bromine (75), N-bromosuccinimide (76), copper(II) plus sulfite (77), and ferricyanide (63). The specificity, however, of these oxidants is much too low to make them practical in most studies of biological samples.

c) Electrochemical Methods

The majority of electrochemical determinations of glutathione and cysteine utilizes classical polarography. Direct determination using the anodic diffusion current of the thiol (78,79) has been applied to the determination of glutathione in fruits (74,80). The polarographic determination of excess reagent such as methylmercuric iodide (81) and N-ethylmaleimide (82) added to the sample has also been reported. Other electrochemical methods include the voltammetric determination of cysteine after collection on a hanging mercury drop electrode (83) and the <u>in vivo</u> determination

of cysteine in rats via cyclic voltammetry (84).

d) Spectroscopic Methods

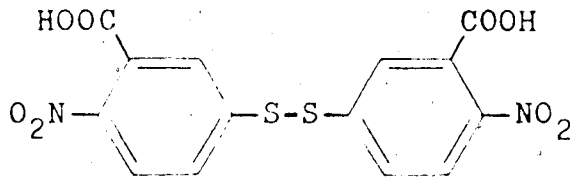
One of the more extensively used color-developing reagents for the detection and determination of glutathione and cysteine is undoubtedly nitroprusside (XI).

$$[Fe(CN)_5NO]^=$$

(XI)

Probably the first mention of this reagent for thiols was by Mörner (5). It has frequently been used in the presence of cyanide in screening tests for cystinuria and homocystinuria (85). Later the toxic cyanide was replaced by the reductant $NaBH_4$ in these tests (86). Bierich and Rosenbohm (87) developed a procedure using nitroprusside to determine glutathione in tissues. This procedure soon was slightly improved (88) by substitution of trichloroacetic acid by metaphosphoric acid for protein precipitation, and it was found to be an improvement on titrations with iodine because of non-interference from ascorbic acid. Even after the addition of cyanide (89) which to some extent stabilizes the developed color, the color formed has been reported to be impractically temperature dependent and unstable (90).

After the introduction by Beutler et al (90) of 5,5'-dithio-bis(2-nitrobenzoic acid), or DTNB (XII),



(XII)

which gives rise to a much more stable color with glutathione, the use of nitroprusside declined rapidly. With the DTNB method, the total of the free sulfhydryl groups in a sample is determined according to Reaction 1

$$RS^- + DSSD \rightleftharpoons RSSD + DS^- \qquad (1)$$

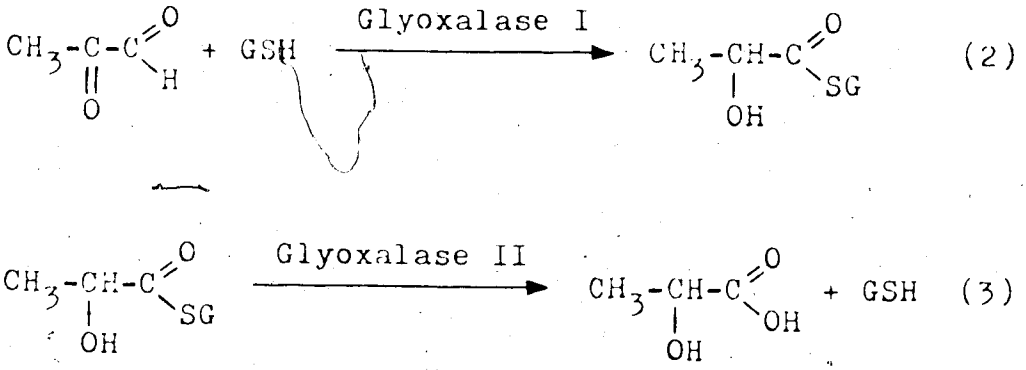
where DTNB is abbreviated DSSD. It is therefore nonspecific for a particular thiol, but nevertheless it is probably the most commonly used method for routine glutathione determinations in biological materials (91-93) and has recently been automated with a Technicon Auto Analyzer (94). It has also been used for cysteine in normal and cystinuric urine (43). A similar reagent, dithio-bis(4-nitrobenzene) was used by Stevenson et al (95) to determine glutathione in red blood cells. A more specific assay for glutathione is obtained with the color-developing reagent alloxan (96), but low recovery and irreproducible results have been reported with this technique (52). Other spectrophotometric methods applied

to glutathione in biological samples include heating with conc. H_2SO_4 (97) to possibly form a thiazoline ring by dehydration, and measuring the decrease in absorbance of a palladium(II)chlorpromazine solution by competitive displacement of the ligand by glutathione (98). The former was reported to be specific but to lack sensitivity. A fluorometric method based on the product formed between glutathione and o-phthalaldehyde has also been reported (99,100). The method showed no interference from cysteine, homocysteine, and ergothioneine. For cysteine in biological materials, several color-developing reagents have been tried. These include 2,6-dichlorobenzoquinone (101), p-aminodimethylaniline (102,103), ninhydrin in acetic acid-HCl solution (104,105), and noradrenochrome (106,107). Penicillamine is generally determined as its blue Fe(III) complex (108). This method, however, has been reported to lack precision (109). For homocysteine, a method based on the absorbance at 230 nm of its thioester has been described (12).

e) Enzymatic Methods

Several enzymatic methods for glutathione have been described (110). These methods are based on reactions in which glutathione functions as a specific activator of enzymes such as glyoxalase (111), formaldehyde dehydrogenase (112), or maleylacetoacetic acid isomerase (113). Woodward (111) described an enzymatic method for

glutathione based on the glyoxalase system represented by Reactions 2 and 3,



in which he measured the rate of evolution of CO_2 from a solution containing bicarbonate. He used a plot of the volume of CO_2 obtained in 20 min. versus concentration of standard glutathione solution as calibration curve. The rate of the reaction has since been measured in several ways. Schroeder and Woodward (114) eliminated manometry by measuring the methylglyoxal disappearance iodometrically. A more convenient modification is probably the titrimetric determination of the rate of lactic acid formation (115). Most common, however, is the spectrophotometric measurement (116) of the intermediate, S-lactoylglutathione, which is formed quantitatively within a few minutes. The other enzyme reactions used for the determination of glutathione (112,113) are also followed spectrophotometrically, either by measuring the decrease in substrate or the increase in product concentration. These techniques are

extremely sensitive and selective, and their only drawback is that they tend to be somewhat tedious.

f) Chromatographic Methods

Glutathione and cysteine in biological samples have been separated from naturally occuring amino acids by paper chromatography (117-121). This is probably the simplest and least expensive of the chromatographic methods, but usually only semiquantitative results or estimates are obtained.

Of the column chromatographic methods, ion-exchange is almost the only technique used. Brigham et al (30) developed a procedure for cysteine in plasma and urine using an automated amino acid analyzer with ninhydrin detection, which at the present time appears to be the method of choice for cysteine (122-124). To stabilize the cysteine against oxidation and disulfide exchange, in which it is the deprotonated sulfhydryl group which is reactive, it is converted into its S-carboxymethyl derivative by reaction with iodoacetic acid before the ion-exchange step. Since the color-developing reagent ninhydrin, which reacts nonspecifically with the amino group of amino acids, is used for detection, the cysteine derivative must be separated from all other amino acids in the sample by the ion-exchange column. This causes the analysis time to be excessively long, i.e. 3 hours for cysteine and 13 hours for cystine. Recently Tabor and Tabor (125) determined glutathione

by automated anion-exchange chromatography after derivatization with N-ethylmaleimide and iodoacetic acid. They also used detection with ninhydrin, and an elution time of about 60 min. was needed. Cysteine, homocysteine, and penicillamine have also been determined by ion-exchange chromatography after oxidation with performic acid (109,126,35), and in one of the procedures (35), detection was performed with sulfur specific iodoplatinate. Resins containing mercury (127,128) and radiolabeled ^{110}Ag (129) have been used to separate glutathione and cysteine, and in the latter, detection with a scintillation counter gave detection limits of 5×10^{-10} M. The quantitative determination by gas-liquid chromatography after suitable derivatization has been reported for glutathione (130,131) and for cysteine, homocysteine, and penicillamine (132).

C. Determination of Small Biologically Active Sulfhydryl-Containing Molecules by Ion-Exchange Chromatography with Electrochemical Detection

If the determination of only one or two amino acids in a complex biological sample is of interest, the major disadvantage of an automated amino acid analyzer with ninhydrin detection becomes the time requirement for a single separation which can amount to several hours. This is, as has already been pointed out, due to the nonselectivity of the ninhydrin detection system

which necessitates separation of the one or two amino acids of interest from all other ninhydrin-positive molecules present in the sample. A way around this problem is to use selective detectors. In this thesis, this approach has been used to develop fast, sensitive, and selective methods for the analysis of biologically active thiols in biological fluids by high performance ion-exchange chromatography. For this purpose, an electrochemical detector based on a mercury pool electrode has been developed.

Several electrochemical detectors (133-146) have been described, and they have been shown to be simple, sensitive, and selective. Carbon paste is the most commonly used electrode material, on which, unfortunately, sulfhydryl-containing molecules are oxidized only with great difficulty. Mercury, however, is easily depolarized by thiols over a wide potential range. Detectors using a dropping mercury electrode (135,143) have been described, but these are rather awkward and mechanically unstable devices. A detector using mercury-plated platinum (143) has also been described, but it is unsuitable for the present application because mercury is consumed in the electrode reaction (4) with thiols.

$$RSH + Hg \rightleftharpoons HgSR + H^+ + e^- \qquad (4)$$

With this in mind, a simple stationary mercury pool

detector with high sensitivity was developed. The use of this detector in combination with modern high performance ion-exchange chromatography for the determination of biologically active sulfhydryl-containing molecules provides to a great extent the sought-for characteristics of simplicity, sensitivity, and selectivity.

Prior to the start of this thesis research, there were no reports in the literature of ion-exchange chromatography of biologically active sulfhydryl-containing molecules with direct electrochemical detection. Kissinger (147) has recently mentioned an unpublished procedure in which post-column addition of ferricyanide is used to oxidize the eluted thiols, and the formed ferrocyanide is quantitated electrochemically with a carbon paste electrode. Very recently Cox and Przyjazny (148) used a carbon paste electrode to detect organic sulfur compounds eluted from a liquid chromatograph, and the performance of the detector was compared to that of a flame photometric detector (149). Specific detection of sulfur-containing molecules has also been obtained with post-column addition of iodoplatinate (35).

D. Overview

Since electrochemical detectors for liquid chromatography are sensitive and selective, a study was undertaken to develop such a detector based on a mercury

pool which could be used for the detection of biologically active sulfhydryl-containing molecules. In Chapter III, the characteristics and potential applicability of the developed detector are discussed. Because the disulfides are electroactive only at very negative potentials, they were assayed indirectly after electroreduction at a mercury pool cathode. The electrolysis cell constructed for this purpose is described in Chapter II. A cation-exchange column was used to separate the sulfhydryl-containing molecules and a study to determine the chromatographic conditions is presented in Chapter IV.

The developed method has been applied to the determination of glutathione, penicillamine, cysteine, homocysteine, and their respective disulfides and mixed disulfides in biological fluids. The detailed sample treatments are given in Chapter II, and the results of these studies are presented and discussed in Chapters V-VIII.

CHAPTER II

EXPERIMENTAL

A. Chemicals

i) Eluents and Sample Treatment

Disodium hydrogen orthophosphate (BDH), citric acid (BDH), boric acid (Fisher Scientific), lithium hydroxide (J. T. Baker Chemicals), phosphoric acid (Terochem. Laboratories Ltd.), $Na_2H_2EDTA \cdot 2H_2O$ (Aldrich), metaphosphoric acid (Mallinckrodt), sodium tetrahydridoborate (Terochem. Laboratories Ltd.), and 5,5'-dithiobis(2-nitrobenzoic acid), or DTNB, (Sigma) were all used as received. All concentrations of metaphosphoric acid were based on the weight of the mixed $NaPO_3$-HPO_3 (58.0-62.0% $NaPO_3$ and 34.0-36.0% HPO_3). The solutions of metaphosphoric acid were stored a maximum of three days in the refrigerator before fresh solutions were prepared.

ii) Standards

Glutathione (Sigma), oxidized glutathione (Sigma), cysteine (Nutritional Biochemicals Corp.), cystine (Aldrich), penicillamine (ICN Pharmaceuticals), homocysteine (Nutritional Biochemicals Corp.), N-acetylcysteine (K&K Laboratories), cysteamine (Aldrich), and ergothioneine (Sigma) were used as received. The purity of glutathione was determined by titration with coulometrically-generated I_2 in acetate buffer using

biamperometric endpoint detection (68). Five replicates gave a purity of 97.3 ± 0.5%. The purities of cysteine and penicillamine were determined by titration with coulometrically-generated Br_2 in 1 M HCl using biamperometric endpoint detection (75). Five replicates gave a purity for cysteine of 95.0 ± 0.5% and for penicillamine of 99.6 ± 0.4%. The purity of homocysteine was determined to be 99.0 ± 0.3% for five replicates by reaction with iodoacetamide followed by titration with NaOH (150). The purity of cystine was determined by first electroreducing it and then comparing it by chromatography with a standard cysteine solution. An average value of only 80% was obtained when cystine from an opened bottle was tested. When, however, an unopened bottle of cystine (nominal purity of 99%) was used, a purity of 101 ± 2% was obtained. The difference is most likely due to adsorbed water in the case of the opened bottle. Oxidized penicillamine (Aldrich) was used as received except in the glutathione-disulfide exchange experiment, where it was recrystallized twice from an approximately 20% aqueous ethanol solution to remove residual reduced penicillamine. The product was washed with ethanol and dried in a vacuum dessicator. The level of reduced penicillamine in the product was 0.03% as compared to about 0.1% before recrystallization. The cysteine-penicillamine mixed disulfide was prepared by a literature proce-

dure (151). The inorganic sulfur compounds sodium sulfite (Schawinigan), sodium thiosulfate (BDH), and sodium sulfide (J. T. Baker Chemicals) were used as received. Doubly distilled water was used throughout.

B. Eluent Preparation

The borate-sodium hydroxide buffers and the phosphate-citrate buffers were prepared according to Perrin (152). In some cases, it was necessary to dilute the phosphate-citrate buffers with doubly distilled water to give the needed ionic strength for optimum chromatographic conditions. The phosphoric acid eluents were prepared by dilution of concentrated phosphoric acid with doubly distilled water. All eluents were deaerated with O_2-free nitrogen (scrubbed with 1 M chromium(II) chloride solution) (153) for at least 15 min. before use, and they were kept under nitrogen during use.

C. Apparatus

i) Chromatography

The chromatographic setup is shown schematically in Figure 1. It consists of a 500-mL eluent reservoir, a Milton Roy minipump (maximum flow rate 3 mL/min.), connecting Teflon tubing, a glass column, an electrochemical detector, and a Fisher Recordall Series 5000 recorder. The eluent was deaerated with O_2-scrubbed

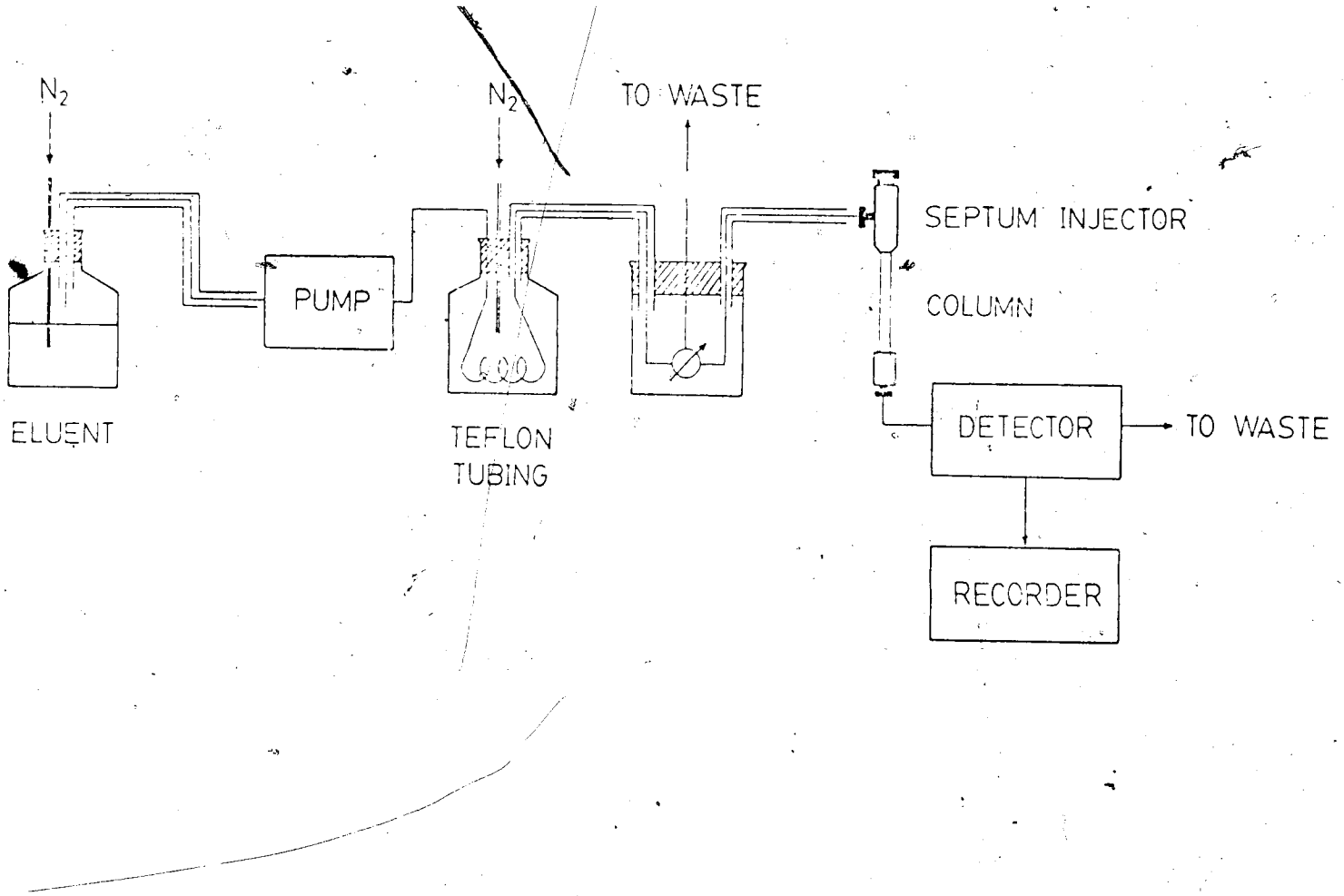


Figure 1. Apparatus for liquid chromatography with electrochemical detection.

nitrogen before use and kept under nitrogen during use. A 1 M $CrCl_2$-0.1 M HCl solution in contact with heavily zinc-amalgamated Hg was used as scrubber (153). The nitrogen was presaturated with water before entering the eluent reservoir. All Teflon tubing, the short piece (1.5-mm i.d.) connecting the eluent reservoir and the pump and the ca. 50 feet (0.8-mm i.d.) connecting the pump and the column, was kept under nitrogen to prevent diffusion of O_2 through the Teflon tubing, the effect of which will be discussed in Chapter III. This was accomplished by surrounding the Teflon tubing with Tygon tubing (0.7-cm i.d.) which was constantly flushed with N_2. It was found unnecessary to keep under N_2 the 1 cm of Teflon tubing connecting the outlet of the chromatographic column to the inlet of the detector. The major portion of the 50 feet of tubing, which served to minimize pulsations from the pump, was coiled up in a 2-L erlenmeyer flask, whereas the switch connecting the pump to waste was kept separately in a 300-mL beaker. The 2-L erlenmeyer flask was connected to the pump with a short piece of stainless steel tubing.

All columns were made of glass with an i.d. of 0.2 cm. A stainless steel filter disc at the bottom served as a bed support. Column lengths varied between 30 cm and 50 cm. The columns were either dry-packed with Zipax 20-35 μM strong acid or strong base ion exchanger (Du Pont) or slurry-packed with Amberlite 200-400

mesh strong acid or strong base ion-exchange resin (Mallinckrodt). In cases where the resin at the top of the column was quickly contaminated by samples such as concentrated urine, a 0.2 x 3-cm precolumn was used. This was easily repacked without wasting too much packing material. A septum injection port was attached to the column, and syringe injection (10 µL) was used for all samples. The columns, tubing, connectors, and valves were obtained from the Cheminert Division of Laboratory Data Control. The electrochemical detector will be described in detail in Chapter III.

ii) Electrolysis Cell

The electrolysis cell used to reduce the disulfides is based on a mercury pool cathode. It (Figure 2) consists of a 1.5 x 5-cm tube with an inverted U-shaped side arm (1-mm i.d.) attached to the side approximately 1 cm from the bottom. A Hg pool of 0.7-cm depth is used. Electrical contact to the Hg is made via a Pt wire sealed into the bottom. The side arm is filled with saturated NaCl-2% Agar and dips into a beaker containing saturated NaCl and a Pt wire counter electrode. The saturated NaCl was made slightly alkaline to avoid evolution of Cl_2. With a small magnetic stirring bar on top of the Hg pool, this cell is suitable for sample volumes down to a few hundred microliters. Samples were electrolyzed at a constant current of 6.43 mA, provided by a Leeds & Northrup constant current coulometer. This

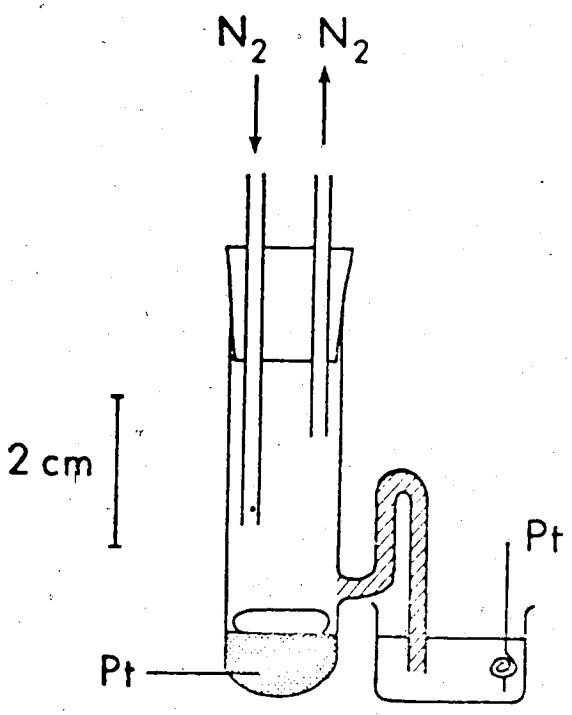


Figure 2. Mercury pool electrolysis cell. See text for details.

gave rise to a voltage drop of about 6 V across the cell. It was found convenient to reduce simultaneously several samples with multiple electrolysis cells connected in series. Oxygen-free nitrogen usually was passed over the solution during electrolysis.

The salt bridge has been attached to the side rather than having it dip into the solution from the top of the tube so that magnetic stirring can be used with samples volumes down to a few hundred microliters. Also, the cell resistance is smaller, permitting the use of several cells in series without exceeding the output limit of the coulometer. Initially, the salt bridge was filled with saturated KCl-2% Agar. This, however, gave rise to decreased retention times for the thiol and caused tailing of the peaks. During electrolysis at 6.43 mA for 10 min., the approximately 4×10^{-5} moles of H^+ which are reduced are replaced by cations from the salt bridge. When these are K^+ ions, they are more strongly retained on the Zipax SCX cation exchanger than are Na^+ ions in the buffer. In high concentrations, they tend to saturate the column, thereby reducing the retention time for the thiol. Saturated NaCl-2% Agar in the salt bridge eliminated this problem. When electrolyzing small samples, care must be taken to ensure that the samples are sufficiently acidic to avoid quantitative reduction of the protons. All samples were made 0.2 M in HCl prior to electrolysis.

iii) Miscellaneous

All pH measurements were performed with a Fisher combination electrode attached to a Beckman Zeromatic analog pH meter. The pH meter was standardized with Fisher Certified Buffers of pH 4.0, 7.0, and 10.0. All measurements were done at room temperature.

A PAR Model 174A Polarographic Analyzer was used for the polarographic experiments and as a potentiostat to control the potential of the electrochemical detector. The detector cell resistances were measured at 1 kHz with a 1650A Impedance Bridge, General Radio Company. All coulometric experiments were done at 6.43 mA with a Leeds & Northrup constant current coulometer. Centrifuging was done with an IEC HN-S centrifuge with a maximum rotation speed of 2800 rpm.

D. Sample Treatment Prior to Analysis

The general outline of the various sample treatments is shown in Figure 3. Common to all treatments is the rapid acidification of the sample to precipitate protein after the sample has been removed from its biological environment. The acidification also stabilizes the thiol towards air-oxidation as well as "freezing" any disulfide exchange reactions because in both reactions the thiol group participates in its deprotonated form. The following describes the details of the treatments for the different samples. All blood samples were ob-

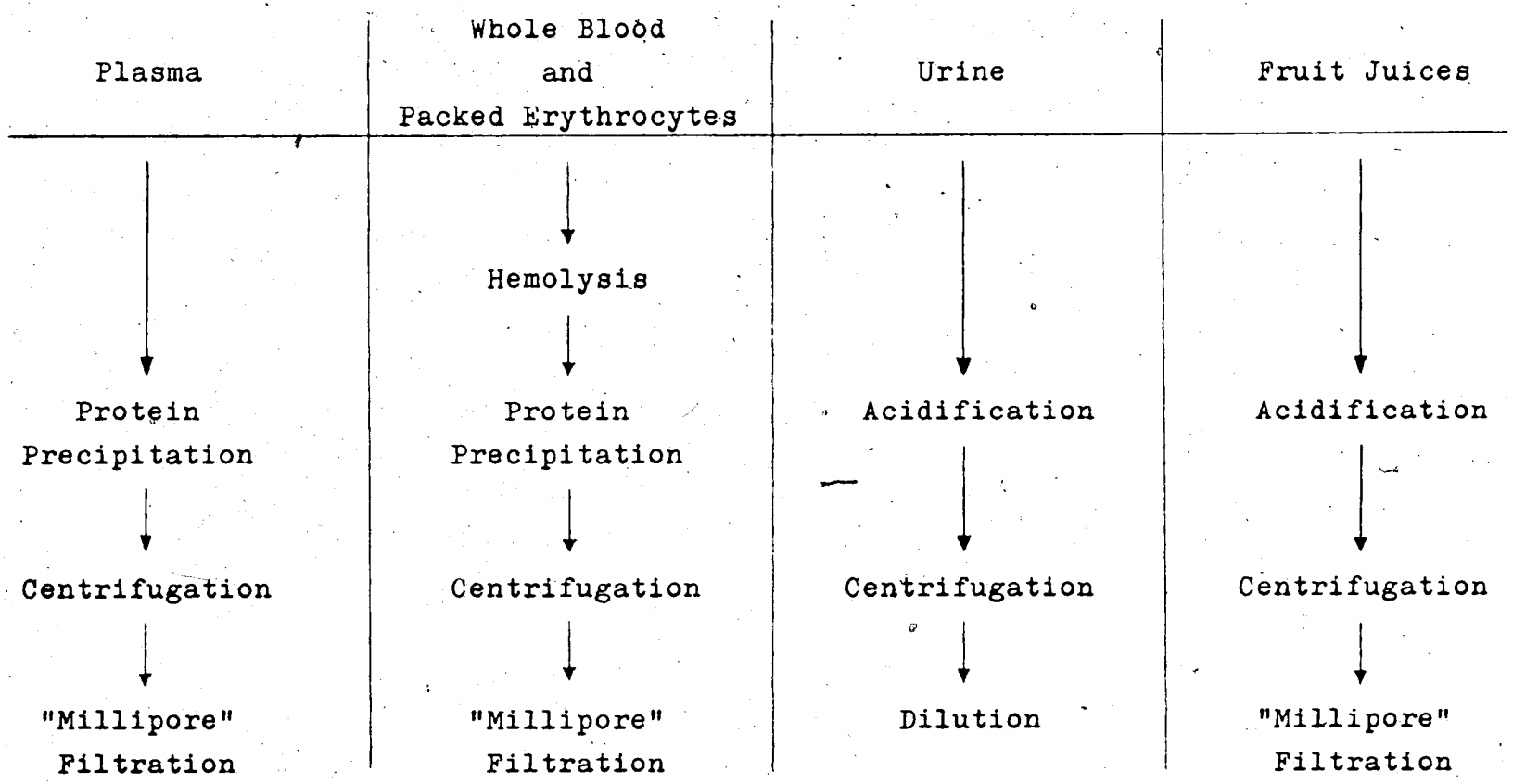


Figure 3. Flow chart for the various sample treatments.

tained from the Rheumatic Disease Unit at the University of Alberta, and the fruits were obtained from a commercial distributor.

Plasma: Approximately 5 mL of blood was collected in a Vacutainer (Fisher Scientific) which contained a K_3HEDTA solution. The K_3HEDTA is present to complex calcium and thus prevent clotting of the blood. EDTA was preferred to other anticoagulent reagents such as heparin because it also complexes heavy metal ions which otherwise catalyze the air-oxidation of the thiols. After collection of the blood, the Vacutainer was gently shaken to mix the contents, and then centrifuged immediately at 2500 rpm for 5 min. With a 1-mL tuberculin syringe, 0.8 mL of the plasma was withdrawn and added to a 10 x 75-mm test tube containing 0.2 mL of 200 to 500 g/L metaphosphoric acid. The smaller amount of metaphosphoric acid was used in the cysteine assay, whereas the larger amount was used in the penicillamine assay. The contents were mixed well and, after about 10 min., the tube was centrifuged at 2500 rpm for 10 min. to remove precipitated protein. The supernatant liquid was withdrawn with a 1-mL tuberculin syringe and forced through a 0.45-µm pore diameter filter, which was held in a filter holder (Millipore) fitted to the syringe. The filtrate was collected in a 10 x 75-mm test tube. Aliquots of 10 µL of the filtrate were used to analyze for nonprotein thiols by HPLC. To

determine the total amount of a specific thiol, i.e. reduced plus the amount present in symmetrical and non-symmetrical disulfide forms, 0.25 mL of the filtrate was diluted with 0.25 mL of 0.4 M HCl. This solution was placed in the electrolysis cell (see Section C) and electrolyzed for 10 min. Aliquots of 10 µL of the electrolyzed solution were then analyzed for total reduced nonprotein thiol by HPLC.

Packed Erythrocytes: Whole blood was collected and centrifuged in the Vacutainer as described above for plasma. After estimating the hematocrit, the plasma was withdrawn with a tuberculin syringe. The packed cells were then twice washed with their volume of normal saline solution (0.9% NaCl). After each washing, the Vacutainer was centrifuged at 2500 rpm for 5 min. The supernatant was withdrawn and, with 100 µL Drummond Microcaps, 0.2 mL of packed cells was transferred to a 10 x 75-mm test tube containing 0.7 mL of EDTA solution (0.2 g Na_2H_2EDTA/L). EDTA is added to complex heavy metal ions present in the water and in the red blood cells and thus inhibit catalytic air-oxidation during hemolysis. The contents were mixed well and, after 1 min., 0.1 mL of 500 g/L metaphosphoric acid was added to the test tube. Again the contents were mixed well. After about 10 min., the tube was centrifuged at 2500 rpm for 5 min. to remove the protein precipitate. The supernatant was forced through a 0.45 µm pore diameter

filter, and the filtrate was collected in a 10 x 75-mm test tube. The reduced and the total nonprotein thiol in the filtrate were determined as described above for the plasma filtrate.

Whole Blood: Whole blood was collected in a Vacutainer (Fisher Scientific) containing a K_3HEDTA solution. With 100 µL Drummond Microcaps, 0.1 to 0.2 mL of the fresh blood was added to 0.7 mL of EDTA solution (0.2 g Na_2H_2EDTA/L) in a 10 x 75-mm test tube and the contents mixed well. After 1 min., metaphosphoric acid was added to give a total volume of 1.0 mL. When 0.1 mL of whole blood was used, for example in the procedure for glutathione analysis, 0.2 mL of 100 g/L metaphosphoric acid was added to the hemolyzed blood. In the case of penicillamine where 0.2 mL of whole blood was used, 0.1 mL of 500 g/L metaphosphoric acid was added. The contents were mixed well and treated further as described above for packed erythrocytes.

Urine: In the determination of penicillamine, 2.25 mL of freshly delivered urine was acidified with 0.25 mL of 2 M HCl. For the assay of cysteine, about 30 mL of urine was collected directly in a beaker containing 3.0 mL of 2 M HCl to avoid any time delay between delivery and acidification. The volume was determined by weighing the beaker with and without the urine. If a precipitate formed, about 5 mL of the urine was centrifuged for 5 min. at 2500 rpm. A small aliquot of the

clear urine was then diluted up to 20-fold with 0.2 M HCl and 10 µL injected onto the column. Total thiol was determined by electrolyzing for 10 to 15 min. an aliquot of the urine diluted at least 2:25 with 0.2 M HCl. The longer electrolysis time was necessary if the dilution was only 2:25. Aliquots of 10 µL of the electrolyzed solution were injected onto the column.

Fruit Juices: With a 1-mL tuberculin syringe, 0.5 mL of fruit juice was added to a 10 x 75-mm test tube containing 0.5 mL of 2% H_3PO_4. The contents were mixed well and centrifuged for 5 min. at 2500 rpm. The supernatant was withdrawn with a 1-mL tuberculin syringe and forced through a 0.45-µM pore diameter filter which was held in a filter holder (Millipore) fitted to the syringe. The filtrate was collected in a 10 x 75-mm test tube. Juice was obtained from the fruits by making a small cut in the skin and squeezing.

CHAPTER III

MERCURY-BASED ELECTROCHEMICAL DETECTORS FOR HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

A. Introduction

Since the early studies of "chromatopolarography" by Kemula and coworkers (154), a variety of electrochemical detectors for liquid chromatography (LC) has been described (133-146). According to the measurement system, these detectors can be classified as either voltammetric, coulometric, potentiometric, or conductometric. The voltammetric detectors are in most cases more accurately referred to as hydrodynamic chronoamperometric detectors or as amperometric detectors since the current measurement is carried out at constant potential. Included in this category would be the mercury-based detector developed in this thesis research. The main advantage of the amperometric detectors over the more commonly used LC detectors such as the differential refractometer and the UV detector is their much higher sensitivity to electroactive compounds. Additionally, selectivity is possible through the choice of electrode potential. The latter characteristic has been used recently by Blank to achieve an instrumental separation with a dual electrode detector (144).

The principle of selectivity can be understood from Figure 4, which shows schematically two polaro-

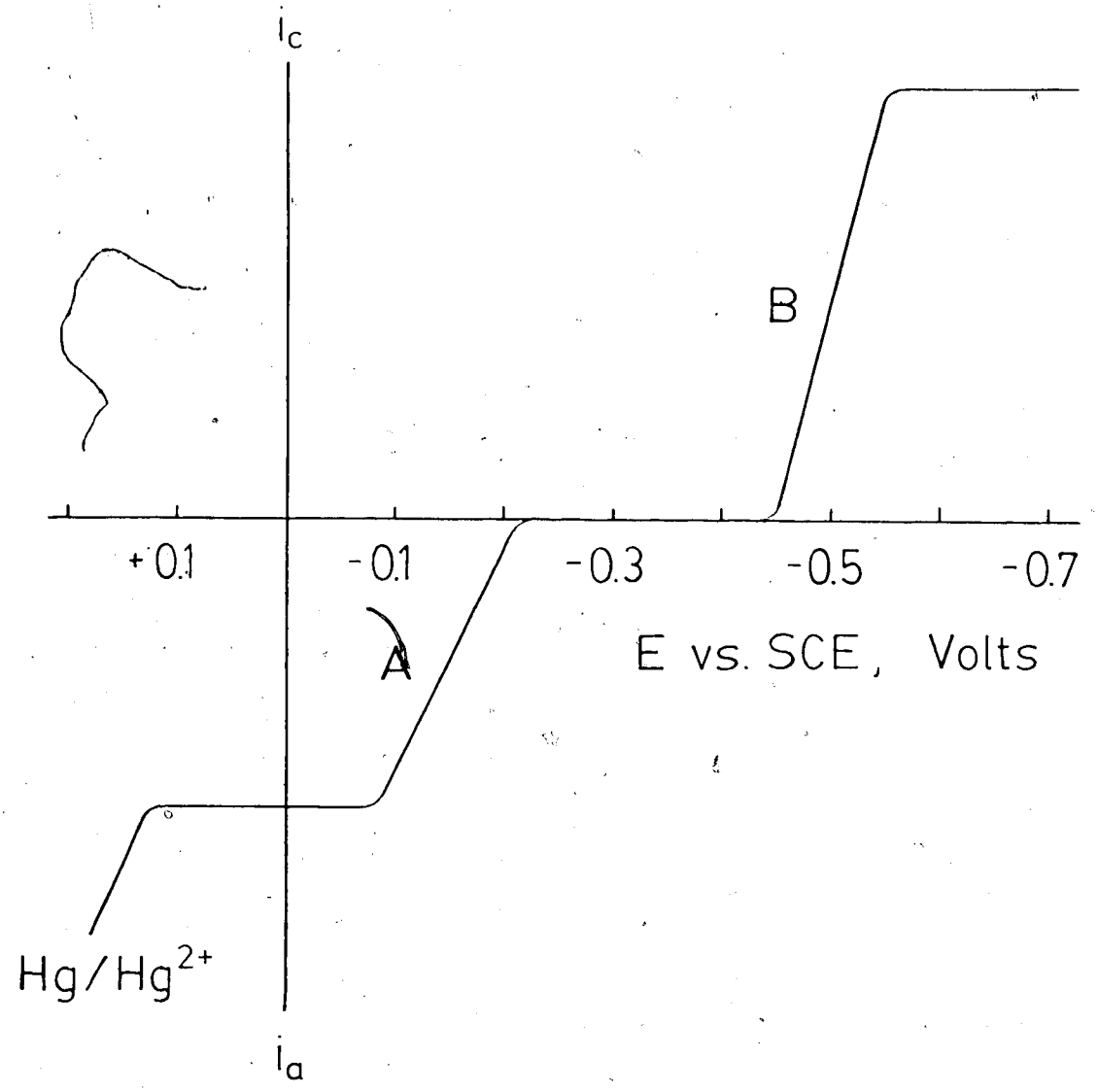


Figure 4. Schematic figure of a polarographic reduction wave (molecule B) and an oxidation wave (molecule A). i_c is cathodic current and i_a is anodic current.

grams; for molecule A an anodic wave with a half-wave potential of about -0.15 V vs. SCE, such as is obtained from a thiol in acidic solution (Equation 4), and for molecule B a cathodic wave. At an applied potential of 0.0 V vs. SCE, only compound A is electroactive, whereas at a potential of -0.7 V vs. SCE, only compound B becomes electroactive. If, for example, A and B are not separated by the column, an electrochemical detector can detect either one of them without interference from the other by operation of the detector at the appropriate potential.

The response of any current measuring detector is dependent on the rate of mass transfer to the electrode surface. According to Levich (155), the limiting current i_L for convective diffusional mass transfer is in general given by Equation 5,

$$i_L = k \cdot n \cdot F \cdot v^{\alpha} \cdot C \qquad (5)$$

where k is a constant dependent on the kinematic viscosity of the eluent, the diffusion coefficient of the electroactive species, and the geometry and area of the electrode, n is the number of electrons, F is the Faraday constant, v is the volume flow rate, and C is the bulk concentration of the electroactive species. The exponent α of the volume flow rate is determined by the specific nature of the hydrodynamic conditions and

generally has a value between 1/3 and 1/2 (156). For an electrode with a conversion efficiency of 100%, a is unity. A detector based on such an electrode is called a coulometric detector since the time integral of the measured current is related by Faraday's law to the number of equivalents of an electroactive species which have passed the electrode. For amperometric detectors, the conversion efficiency is usually less than 10%.

A variety of electrode materials has been used (133-146), including carbon, carbon paste, glassy carbon, mercury (DME), mercury-plated platinum, and platinum. Detectors based on a carbon electrode respond to thiols only at the very positive end of their working potential range (147), and there are no reports of their use for direct detection of biologically active sulfhydryl-containing molecules. Mercury-based detectors, on the other hand, respond to sulfhydryl-containing molecules over a wide potential range; the range depending on the solution pH. In the development of a mercury-based electrochemical detector, neither the DME nor the mercury-plated platinum electrode were investigated because of the inherent mechanical limitations of the DME and the expected limited lifetime of the mercury-plated platinum due to the oxidation of mercury in the electrode process (Equation 4). The polarography of thiols is complicated by adsorption and is still not completely understood. If, however, adsorption occurs on the Hg pool of the

detector, it does not limit its use.

B. Experimental

The heart of the electrochemical detector developed in this thesis is a small mercury pool working electrode. Of the several designs studied, that shown in Figure 5 was found to be the most satisfactory. The body of the detector is machined from a block of transparent Plexiglas, and has a channel through which the column effluent passes. A channel perpendicular to and in contact with the effluent channel contains the Hg electrode and a well holds a reference electrode. Electrical contact between the reference and working electrodes is accomplished by means of the channel labeled B in Figure 5. The diameter of the effluent and Hg channels is the same as the internal diameter of the Teflon tubing (0.8 mm). As shown in Figure 5, the Hg channel is connected to a short piece of Teflon tubing, the other end of which may be attached to the side of the Plexiglas block and filled with mercury. The filling can be accomplished easily with a 2-mL syringe. Sufficient Hg is added to bring the outer edge of the mercury column flush with the bottom of the effluent channel. Electrical contact between the Hg working electrode and the PAR Model 174A Polarographic Analyzer is accomplished via a platinum wire inserted into the mercury column in the Teflon tubing. The diameter of the Pt

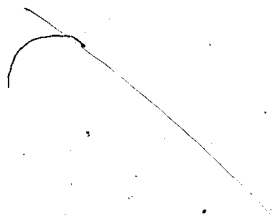
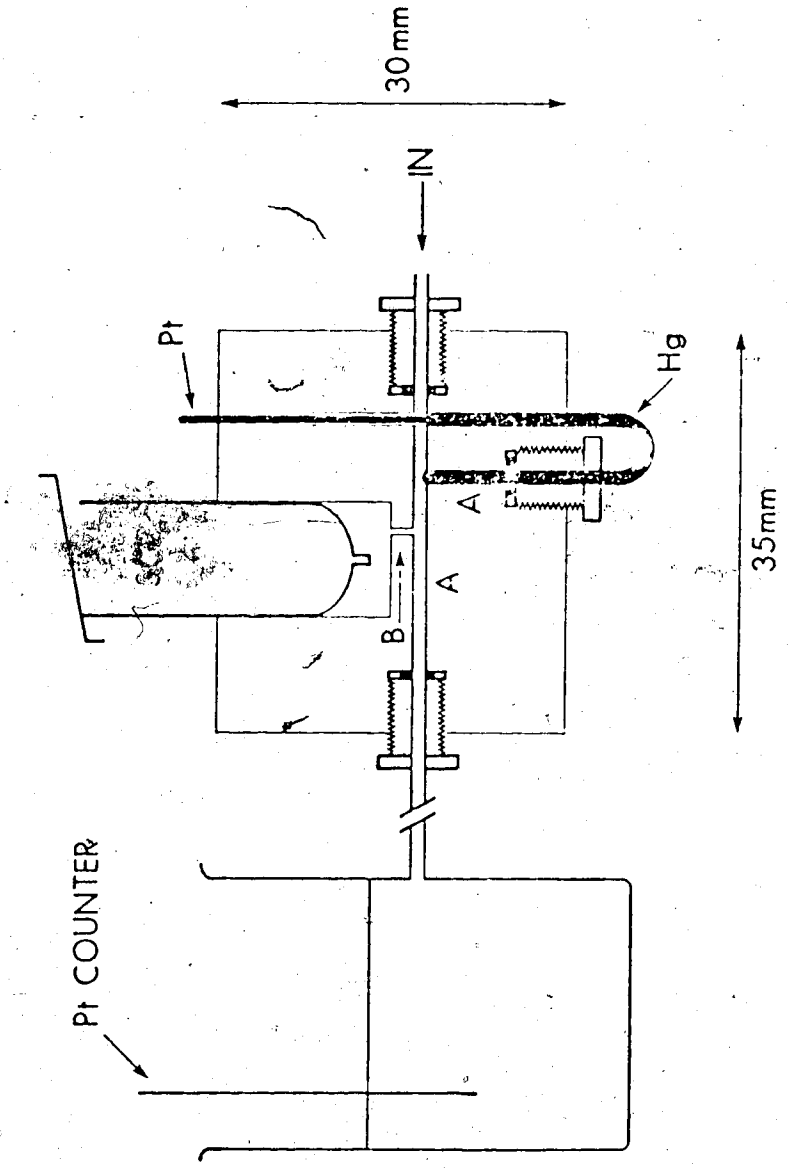


Figure 5. Cross-section of mercury pool electrochemical detector constructed from a Plexiglas block. The A channels have an internal diameter of 0.8 mm. The B channel has an internal diameter of 0.2 mm. See text for other details.

MERCURY-BASED ELECTROCHEMICAL DETECTOR



wire is of the same order as the internal diameter of the Teflon tubing. The calculated volume of the plug of effluent in contact with the Hg pool is < 0.5 μL. The volume of the plug can be varied by changing the depth to which the Pt wire is inserted in the Hg column, which raises the surface of the Hg pool, or by changing the diameter of the bore hole containing the mercury column. To simplify the adjustment of the level of the Hg pool, the Hg column can be attached to a separate Plexiglas block such as that shown in Figure 6. A stainless steel screw with an attached stainless steel rod inserted into the end of the Hg column is used to control the level of the Hg pool. A Pt wire is glued into the Plexiglas block to make electrical contact with the bottom end of the Hg column.

A saturated calomel electrode with a fritted glass junction is used as reference electrode. The well in which the SCE is placed is filled with saturated KCl. A counter electrode (Pt wire) is placed downstream in an overflow beaker and serves as a current sink, i.e. the measured current flows between the counter electrode and the working electrode. In a three-electrode system consisting of a working electrode, a counter electrode, and a reference electrode, essentially no current flows through the reference electrode thus eliminating any internal IR drop during current flow. Consequently, the control of the working electrode poten-

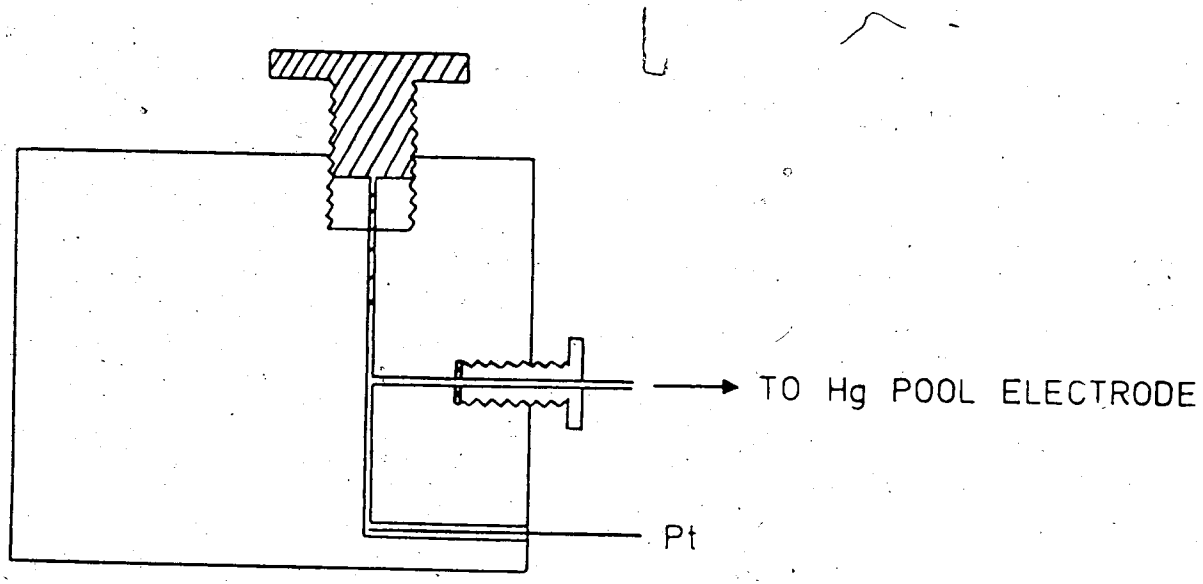


Figure 6. Cross-section of Plexiglas unit used to adjust the mercury pool of the electrochemical detector. See text for details.

tial is more accurate than in a classical two-electrode system.

The distance between the reference electrode and the Hg pool is approximately 0.5 cm. Because the reference and working electrodes are separated by a small diameter channel, and because the reference electrode is positioned in the current path between the working and counter electrodes, there will be an IR drop between the tip of the SCE and the working electrode which is not compensated for by the potentiostat (157, 158). The magnitude of the IR drop will depend on the distance between the working electrode and the reference electrode, the ionic strength of the eluent, and the magnitude of the current flowing. As discussed in Section C, a large resistance between the tip of the SCE and the Hg pool (uncompensated cell resistance) can cause nonlinear calibration curves. With a distance of 0.5 cm between the fritted glass junction SCE and the Hg pool, the cell resistance was less than 100 kΩ for eluents with an ionic strength greater than 0.01.

A Hg-based detector was also constructed from standard, commercially available tee connectors. Such a detector is depicted in Figure 7. In the same way as described above, the bottom opening of the tee is connected to a short piece of Teflon tubing which is attached to the side of the tee and filled with Hg. With a detector of this design, the reference and

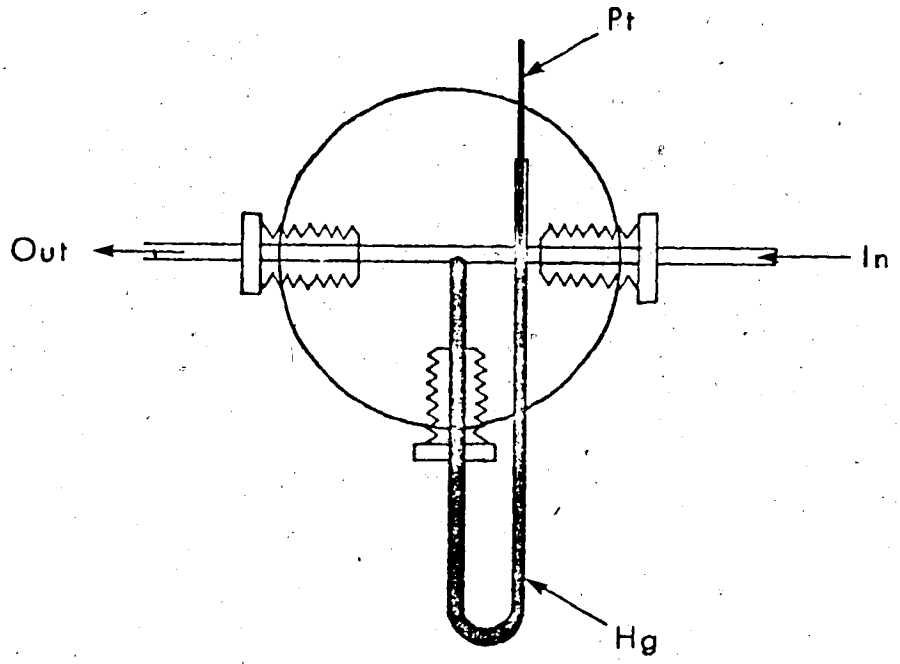


Figure 7. Cross-section of a mercury pool electrochemical detector constructed from a standard chromatography connector. See text for details.

counter electrodes are both placed downstream in an overflow beaker. Unfortunately, with this simple arrangement, the uncompensated cell resistance between the reference and working electrodes can be large and the advantage of a three-electrode system is lost. In such a case, equivalent performance can be obtained from a classical two-electrode configuration using the SCE as a counter electrode. To minimize the uncompensated cell resistance with this detector design, the length of tubing leading from the detector outlet to the overflow beaker should be as short as possible. For tube lengths of 10 cm or more, a cell resistance as large as several megohms was observed.

Variations on the detector designs in Figures 5 and 7 also have been studied. For example, a detector in which the tee connector is rotated 90° from the orientation shown in Figure 7 was investigated. The bore hole at the bottom was filled with Hg and the top bore hole was connected to the outlet of the ion-exchange column. In this design, the mobile phase impinges directly on the electrode surface. A detector in which the angle between adjacent bore holes is 120° also was studied. The characteristics of these detectors were similar to the detector in Figure 7. As dicussed in Section C, to eliminate uncompensated cell resistance, a detector design based on the wall-jet configuration (159) was briefly investigated.

The detectors were characterized partly with a chromatographic column in the flowing stream and partly without a column. A 0.2 x 50-cm column, slurry-packed with Amberlite 200-400 mesh strongly acidic cation-exchange resin in phosphate-citrate pH 7.0 buffer and a 0.2 x 50-cm column, dry-packed with Zipax SCX were used. Samples were injected via a six-position rotary valve or through a septum with a syringe. Sample volumes ranging from 1 µL to 100 µL were used; the smaller volumes were injected with a syringe. As described in Chapter II, Section C, all buffers were deaerated and all the Teflon tubing was kept under N_2.

C. Characterization of the Mercury-Based Electrochemical Detectors

i) Uncompensated Cell Resistance

The three-electrode system used in the electrochemical detector is controlled by a potentiostat (PAR Model 174A) which keeps the applied potential difference between the working electrode and the reference electrode constant. This potential difference between the working and reference electrodes is composed of an interfacial potential difference at the Hg surface-electrolyte interface and, depending on the amount of current flowing between the working and counter electrodes and the electrolyte resistance (uncompensated cell resistance), a potential gradient through the

electrolyte between the working electrode and the tip of the reference electrode. Since there is only an extremely small current flow through the reference electrode at any time, there will be virtually no potential drop across it. For the electrochemical detector shown in Figure 5, the reference electrode is positioned in the current path between the working and counter electrodes. The potential gradient through the electrolyte between the reference and working electrodes will thus decrease and increase as the current flow decreases and increases. As this potential changes, the potential at the Hg surface-electrolyte interface also changes, since the potentiostat operates to keep the sum of both constant. In cases when the detector is used to detect compounds having a narrow limiting current plateau or is operated at a potential close to the polarographic half-wave potential of the compound to achieve selectivity, highly nonlinear calibration curves can be obtained. If, for example, compound A in Figure 4 were to be determined, a potential of 0.0 V vs. SCE, which is in the middle of the limiting current plateau, might be chosen. With a large detector cell resistance, the Hg surface-electrolyte interface potential would be changed toward more negative potentials as the concentration and thus the measured current of compound A increases. At some concentration, the potential on the electrode moves off the limiting current plateau, and

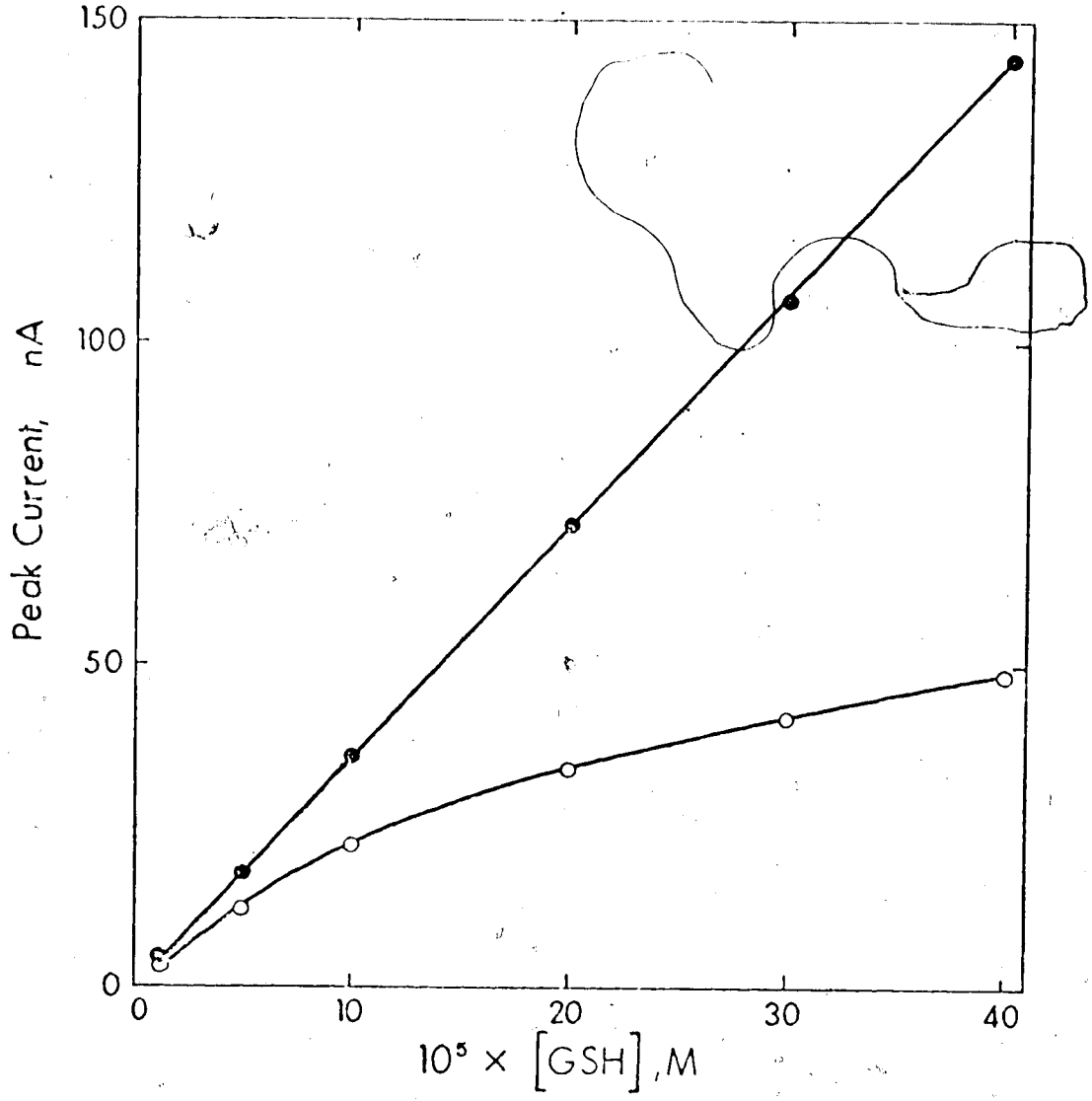
the response of the detector to concentration changes becomes gradually nonlinear. A plot of current vs. concentration of compound A would under these conditions become nonlinear as soon as the detector no longer effectively operates on the limiting current plateau. At low pH, thiols have a narrow limiting current plateau such as indicated for compound A in Figure 4. It is therefore especially important that the control of the Hg electrode potential is accurate for the determination of thiols.

The effect of uncompensated cell resistance on calibration curves obtained for glutathione using an applied potential of 0.0 V vs. SCE is shown in Figure 8. These data were obtained from 10-μL injections onto the 50-cm Zipax SCX column using the pH 2.5 phosphate-citrate buffer mobile phase. Data are shown for a concentration range centered around 10^{-4} M GSH since this is the range for treated blood samples. With the detector in the configuration shown in Figure 5, the uncompensated cell resistance was 75 kΩ a[illegible]he linear calibration curve in Figure 8 was obtained. When the SCE was placed 10 cm downstream in the overflow beaker, the uncompensated cell resistance increased to about 2 megohms, and the nonlinear calibration curve in Figure 8 was obtained. As discussed above, this nonlinearity is due to a shift of the effective working electrode potential in the negative direction when the

Figure 8. Calibration curves for glutathione obtained from 10-µL injections onto a 0.2 x 50-cm column of Zipax SCX cation exchanger with a pH 2.5 phosphate-citrate mobile phase.

●—●—● : uncompensated cell resistance = 75 kΩ

o—o—o : uncompensated cell resistance = 2 MΩ


150
100
50
0
Peak Current, nA
0
10
20
30
40
10⁵ × [GSH], M

current in the circuit increases anodically. This causes the cathodic background current to increase slightly (Section C, iii) and the anodic GSH current to decrease because of its voltammetric behavior at pH 2.5 Polarographically a half-wave potential of -0.16 V vs. SCE is obtained at this pH with no extended limiting current plateau between the GSH wave (Wave A, Figure 4) and the Hg dissolution wave.

The problems associated with uncompensated cell resistance can be minimized by for example increasing the ionic strength of the eluent. The ionic strength, however, will be determined in part by the requirements of the separation step. When low ionic strength eluents are needed, a means for post-column electrolyte addition might be attached in front of the detector. This makes the setup somewhat more involved and, consequently, this approach has not been studied. The uncompensated cell resistance can also be minimized by placing the reference electrode as close as possible to the indicating electrode. For the applications described in Chapters V-VIII, it was satisfactory to position the SCE 0.5 cm downstream of the Hg pool. At this distance, no back diffusion of chloride from the SCE was encountered. To completely eliminate the uncompensated cell resistance, a detector design based on the wall-jet electrode configuration first described by Yamada and Matsuda (159) was briefly investigated. In this design,

the LC effluent impinges normally on the Hg pool. The solution then exits to waste via two diametrically opposed channels, one containing the reference electrode, the other the Pt counter electrode. Because the effluent tended to exit preferentially via one of the channels and because bubbles, once trapped over the Hg pool, were difficult to remove, the study of this approach to eliminating uncompensated cell resistance was discontinued.

ii) Charging-Discharging Current

The phase boundary between the Hg pool surface and the electrolyte can, in its simplest form, be represented according to Helmholtz as a parallel plate capacitor (160). One phase carries a negative charge and the other a positive charge forming a Helmholtz electrioal double layer. The double layer capacity is a complex function of electrolyte concentration and composition. Since the potential difference across the double layer is maintained at a constant value by the potentiostat, a change in electrolyte concentration or composition causes a change in the charge density on the Hg surface. This gives rise to a non-Faradaic current flow which cannot be distinguished by the recording system from a Faradaic current.

Whenever a real sample is analyzed chromatographically, it very rarely has a composition identical to the eluent. Consequently, in ion-exchange

chromatography there will always be an unretained component (the sample matrix) which gives rise to a non-Faradaic current flow i.e. a charging-discharging current. These will be pointed out in several chromatograms shown in Chapters V-VIII.

The charging-discharging phenomenon can also cause retained nonelectroactive ions to give an apparent Faradaic detector response, if these ions are present in high enough concentration. Figure 9 shows how the Hg-based electrochemical detector responds to potassium and sodium ions in 10 μL of 0.1 M KNO_3 and 0.1 M $NaNO_3$ injected onto the 0.2 x 50-cm Zipax strong cation-exchange column. The eluent used was 0.5% H_3PO_4. In the applications described in Chapters V-VIII, these retained artifacts did not present any problems.

When chromatographically separating several components in a sample, a gradient elution system may be needed to overcome the general elution problem (161). In such a case, the charging or discharging of the electrode-solution interface due to eluent change will cause a baseline drift until the eluent composition does not change any more. The severity of the drift will obviously depend upon the difference in composition between the eluents and the slope of the gradient. An example of a stepwise gradient performed with the chromatographic system described in Chapter II, Section C, is shown in Figure 10. Initially a phosphate-citrate

Figure 9. Response of the mercury pool electrochemical detector to 10-μL injections of solutions containing 0.1 M $NaNO_3$ and 0.1 M KNO_3, respectively. A 0.2 x 50-cm column of Zipax SCX cation exchanger was used with a 0.5% H_3PO_4 mobile phase. The peak appearing at about 1.5 min. after injection is due to double layer capacitance effects.

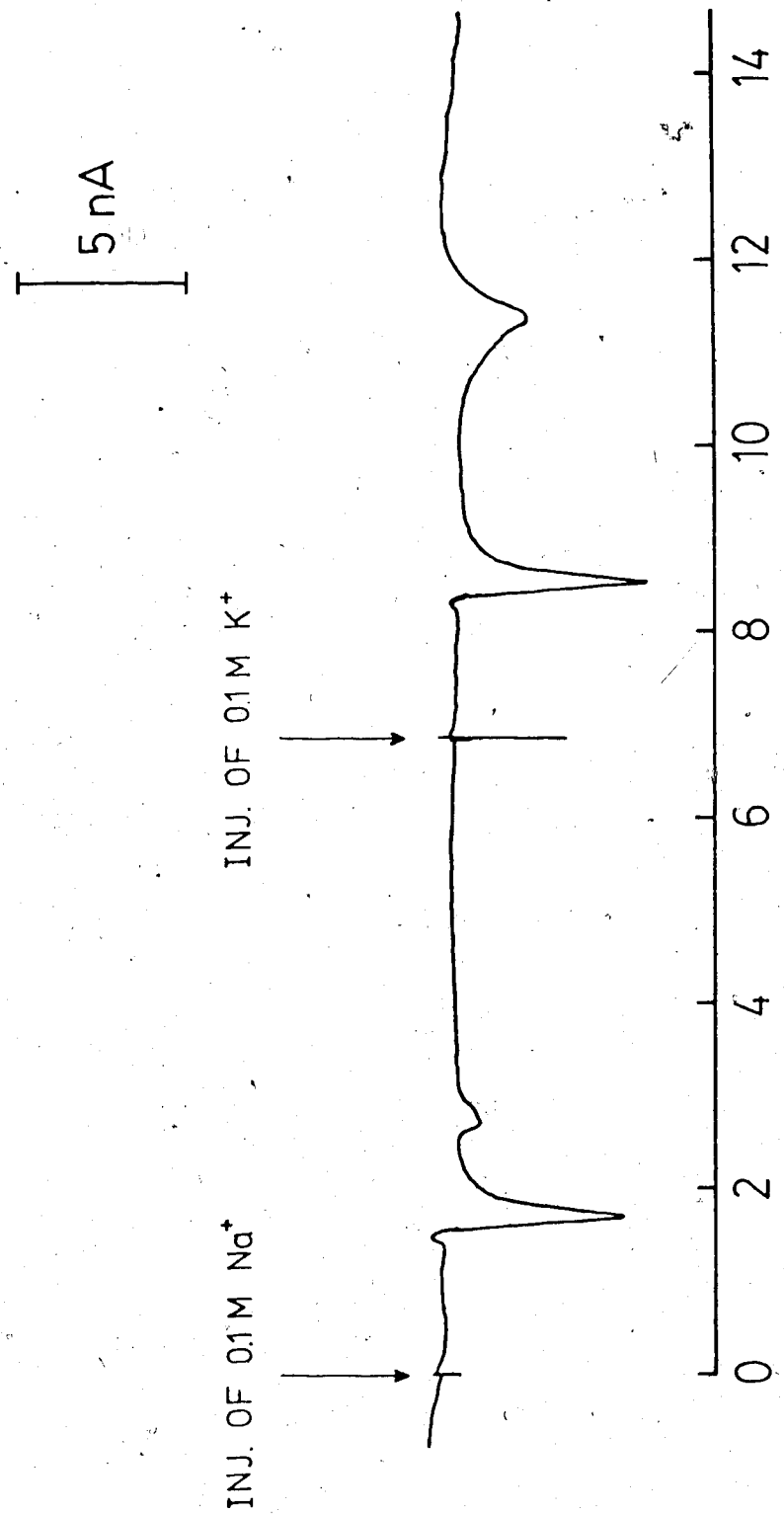
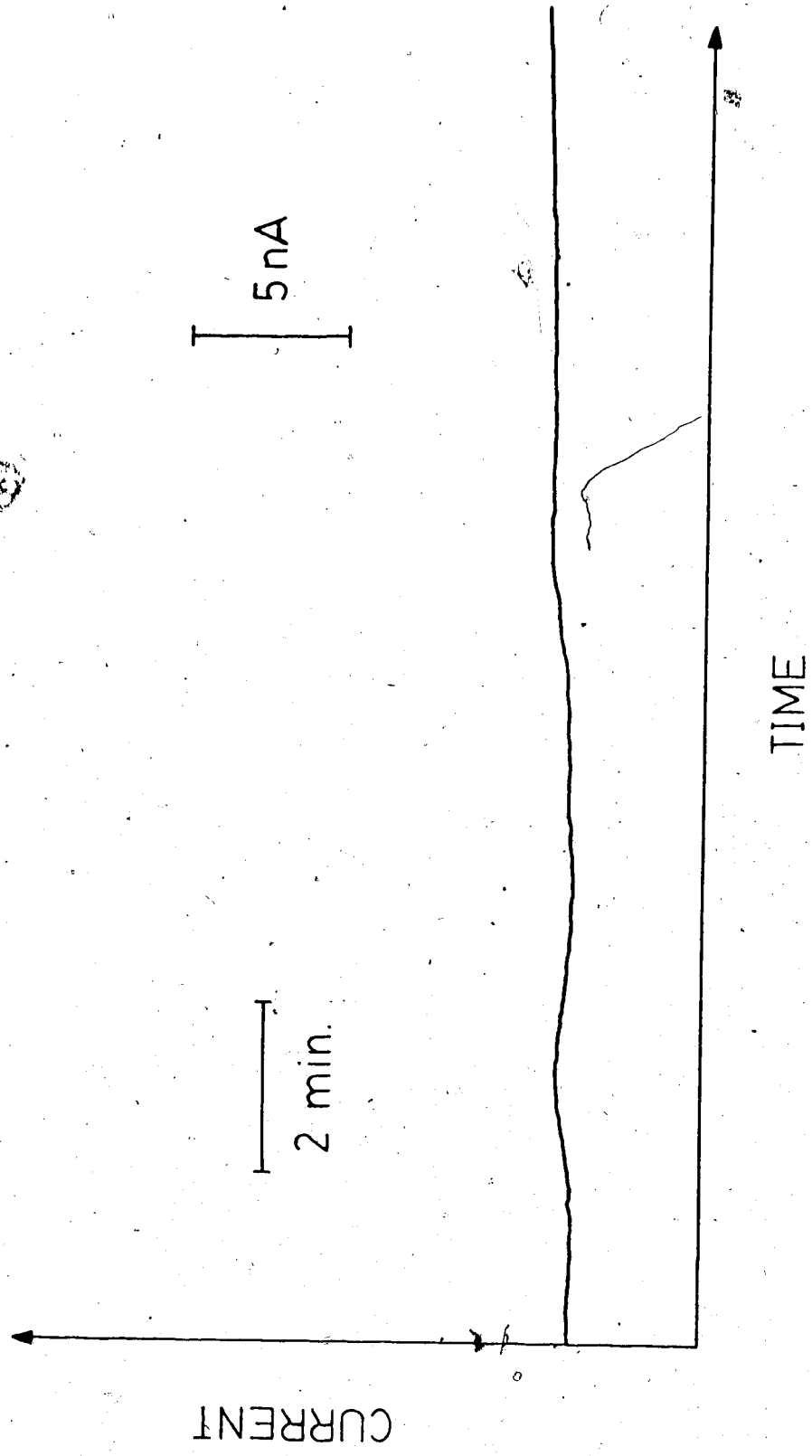

5 nA
INJ. OF 0.1 M Na+
INJ. OF 0.1 M K+
0
2
4
6
8
10
12
14
Minutes

Figure 10. Response of the mercury pool electrochemical detector to a stepped gradient elution.

Initial eluent : phosphate-citrate buffer of pH 2.5 and ionic strength of 0.04.

Final eluent : phosphate-citrate buffer of pH 3.0 and ionic strength of 0.08.


5 nA
2 min.
TIME
CURRENT

buffer of pH 2.5 and ionic strength of 0.04 was pumped through the system. The eluent reservoir was then switched to a phosphate-citrate buffer of pH 3.0 and ionic strength of 0.08. Figure 10 shows the detector response for the transition period between the two eluents. As can be seen, the baseline drift is about 1.5 nA. This is acceptable for practical applications since typical peak currents range from 1 to 50 nA.

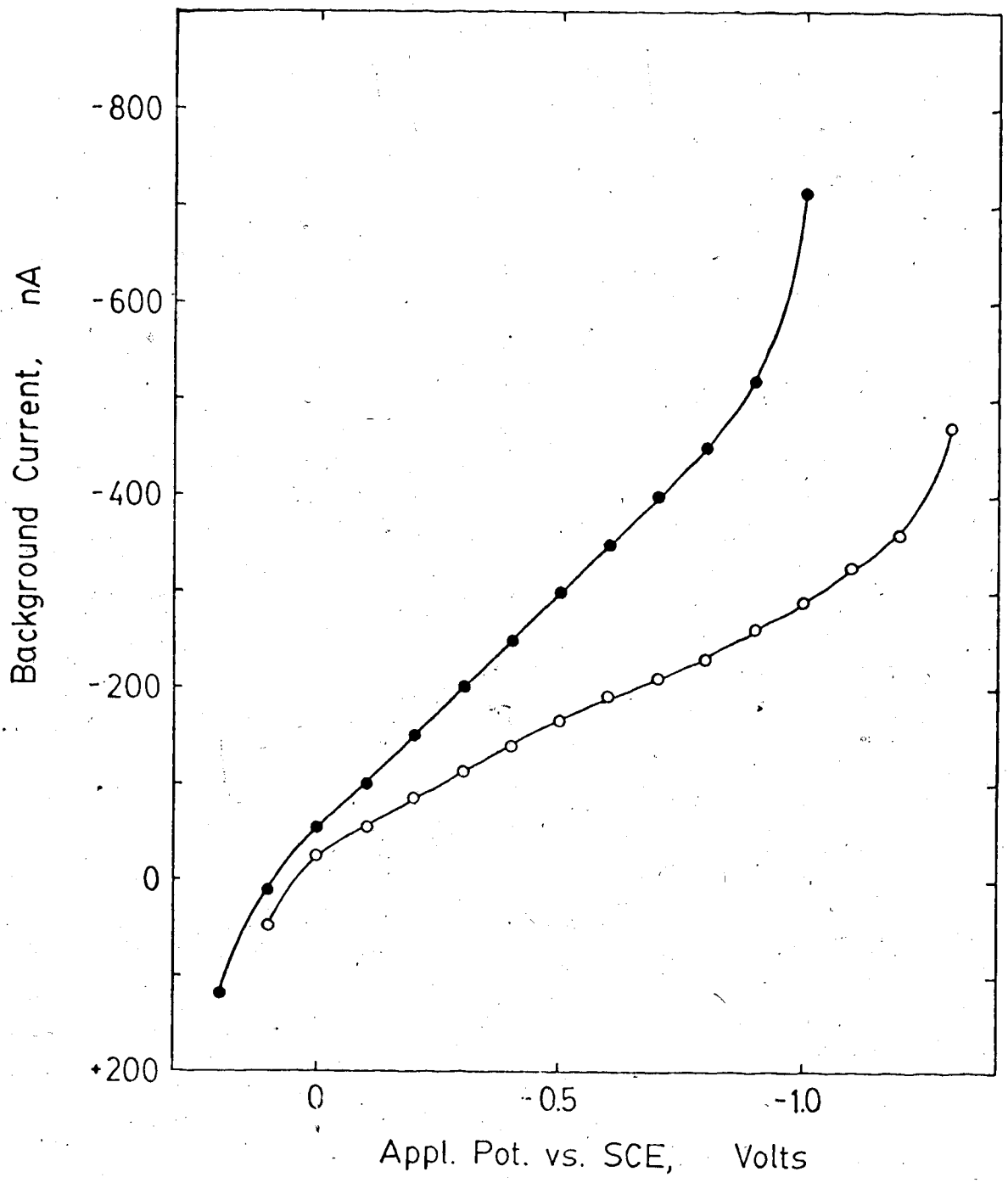
iii) Background Current

The background current of the Hg-based electrochemical detector is a function of pH as well as of ionic strength of the mobile phase, but shows no significant flow rate dependence in the range from 0.3 to 0.9 mL/min. Figure 11 shows how the background current changes with applied potential at pH 2.5 and pH 7.1. Both electrolytes had an ionic strength of approximately 0.35 and gave a total cell resistance of 8 kΩ ; most of which is the resistance of the SCE since no further decrease in cell resistance was measured at higher ionic strength. The most significant difference is the smaller potential range at lower pH which is due to the evolution of hydrogen at less negative potentials. At pH 2.5, the operating range is from +0.2 V to about -1.0 V vs. SCE, whereas at pH 7.1, the negative limit is about -1.3 V vs. SCE. At the positive end of the potential range, the background current is anodic and increases rapidly as the electrode potential is made

Figure 11. Background current of the detector in Figure 5 as a function of electrode potential.

●——●——● : citrate buffer of pH 2.5 and ionic strength of 0.35.

o——o——o : citrate buffer of pH 7.1 and ionic strength of 0.35.


-800
-600
-400
-200
0
+200
Background Current, nA
0
-0.5
-1.0
Appl. Pot. vs. SCE, Volts

more positive, due to Hg oxidation. At any given potential, the background current is constant with time (i.e. has a low noise level). Consequently, even though the peak current may be much less than the background current, it still can be measured precisely. In Figure 12, the background current vs. applied potential is shown for two citrate buffers having ionic strengths of 0.3 and 0.03. The pH's were 5.0 and 5.1, respectively. As can be seen, the background current decreases by about a factor of four with a decrease in ionic strength from 0.3 to 0.03, which indicates that it is due to electroactive impurities as well as traces of residual oxygen.

Since O_2 is electroactive over almost the entire working range of the detector, it is essential to keep its concentration at a negligible level. This is achieved by deaeration of the mobile phase and by keeping all the Teflon tubing in a nitrogen atmosphere. To indicate the severity of O_2 diffusion through the Teflon tubing, Figure 13 shows the background current vs. applied potential for the pH 7.1 buffer (ionic strength 0.35) with and without nitrogen surrounding the Teflon tubing. It is apparent that with the Teflon tubing in an air atmosphere, sufficient O_2 diffuses through to cause intolerable background currents at negative potentials as low as -0.2 V vs. SCE.

Figure 12. Background current of the detector in Figure 5 as a function of electrode potential.

●——●——● : citrate buffer of pH 5.0 and ionic strength of 0.3.

o——o——o : citrate buffer of pH 5.1 and ionic strength of 0.03.

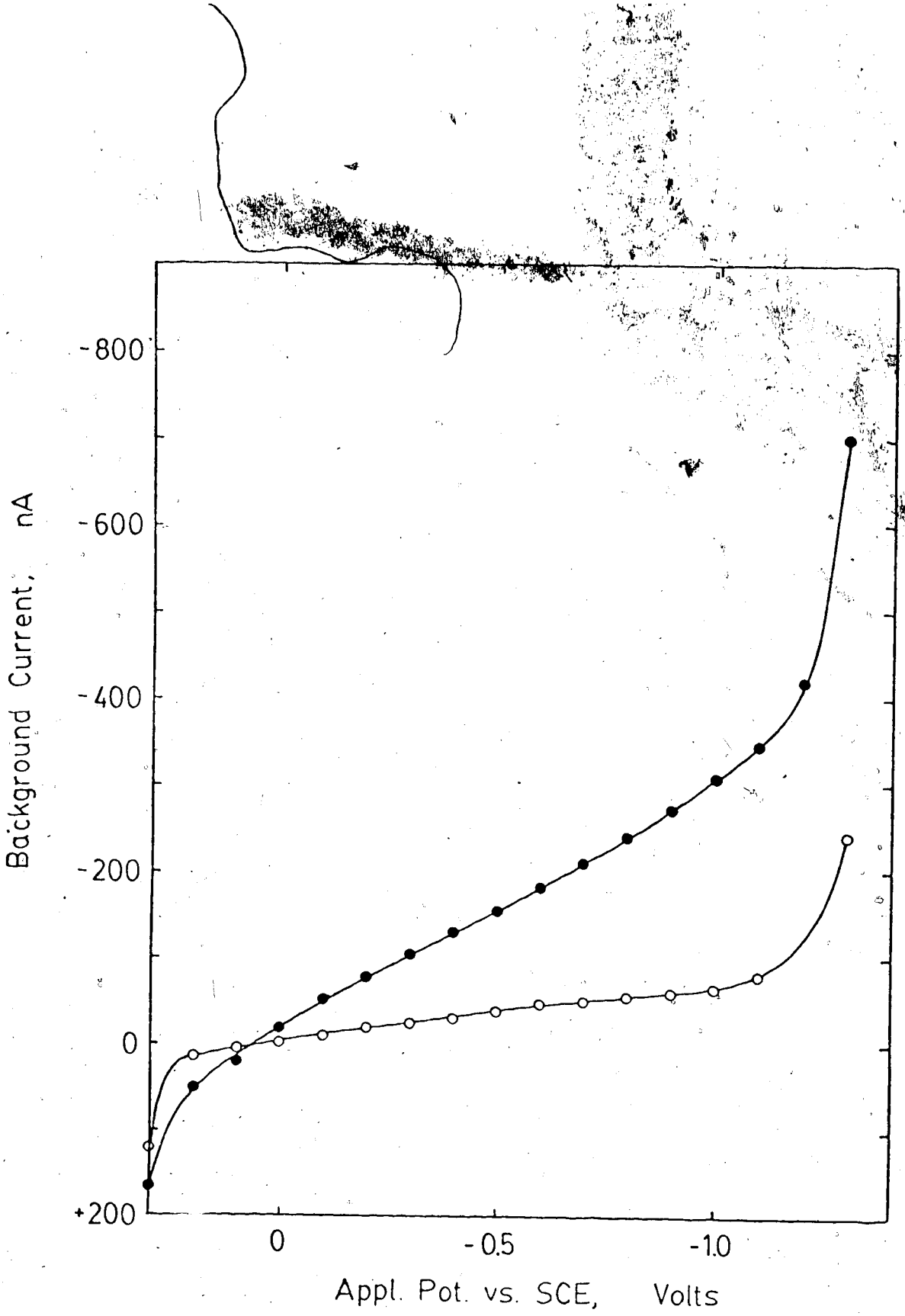
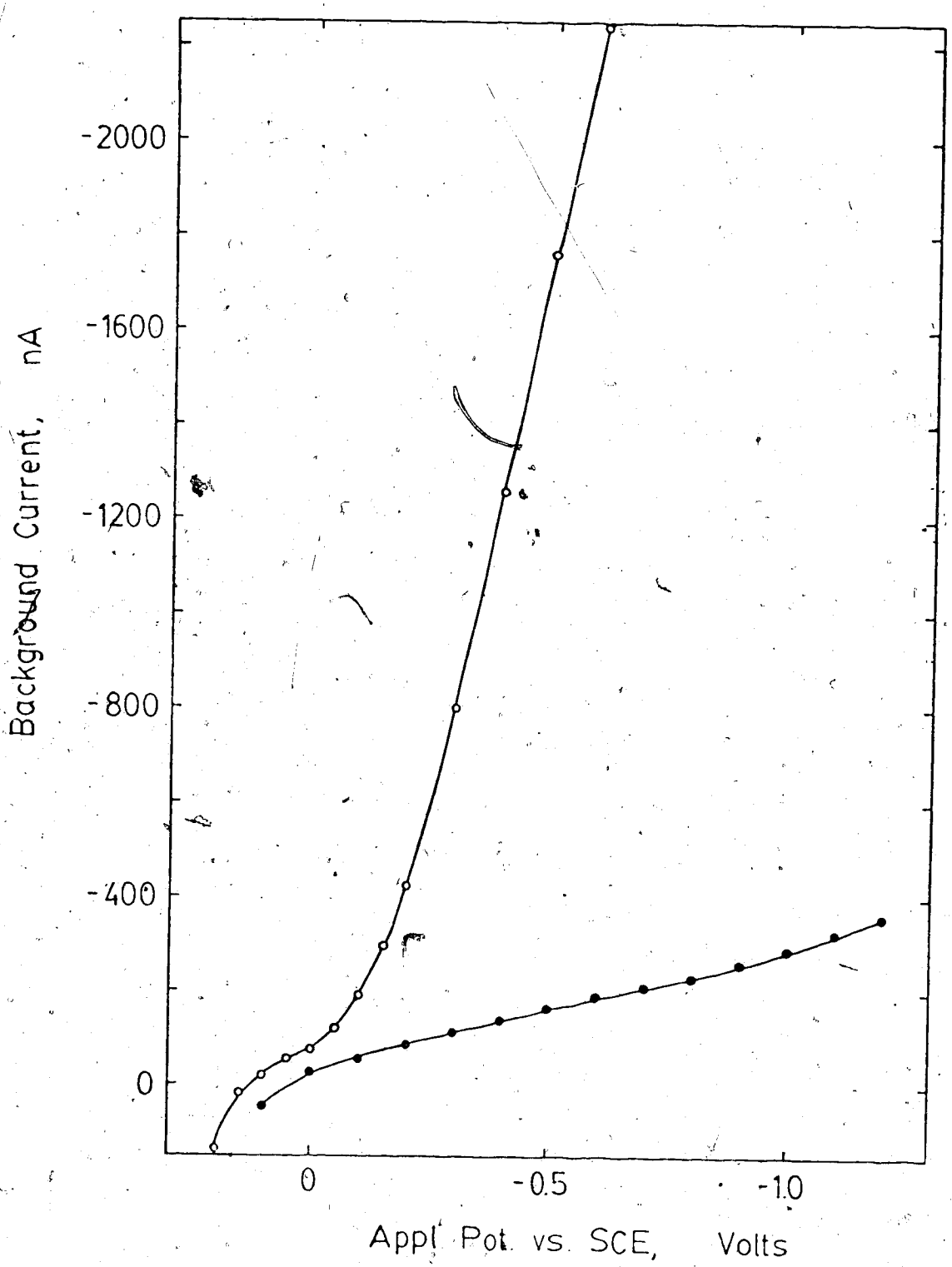

-800
-600
-400
-200
0
+200
Background Current, nA
0
-0.5
-1.0
Appl. Pot. vs. SCE, Volts

Figure 15. Background current of the detector in Figure 5 as a function of electrode potential for a citrate buffer of pH 7.1 and ionic strength of 0.35.

●——●——● : Teflon tubing kept under N_2.

o——o——o : Teflon tubing kept in air atmosphere.

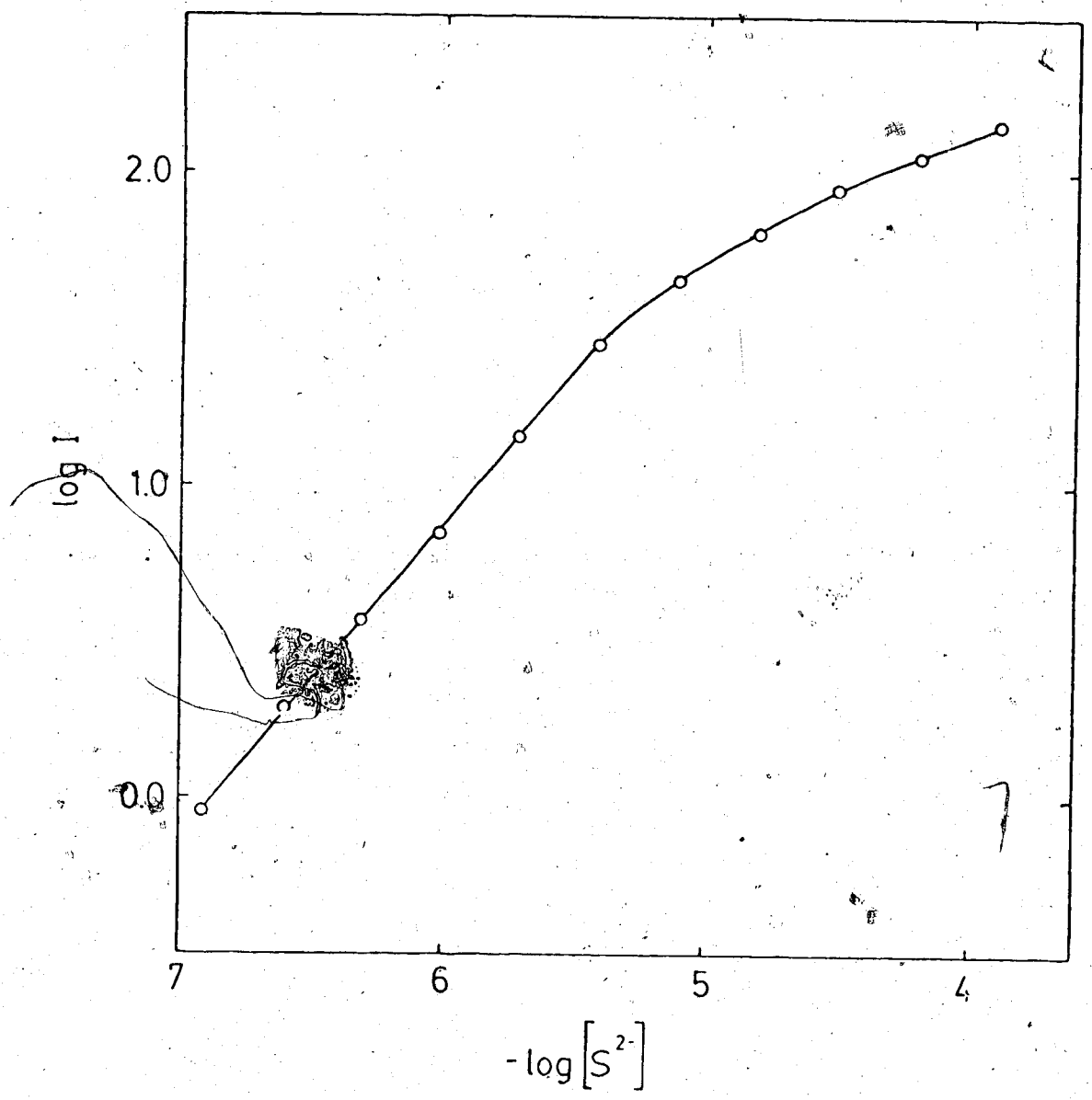

-2000
-1600
-1200
-800
-400
0
Background Current, nA
0
-0.5
-1.0
Appl Pot. vs. SCE, Volts

D. Detector Response to Electroactive Molecules

In principle, any compound electroactive within the working range of a Hg electrode is detectable with the Hg-based detector. However, at high enough concentration, some compounds may foul up the surface of the stationary Hg pool to such an extent that practical use is very limited. Species included in this category might be heavy metal ions which form amalgams after reduction, and sulfide which might cover the Hg surface with insoluble Hg(II) sulfide. To evaluate the general usefulness of the detectors, the response to silver ions and sulfide ions was briefly investigated by injection of approximately 100 µL of standard solutions with a six-position rotary valve injector. The detector was operated at 0.0 V vs. SCE and no column was used. Silver standards were prepared in 0.1 M $NaClO_4$ which was also used as eluent. The standards gave linear response below a silver ion concentration of about 10^{-4} M. No electrode contamination was observed within the time period of the calibration which indicates that the detector does have some application to the detection of electroactive metal ions in eluent streams.

The calibration curve obtained for sulfide, which is of more interest, at least from an environmental point of view, is shown in Figure 14. The solvent used was a borate-NaOH buffer of pH 9.6 which was also used for the preparation of the sulfide standards. A fresh Hg pool was

Figure 14. Calibration curve for sulfide obtained from 100-μL injections without using a column. A pH 9.6 borate-NaOH buffer was used as solvent, and the detector in Figure 7 was operated at 0.0 V vs. SCE. Current I is anodic peak current in nA. Concentrations are in units of moles/L.


2.0
1.0
0.0
log I
7
6
5
4
-log [S2-]

used, and the standards were injected in order of increasing concentration. As can be seen from Figure 14, the detector is very sensitive to sulfide with a limit of detection in this experiment of about 10^{-7} M sulfide. The calibration curve makes a break at about 5×10^{-6} M sulfide which is probably due to Hg surface effects. At higher concentrations of sulfide, the surface of the mercury slowly became covered with a black film of Hg(II) sulfide. These results indicate that a semi-continuous detection system for low levels of sulfide in aqueous solutions should be possible. A short anion-exchange column may be needed in front of the detector to sufficiently retain sulfide to avoid interference from charging-discharging currents, and an automated six-position rotary valve could inject samples at regular intervals.

The second category of compounds to which the detector will respond includes all molecules which form a Hg complex with a sufficiently high formation constant to depolarize the Hg electrode within its working range without fouling up its surface, for example thiols, and any electroactive species for which the Hg behaves as an inert electrode. To illustrate the response of the detector to biologically active sulfhydryl-containing molecules which have been of primary interest in this research, its response to GSH will be described.

Based on polarographic results for GSH (as

summarized by wave A in Figure 4), an electrode potential of 0.0 V vs. SCE is used for GSH detection. The polarography of GSH is complicated by adsorption and is still not completely understood. It is accepted, however, that the anodic current at 0.0 V vs. SCE is due to oxidation of the Hg to form HgSG and not to oxidation of the sulfhydryl group itself (78,79,162,163). If adsorption occurs on the Hg pool of the detector, it does not limit its use. Excellent reproducibility is obtained from successive injections of a given GSH concentration and, when the concentration is increased or decreased, the first peak from replicate injections is identical to others in the set. For example, the average peak current from four 8-μL injections of 10^{-3} M GSH in pH 7.0 buffer onto the 50 cm Amberlite cation-exchange column with a pH 7.0 mobile phase was 123.0 nA with a standard deviation of 0.9 nA.

The reproducibility can be affected by the sample medium if it is different from the mobile phase. For example, the procedure described by Ladenson and Purdy (52) has been used to prepare whole blood for analysis. The sample medium contains 50 g/L metaphosphoric acid which, when eluted from the column, gives rise to a large, unretained peak due to double layer capacitance effects. The GSH peak can be adequately resolved from this peak and from peaks due to other sulfhydryl compounds which might be present by using the

Zipax SCX column with a pH 2.5 mobile phase of low ionic strength (about 8×10^{-3}). However, with this sample medium, the reproducibility is poor, presumably due to irreproducible changes of the Hg surface. By application of a cleaning pulse of +0.6 V vs. SCE for 5 s between each injection, the reproducibility is improved. For example, the average peak current from fifteen 10-μL injections of 10^{-4} M GSH was 21.2 nA with a standard deviation of 0.17 nA. Using a lower concentration of metaphosphoric acid (20 g/L) made the use of a cleaning pulse unnecessary. Also, when mobile phases of higher ionic strength are permitted for the chromatographic separation, as for example for penicillamine and cysteine, no cleaning pulse is needed between sample injections.

To give some indication of the sensitivity of the Hg pool detector to organic sulfhydryl compounds when used with the PAR 174A Polarographic Analyzer, 2 μL of 5×10^{-6} M GSH, which corresponds to 3 ng of GSH, was injected onto the 50-cm Amberlite cation-exchange column. A peak current of 0.2 nA was obtained with a signal-to-noise ratio of 14:1, where S/N = average signal amplitude/RMS noise with RMS noise = average peak-to-peak noise/2.5. Calculations indicate approximately 0.5% of the GSH is consumed in the electrode reaction at a flow rate of 0.5 mL/min.

At an electrode potential of 0.0 V vs. SCE, the

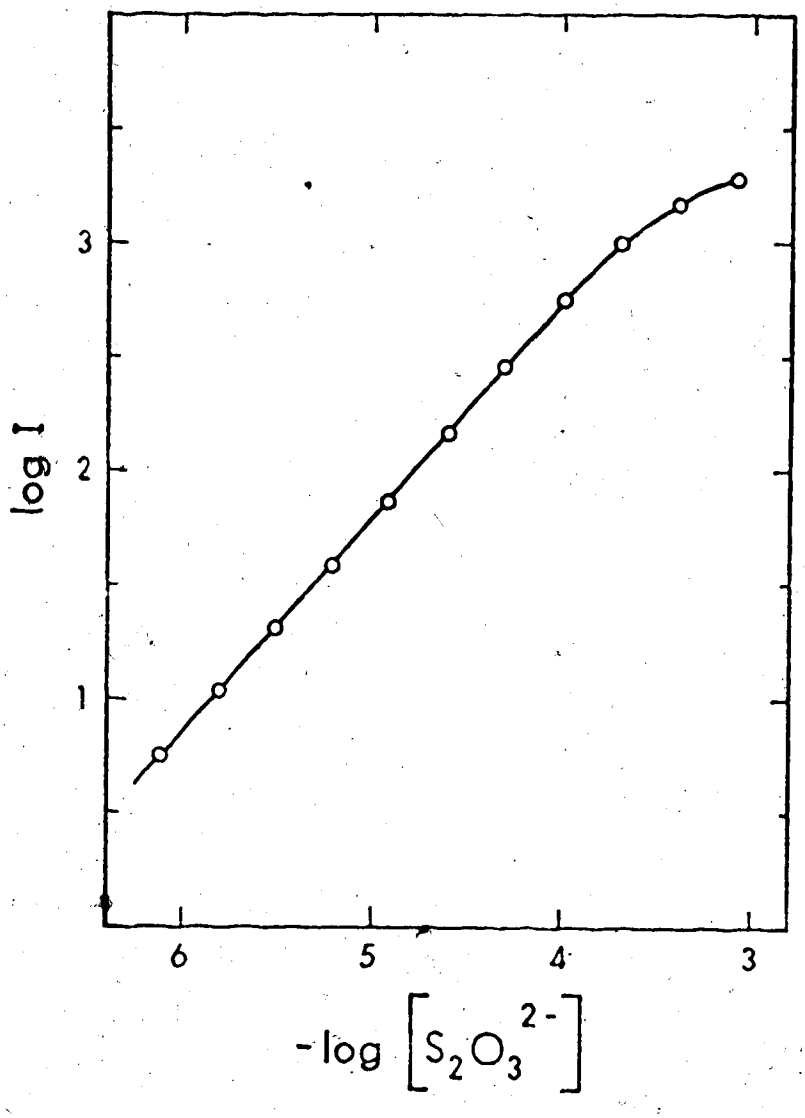
Hg pool detector is highly selective towards the sulfhydryl-containing amino acids in an amino acid mixture. Injection of 1 x 10^{-3} M solutions of the amino acids glycine, lysine, aspartic acid, glutamic acid, and histidine, caused no detector response at a 20-nA full scale sensitivity. As a result of this high selectivity, it is only necessary that the ion-exchange column separate the sulfhydryl compounds from each other and not necessarily from the other amino acids and peptides in the sample.

The response of the detector to disulfides at a potential of -1.2 V vs. SCE was briefly investigated. As pointed out in Chapter VI, the detection limit turned out to be too high to be applicable to biological samples.

The detector also responds to inorganic sulfur-containing molecules such as sulfite and thiosulfate, as illustrated by the calibration curve for thiosulfate in Figure 15. Sulfite showed a very similar calibration curve. Aliquots of 10 µL were injected onto a 0.2 x 50-cm column of 200-400 mesh Amberlite strongly acidic cation-exchange resin. The detector was operated at + 0.1 V vs. SCE. Figure 15 shows that the detector is extremely sensitive towards these ions.

To see how the detector behaved towards electroactive metal ion species which are reduced to soluble species of a lower oxidation state, a calibration curve

Figure 15. Calibration curve for thiosulfate using a pH 8.1 phosphate-citrate mobile phase. Aliquots of 10 µL were injected onto a 0.2 x 50-cm column of 200-400 mesh Amberlite strongly acidic cation-exchange resin. The electrochemical detector was operated at +0.1 V vs. SCE. Current I is anodic peak current in nA. Concentrations are in units of moles/L.


log I
3
2
1
6
5
4
3
-log [S2O3 2-]

for ferricyanide was obtained. The supporting electrolyte was 0.1 M KNO_3. About 100 µL of standard was injected with the six-position rotary valve without using a column. The detector which was operated at 0.0 V vs. SCE gave a linear response below 10^{-3} M ferricyanide. No significant flow rate dependence of sample peak current was observed in the range from 0.3 to 0.9 mL/min. Other metal ions or metal complexes which are not reduced to the metallic state, would be expected to show similar behavior.

CHAPTER IV

ION-EXCHANGE CHROMATOGRAPHY

A. General Principles

Small molecules which are present in solution as ions, are normally most conveniently separated by ion-exchange chromatography (164). The separation is based on a reversible stoichiometric exchange of ions between a stationary ion-exchange phase and an external liquid mobile phase. The cation-exchange process is represented by Equation 6

$$(R^-A^+)_r + B^+_{aq} \rightleftharpoons (R^-B^+)_r + A^+_{aq} \qquad (6)$$

and the anion-exchange process by Equation 7

$$(R^+X^-)_r + Y^-_{aq} \rightleftharpoons (R^+Y^-)_r + X^-_{aq} \qquad (7)$$

where A^+, B^+, X^-, and Y^- are counter-ions and $R^\pm$ is the fixed charge within the exchanger. The subscripts r and aq refer to the exchanger phase and the external solution phase (usually aqueous), respectively. A separation is obtained because of the different affinities of the solute ions for the ion-exchange phase.

Compounds such as amino acids which, depending on the solution pH, may have a positive or negative overall charge, can in principle be separated by

cation- or anion-exchange chromatography. The choice depends primarily on the distribution of the fractionally ionized forms of the amino acids to be separated. In this thesis, major interest has been focused on the separation of glutathione, penicillamine, and cysteine. The macroscopic ionization schemes for glutathione and penicillamine or cysteine are shown in Figures 16 and 17, respectively. For the fully protonated form of glutathione, four macroscopic acidity constants are obtained, i.e. $pK_1 = 2.12$, $pK_2 = 3.53$, $pK_3 = 8.66$, and $pK_4 = 9.62$ (165). The macroscopic deprotonation constants for penicillamine (166) and cysteine (167) are presented in Table 1. Using these constants, the distribution of the fractionally ionized forms of these molecules was calculated according to Laitinen and Harris (168) (Figures 18-20). From a comparison of the distribution of the anionic forms of cysteine and penicillamine as a function of pH, it is apparent that a separation of these two amino acids on an anion-exchange column might be expected to be very difficult without rigorous pH control. The more pronounced difference in species distribution at low pH would seem to favor a separation by cation-exchange chromatography with penicillamine being the more strongly retained.

The concentration equilibrium constant K_A^B for a cation-exchange process (Reaction 6) is given by:

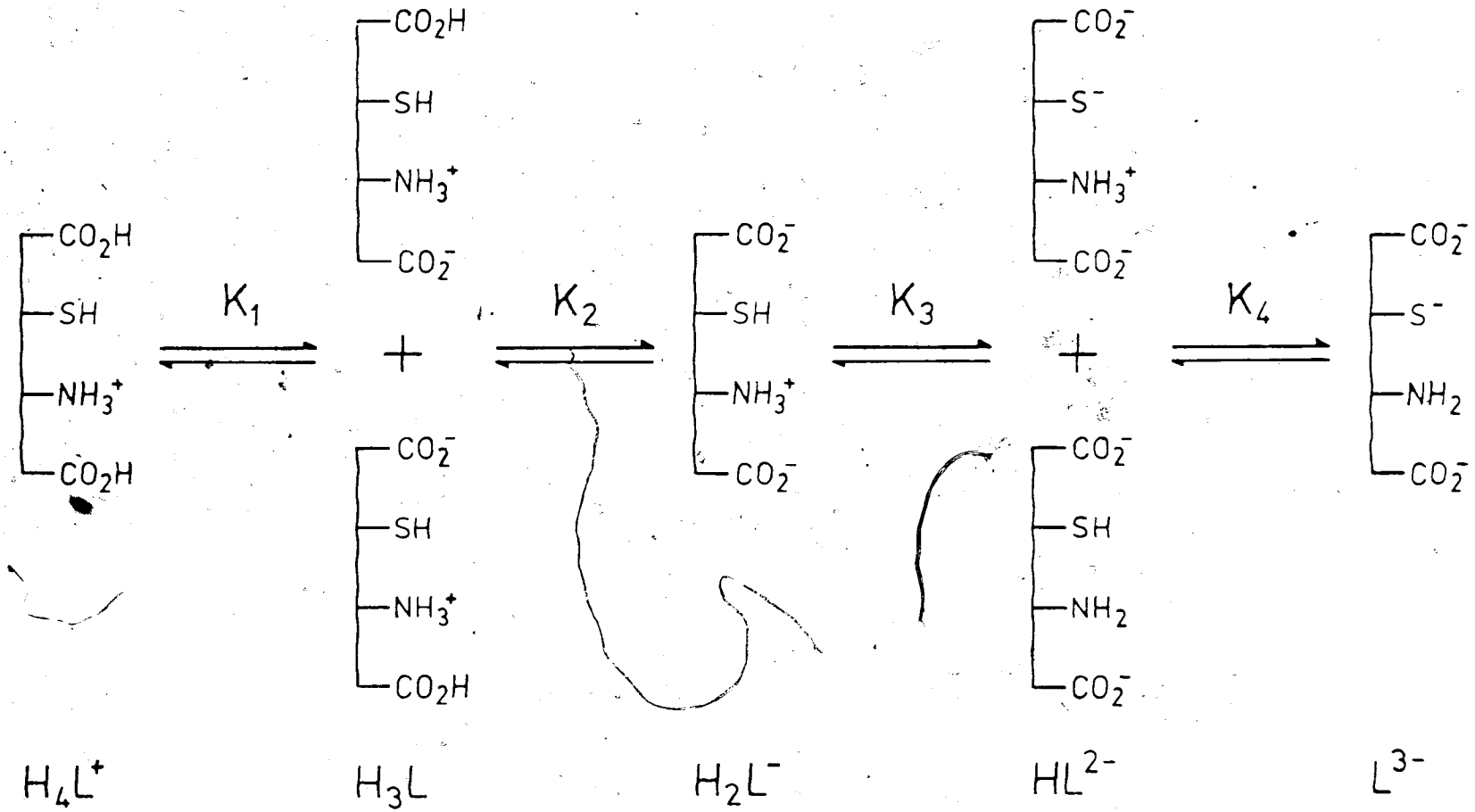


Figure 16. Macroscopic ionization scheme including the protonation isomers of the H_3L and HL^{2-} forms for glutathione.

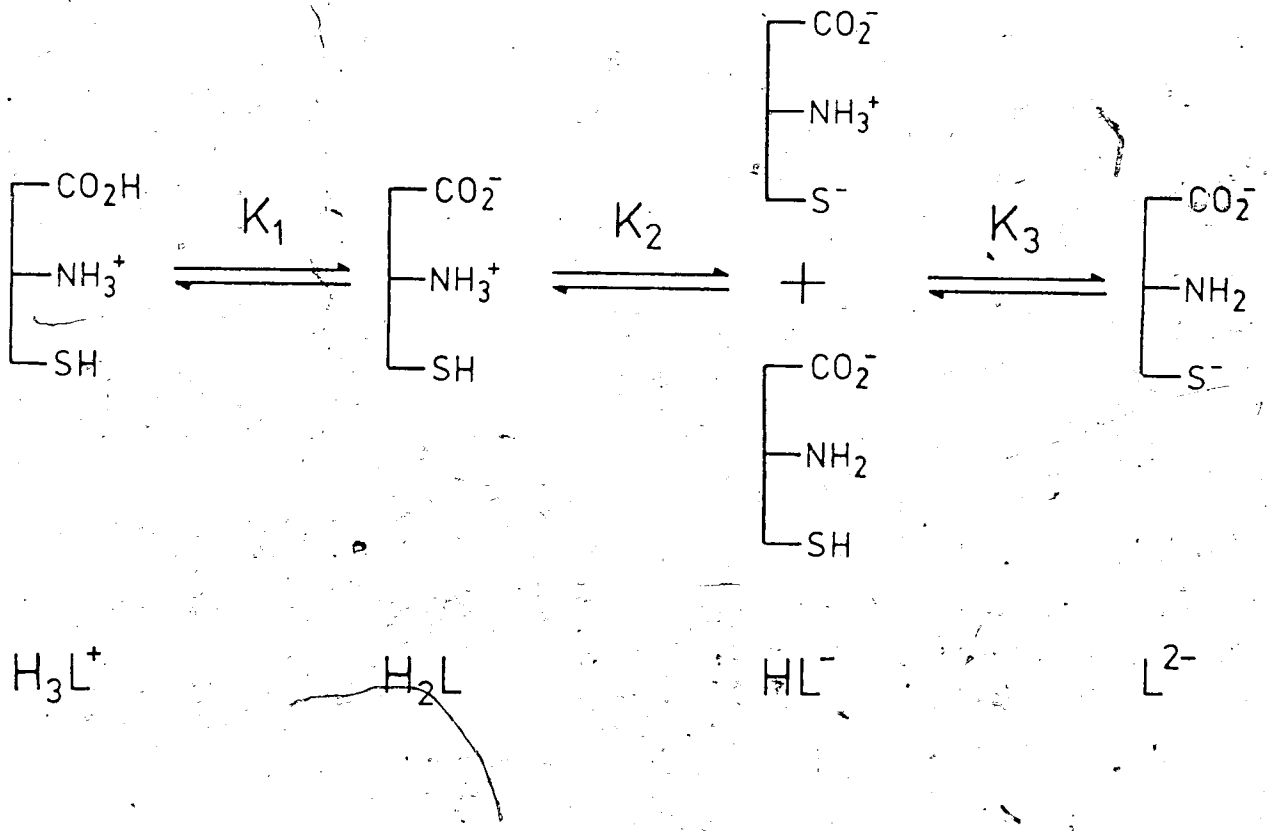


Figure 17. Macroscopic ionization scheme including the protonation isomers of the HL^- form for penicillamine or cysteine.

TABLE 1

Macroscopic Acid Dissociation Constants of Penicillamine and Cysteine

	Penicillamine	Cysteine
pK_1	2.44	1.77
pK_2	7.97	8.33
pK_3	10.46	10.78

Figure 18. pH dependence of the distribution of the fractionally ionized forms of glutathione.

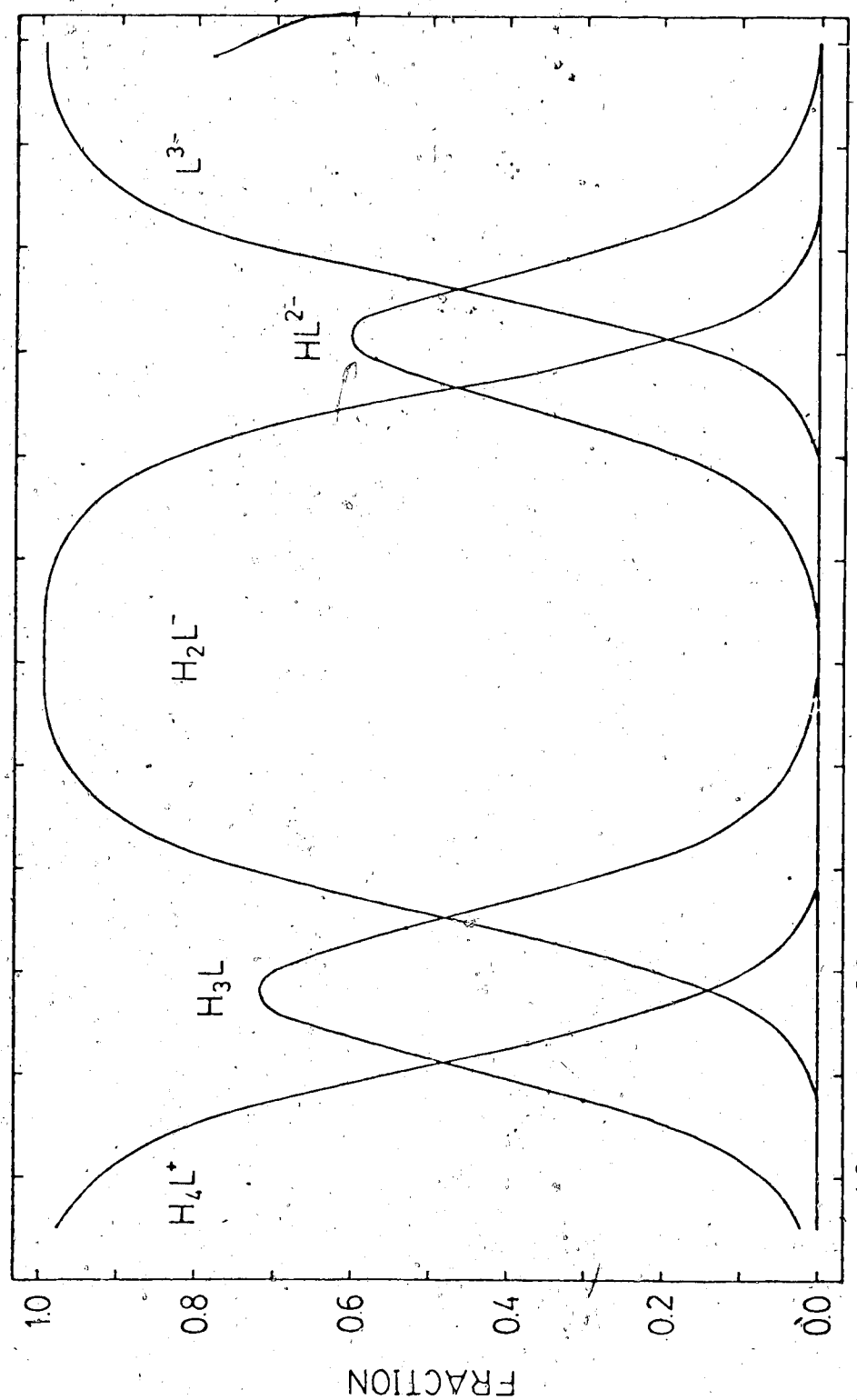

FRACTION
1.0
0.8
0.6
0.4
0.2
0.0
1.0
3.0
5.0
7.0
9.0
11.0
pH
H_4L^+
H_3L
H_2L^-
HL^{2-}
L^{3-}

Figure 19. pH dependence of the distribution of the fractionally ionized forms of penicillamine.

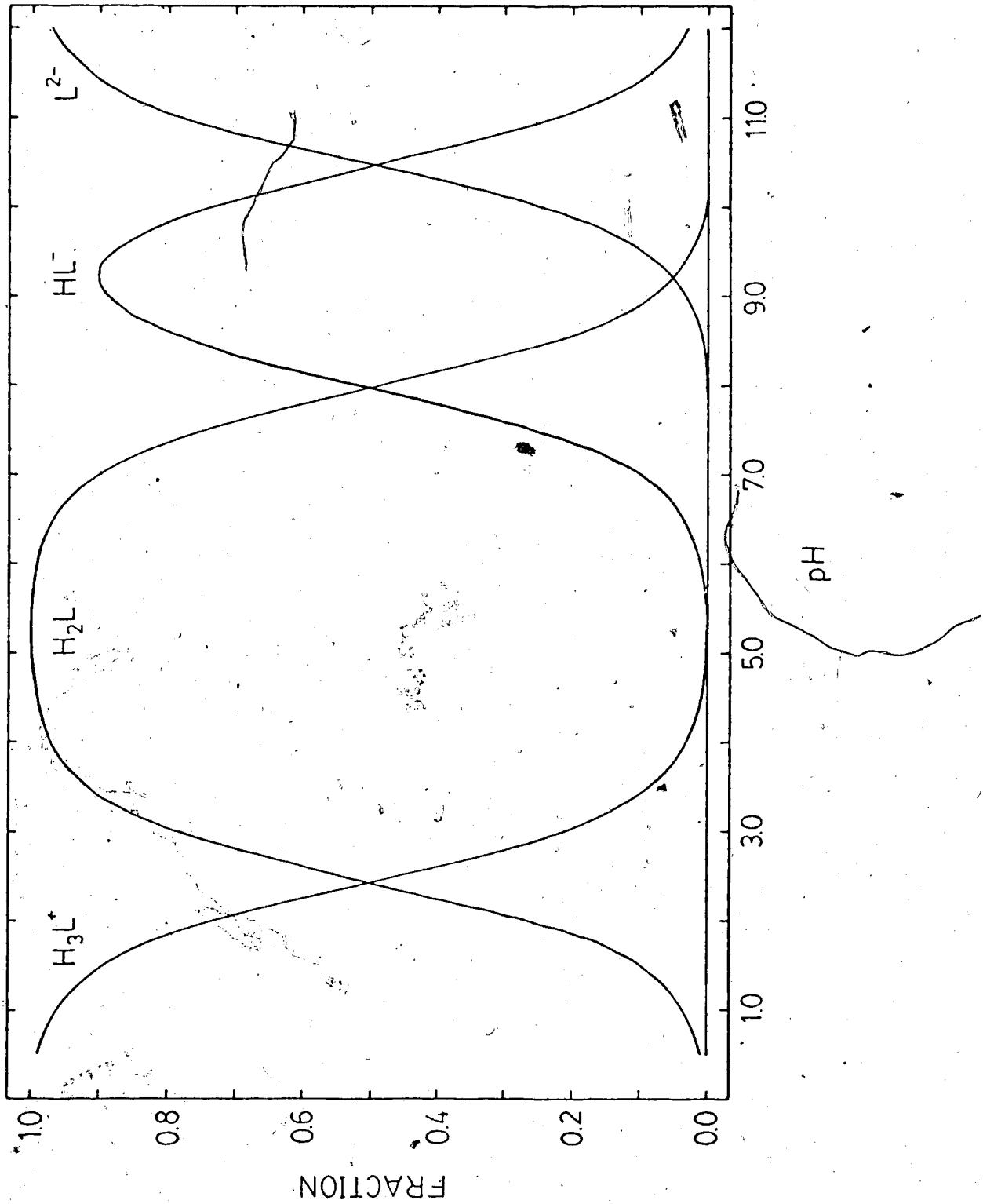
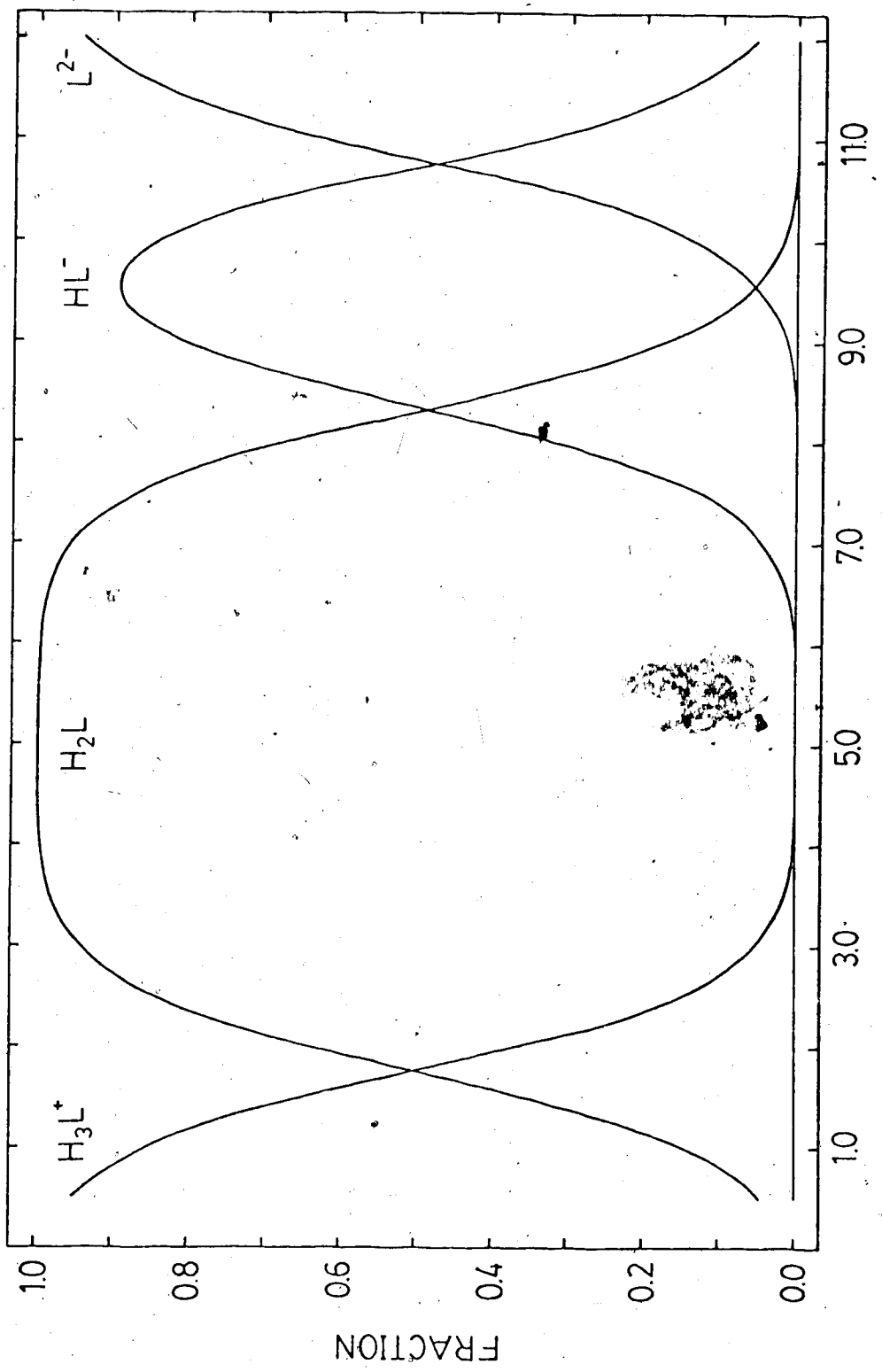

H_3L^+
H_2L
HL^-
L^{2-}
FRACTION
0.0
0.2
0.4
0.6
0.8
1.0
1.0
3.0
5.0
7.0
9.0
11.0
pH

Figure 20. pH dependence of the distribution of the fractionally ionized forms of cysteine.


FRACTION
1.0
0.8
0.6
0.4
0.2
0.0
H_3L^+
H_2L
HL^-
L^{2-}
1.0
3.0
5.0
7.0
9.0
11.0
pH

$$K_A^B = \frac{[R^-B^+]_r [A^+]_{aq}}{[R^-A^+]_r [B^+]_{aq}} \qquad (8)$$

If the resin is nearly completely in the A^+ form, and if $[A^+]_{aq} \gg [B^+]_{aq}$, trace exchange conditions prevail and the distribution coefficient K_B (Equation 9) becomes essentially constant, independent of $[B^+]_{aq}$ (169).

$$K_B = \frac{[R^-B^+]_r}{[B^+]_{aq}} = K_A^B \frac{[R^-A^+]_r}{[A^+]_{aq}} = \text{const.} \qquad (9)$$

As soon as $[B^+]_{aq}$ approaches the concentration of A^+ in the aqueous phase, K_B will decrease with increasing $[B^+]_{aq}$. Consequently, trace exchange conditions, which are necessary for linear elution chromatography, require low sample loading compared to the exchange capacity of the ion exchanger and moderately high ionic strength in the mobile phase.

The capacity factor (170),

$$k' = \frac{t_r - t_o}{t_o} = \frac{n^s}{n^m} = \frac{[R^-B^+]_r V_s}{[B^+]_{aq} V_m} \qquad (10)$$

of a sample component expresses the affinity of the resin for that component. In Equation 10, t_r is the retention time for a sample component, t_o the retention time for an unretained component, and n^s the total

number of moles in the stationary phase of the sample component in equilibrium with the total number of moles n^m of the component in the mobile phase. V_s and V_m are the volumes of the stationary and mobile phases, respectively. In the experiments described in Chapters V-VIII, the retention time for the unretained component was determined from the charging-discharging peak. For cation-exchange chromatography, the capacity factor of a weak acid (HA) with a dissociation constant

$$K_{eq} = \frac{[A^-]_{aq}[H^+]_{aq}}{[HA]_{aq}} \qquad (11)$$

may be influenced markedly by changes in pH. By combining Equations 9, 10, and 11, it can easily be shown that, at constant ionic strength, a decrease in pH will increase k' due to the increase in the concentration of the protonated form of the sample component (171). It can also be shown that, at constant pH, k' theoretically is proportional to the reciprocal of the counter-ion concentration in the eluent (171). The value of k' can also be adjusted by the type of counter-ion used in the mobile phase, i.e. mobile phases containing Li^+, H^+, or Na^+ would represent weak eluents, whereas mobile phases containing divalent metal ions would be strong eluents (172).

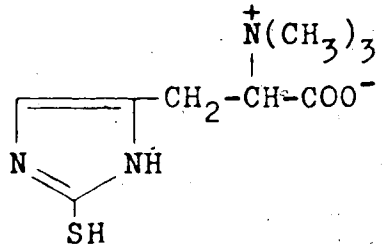
In most processes which involve columns with conventional ion-exchange resins such as the Amberlite resins, the rate determining step is solute diffusion inside the resin particle (173-175). In modern HPLC, this problem of long diffusion paths has been minimized by the introduction of ion-exchange resins confined to a thin layer on a suitable solid core. The pellicular ion exchangers or controlled surface porosity ion exchangers (173-175) such as Corasil (Waters), Vydac (Separations Group), and Zipax (Du Pont) comprise one group of these nonconventional ion exchangers. With these stationary phases, the speed with which a separation can be performed is increased, and the band spreading due to resistance to mass transfer in the stationary phase is decreased. Because of the presence of a solid core which may give rise to adsorption-desorption phenomena, their behavior is rarely exactly predictable from conventional ion-exchange chromatography. The exchange capacity of the pellicular ion exchangers is low (typically 1-10 microequivalents/g), and thus a sensitive detector is required.

B. Results

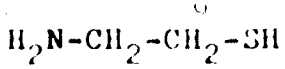
In this section, the results of an investigation of the chromatographic behavior of glutathione, penicillamine, cysteine, and several other thiols on cation exchangers are presented. Their separation on Amber-

lite strong anion-exchange resin and on Zipax SAX strong anion exchanger was also investigated, but only briefly because the species distribution diagrams in Figures 18-20 indicate that the separation should be considerably easier on cation-exchange resins. Also, oxidation of the thiols is expected to be less of a problem at the low pH of the eluents needed for separation by cation-exchange as indicated by the results in Figures 18-20.

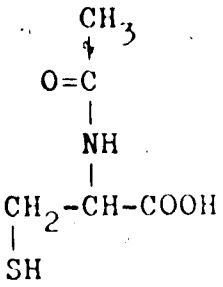
For the initial separation studies with cation exchangers, Amberlite 200-400 mesh strong cation-exchange resin was used. For reasons discussed in Section A, the resin was soon replaced with Zipax SCX strong cation exchanger. To obtain a general indication of its retention characteristics, several biologically active sulfhydryl-containing molecules were studied under different conditions on a 0.2 x 30-cm Zipax SCX column. The mobile phases were all phosphate-citrate buffers. Net retention times vs. mobile phase pH for glutathione, cysteine, penicillamine, ergothioneine (XIII), homocysteine, cysteamine (XIV), and N-acetyl-cysteine (XV)



(XIII)



(XIV)



(XV)

are presented in Figure 21. The analytical procedures described in Chapters V-VIII are based on these results. Since the ionic strength of the mobile phase decreased from 0.2 to about 0.02 when changing the pH from 4.1 to pH 2.2, the increase in retention time is a result of a decrease in pH as well as in ionic strength. As can be seen, N-acetyl-cysteine, which is not protonated to form a cationic species, is unretained under all tested conditions, and an anion-exchange resin would probably be a better choice for this molecule. To illustrate the ease with which some of the studied thiols can be separated under isocratic conditions on the 0.2 x 50-cm Zipax SCX column, Figure 22 shows a chromatogram of a mixture of N-acetyl-cysteine, glutathione, cysteine, and homocysteine. The

Figure 21. Net retention times vs. mobile phase pH for several biologically active sulfhydryl-containing molecules.

o —— o —— o : Glutathione

Δ —— Δ —— Δ : Cysteine

□ —— □ —— □ : Penicillamine

× —— × —— × : Ergothioneine

● —— ● —— ● : Homocysteine

▲ —— ▲ —— ▲ : Cysteamine

■ —— ■ —— ■ : N-acetyl-cysteine

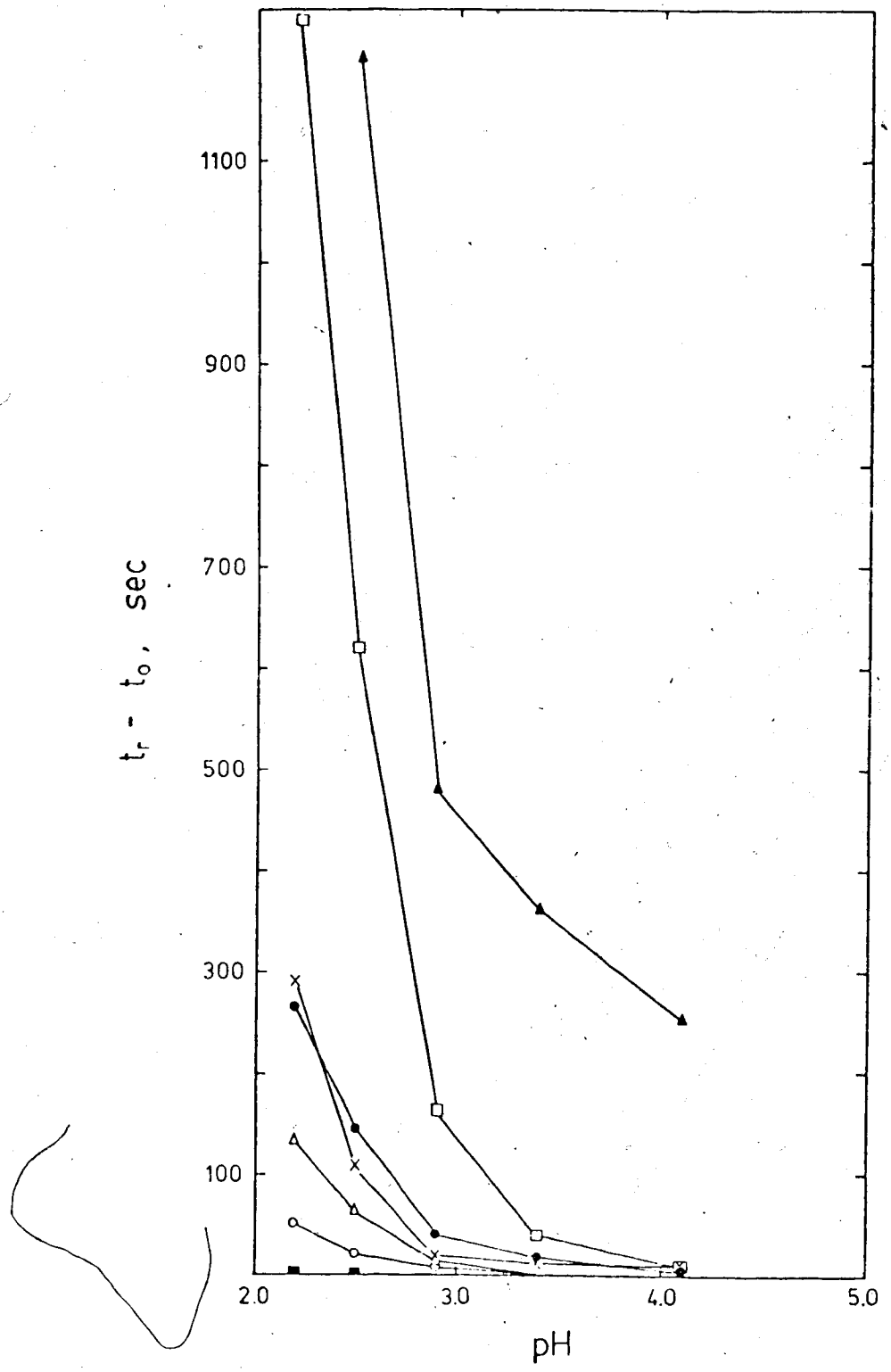
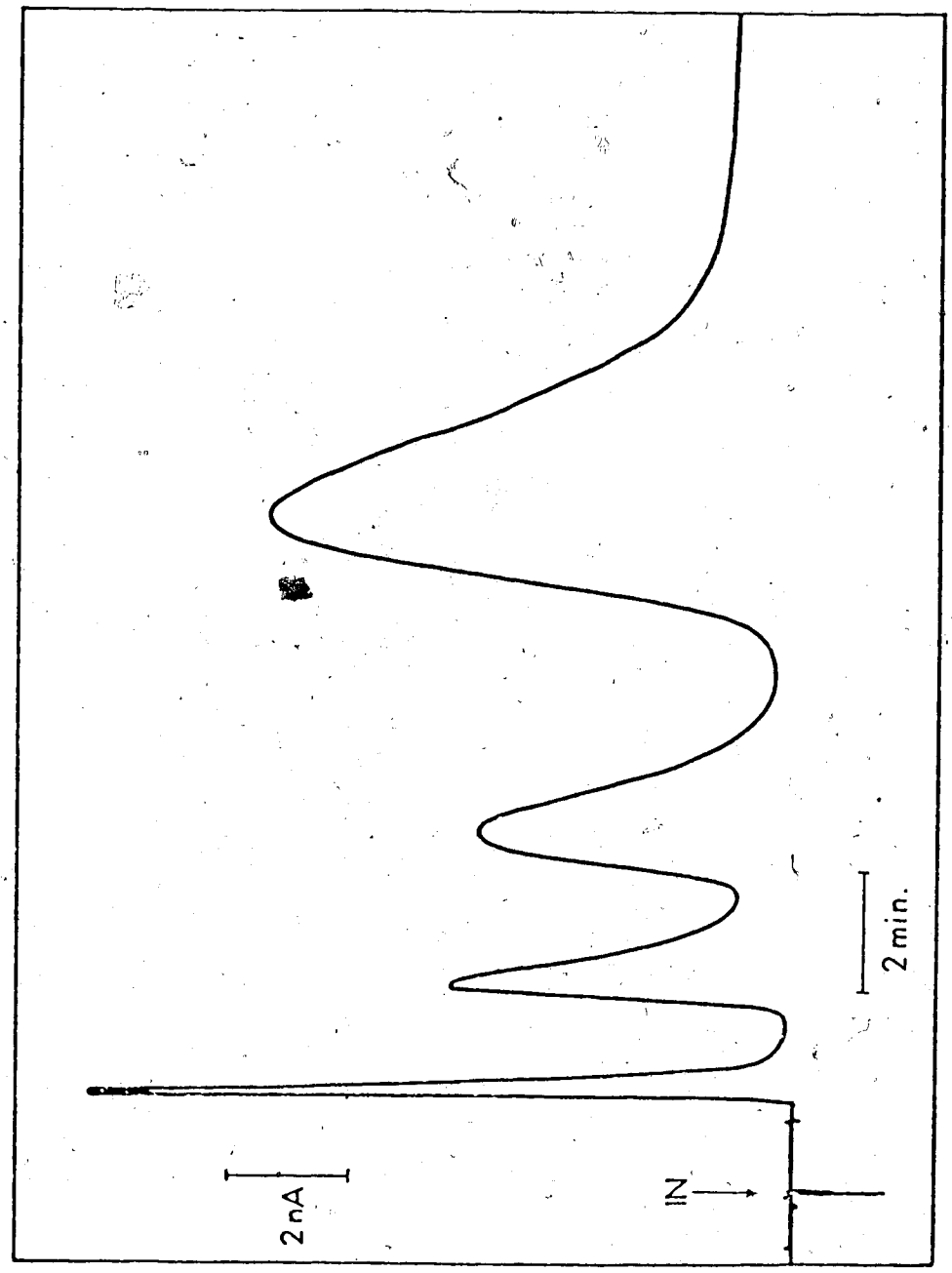

1100
900
700
500
300
100
$t_r - t_o$, sec
2.0
3.0
4.0
5.0
pH

Figure 22. Response of the mercury pool electrode detector to a 10-μL injection of a solution containing 1×10^{-5} M N-acetyl-cysteine, 4×10^{-5} M glutathione, 4×10^{-5} M cysteine, and 1×10^{-4} M homocysteine. A 0.2 x 50-cm column of Zipax SCX cation exchanger was used with a pH 2.5 phosphate-citrate mobile phase. The flow rate was 0.5 mL/min. Order of elution: N-acetyl-cysteine, glutathione, cysteine, and homocysteine.


2 nA
N
2 min.

mobile phase was a pH 2.5 phosphate-citrate buffer with an ionic strength of 0.01. N-acetyl-cysteine appeared unretained, but no injection artifacts were observed in the chromatogram since the mixture was prepared in mobile phase.

As can be seen from Figure 21, glutathione is rather weakly retained on the Zipax SCX resin beads. A mobile phase of pH 2.5 gives a k' of about 1.4 at the low ionic strength of about 8×10^{-3}. Since the blood matrix in which glutathione is to be determined gives rise to an unretained charging-discharging peak with the electrochemical detector, a k' value of this order is needed to avoid any interference from the unretained peak. It would, however, be desirable to increase the ionic strength of the mobile phase from the point of view of small uncompensated detector cell resistance and maintenance of trace exchange conditions. A decrease in pH below 2 did not change k' significantly. This is mainly due to the counteracting effects of an increase in ionic strength (proton ion concentration) and an increase in the concentration of the fully protonated form of glutathione (H_4L^+). The effect of changing the mobile phase counter-ion from Na^+ to H^+ which should make the eluent weaker, is also counteracted by the increased ionic strength. The possibility of changing the mobile phase cation from Na^+ to Li^+

for which the resin should have the lowest selectivity (172), was therefore investigated. Several buffers made up of LiOH and citric acid or phosphoric acid were tested. The observed k' values increased only slightly as compared to the values obtained with the eluents containing Na^+. In addition, non-Faradaic detector response to retained Na^+ ions in the sample became a problem as the Na^+ ions happened to have approximately the same retention time as glutathione.

The number of theoretical plates N (176) calculated from

$$N = 16\left(\frac{t_r}{W_t}\right)^2 \qquad (12)$$

was about 300 for the 0.2 x 50-cm Zipax SCX column, which corresponds to a height equivalent of a theoretical plate (HETP) of 0.17 cm. In Equation 12, t_r is the retention time of a sample component and W_t is the base width in units of time of the peak obtained for that component.

CHAPTER V

DETERMINATION OF GLUTATHIONE IN WHOLE BLOOD

A. Introduction

A variety of assay methods has been reported for glutathione in erythrocytes. Apart from the enzymatic assays (110-116) which tend to be somewhat tedious and involved, all methods for the determination of glutathione either lack selectivity or sensitivity. The success of many of them for the determination of glutathione in whole blood is solely due to the high concentration of glutathione as well as its large excess over other nonprotein thiols.

In this chapter, a highly selective and sensitive method for the determination of glutathione in whole blood is described. The method is based on a separation of glutathione from other nonprotein thiols by high performance cation-exchange chromatography followed by detection with a mercury-based electrochemical detector (LCEC). Results for several normal blood samples are presented and compared to the results obtained with the DTNB method.

B. Chromatographic Conditions

A pH 2.5 phosphate-citrate buffer with an ionic strength of about 8×10^{-3} was used as eluent. For the separation step, the 0.2 x 50-cm Zipax SCX column was

used, and the flow rate was 0.5 mL/min. The electrochemical detector was operated at 0.0 V vs. SCE. With these conditions, a capacity factor of 1.4 was obtained for glutathione.

C. Results

To find suitable conditions for the storage of standard glutathione (GSH) solutions, the stability of several GSH solutions was studied. The concentration of GSH was made 1.00×10^{-3} M and, in one experiment, the solvent was doubly distilled water (solution pH 3.5), whereas in a second experiment, the water contained 1 g/L $Na_2H_2EDTA \cdot 2H_2O$ (solution pH 3.8). All solutions were kept in 10-mL volumetric flasks and stored at room temperature or in a refrigerator at 4° C. The stability of the GSH in the two solutions stored at room temperature and in the EDTA-containing solution stored at 4° C is shown as a function of time in Figure 23. The concentrations in Figure 23 were obtained by comparing the peak height obtained chromatographically to that obtained for a freshly prepared GSH solution. The GSH concentration in the solution containing no EDTA which was stored at 4° C, decreased by 2-3% after two weeks. Apparently, the added EDTA complexes trace heavy metal ions which otherwise catalyze the air-oxidation of GSH.

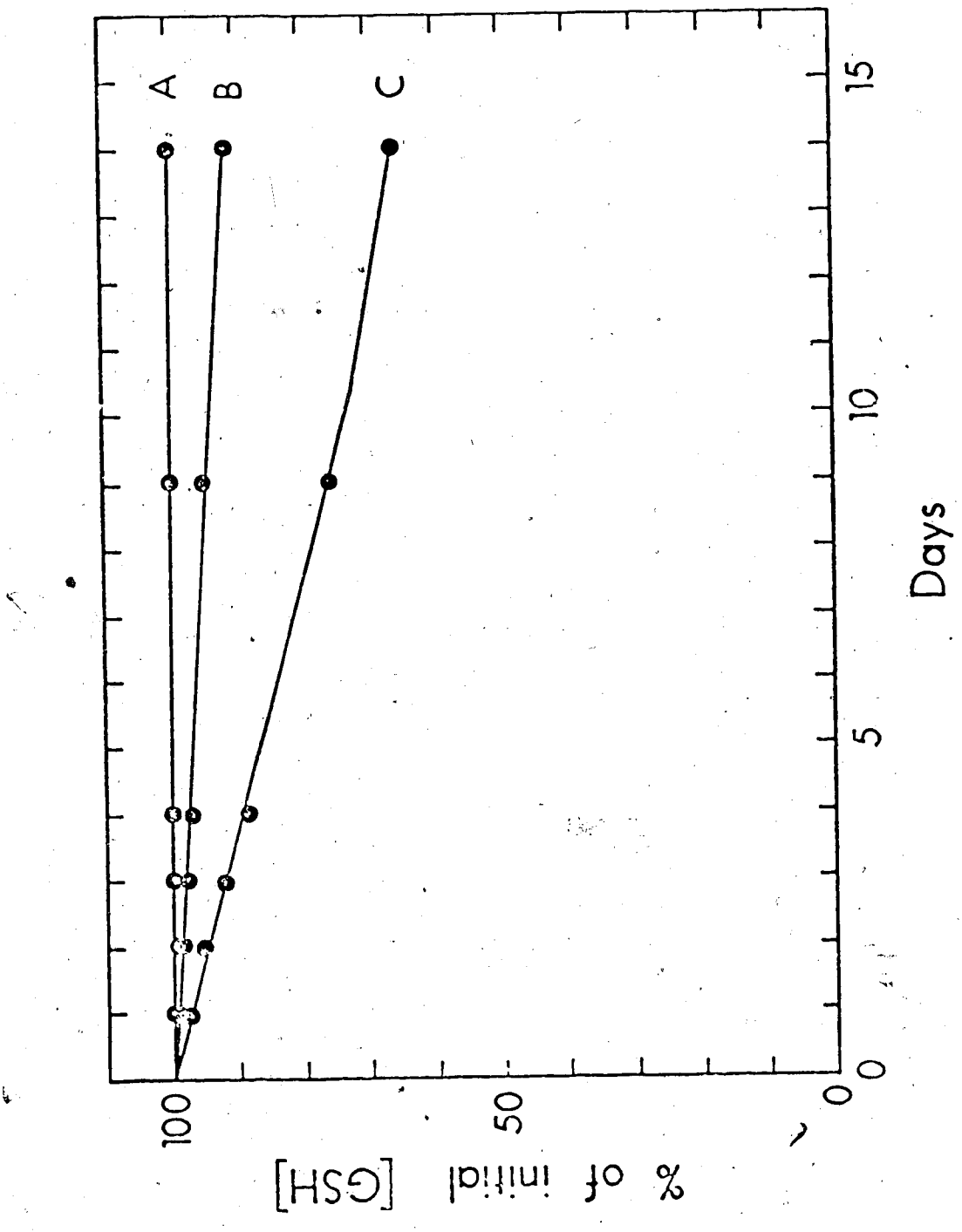
The procedure used for the preparation of the whole blood filtrates was a slightly modified version

Figure 23. Time course for the stability of 1.00×10^{-3} M GSH

(A) in distilled water containing 1 g/L $Na_2H_2EDTA \cdot 2H_2O$ when stored at 4° C

(B) in distilled water containing 1 g/L $Na_2H_2EDTA \cdot 2H_2O$ when stored at room temperature

(C) in distilled water when stored at room temperature.


A
B
C
100
50
0
% of initial [GSH]
0
5
10
15
Days

of that described by Ladenson and Purdy (52). After the protein precipitation step, most of the samples were filtered through a medium fast 4.25-cm filter rather than centrifuged. Both methods gave identical results when compared, and since the filtration step is a few minutes faster than centrifugation, a significant amount of time might be saved when performing routine GSH analyses.

An EDTA solution of 100 mg/L was used by Ladenson and Purdy for the hemolysis step. When using this solution, however, some of the samples tested showed a slow decrease in GSH concentration after having been filtered. Increasing the concentration of EDTA to 1 g/L stabilized the filtrates, probably because of a more complete complexation of heavy metal ions present in the sample.

For the protein precipitation step, a metaphosphoric acid concentration of 100 g/L was used rather than the 250 g/L used by Ladenson and Purdy. This did not affect the stability of the treated samples, and the double layer capacitance effect of the electrochemical detector for the unretained component became less serious. It also eliminated the need for a "cleaning pulse" as discussed in Chapter III, Section D.

A typical chromatogram of GSH in a whole blood filtrate is shown in Figure 24. As can be seen, the elution of GSH takes about 5 min. Ergothioneine, which

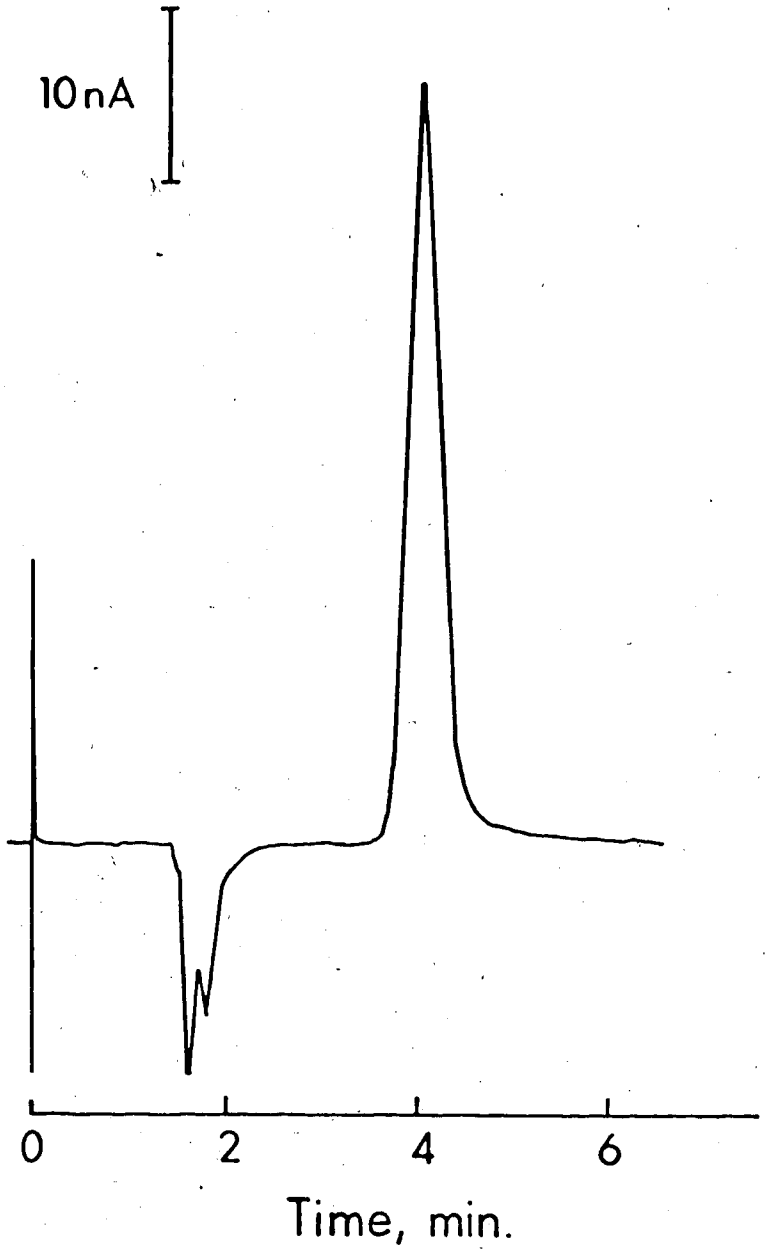


Figure 24. Representative chromatogram of a treated whole blood sample. Injected volume was 10 µL containing 290 ng GSH.

has been reported to interfere in all methods except the enzymatic ones, gave a capacity factor of 4.6 and thus did not interfere. However, no ergothioneine peak was observed in any of the tested blood samples. A calibration curve was used to quantify glutathione, with standard GSH solutions injected between every five samples. As shown in Figure 8, the calibration curve is linear up to about 5×10^{-4} M GSH at which concentration the curve becomes slightly concave due to the effects of uncompensated detector cell resistance. Standard GSH solutions were prepared in 20 g/L metaphosphoric acid. Since cysteine is eluted shortly after GSH, the possibility of improving the reproducibility of the results from replicate injections using this amino acid as internal standard was briefly investigated. The reproducibility turned out to be slightly worse than without the internal standard, and since the elution time per sample increased considerably, the approach was discontinued.

The concentrations determined by the LCEC method for the GSH in fresh whole blood samples obtained from five normal adults are shown in Table 2, together with the results obtained on the same samples by the DTNB method (90). Also included are the red blood cell (RBC) concentration of the blood samples, from which the quantity of GSH per million erythrocytes was calculated. The concentrations of GSH are averages with their

TABLE 2

Glutathione Values for Whole Blood

Sample	[GSH] [a]		RBC [b]	[GSH] [c]	
	DTNB	LCEC		DTNB	LCEC
1	1.21 ± 0.01	1.18 ± 0.03	5.7	2.12×10^{-10}	2.07×10^{-10}
2	1.17 ± 0.02	1.10 ± 0.02	5.6	2.09×10^{-10}	1.96×10^{-10}
3	0.94 ± 0.01	0.88 ± 0.02	3.8	2.47×10^{-10}	2.32×10^{-10}
4	0.96 ± 0.01	0.94 ± 0.02	4.6	2.09×10^{-10}	2.04×10^{-10}
5	1.27 ± 0.03	1.17 ± 0.01	5.2	2.44×10^{-10}	2.25×10^{-10}

a) 10^3 x moles/L.
b) million erythrocytes/mm^3.
c) moles/million erythrocytes.

standard deviations for five replicates. Recovery experiments of GSH were performed by addition of a known amount of GSH to the EDTA solution used for hemolyzing the samples. For the LCEC method, a recovery of 102 ± 4% was obtained, and for the DTNB method, the recovery was 98 ± 4%.

D. Discussion

The normal range for GSH concentrations in blood is 0.9×10^{-3} - 1.7×10^{-3} moles/L of whole blood, all of which is in the erythrocytes (110). Since there is a considerable variation in the RBC count from individual to individual, the GSH level in blood should preferably be expressed in units of, for example, moles per million erythrocytes. This gives, as shown in Table 2, a much narrower range for the actual intracellular levels of GSH. It should be noted that the erythrocyte level of that sample (sample 3 in Table 2) falling outside the normal range is considerably lower than those of the other samples and that the actual intracellular GSH level is very high.

From Table 2, it is apparent that the chromatographic procedure gives consistently slightly lower results than the DTNB method which is probably due to the much higher discrimination of the former against potential interferences such as cysteine and ergothioneine. Table 2 also shows that the standard deviations

obtained with the chromatographic method are approximately the same as those obtained with the DTNB method. The major disadvantage of the DTNB method is the large number of reagents needed in the sample treatment which consequently demands more time and care per sample.

The LCEC method for the determination of GSH in whole blood has several advantages over the multitude of analytical techniques described for GSH. It is fast and sensitive, and shows a high degree of discrimination against other sulfhydryl-containing molecules. The speed and sensitivity are due largely to the characteristics of the Hg-based electrochemical detector. In this determination, the detector was operated at 0.0 V vs. SCE, and signals are obtained only as those compounds which are electroactive at this potential are eluted through the detector. Since the thiols are the only components of whole blood which are electroactive at this potential, this reduces considerably the requirements and the time of the chromatographic step. For example, in a recently described anion-exchange procedure for GSH with ninhydrin detection (125), approximately 60 min. were needed for elution as compared to about 5 min. with the LCEC method. Because of the high selectivity of the LCEC method for sulfhydryl-containing molecules, other biologically active thiols such as cysteinylglycine and glutamylcysteine can also be assayed by appropriate choice of chromatographic

conditions. This should make the LCEC method attractive in studies of biosynthesis and metabolism of GSH.

CHAPTER VI

DETERMINATION OF PENICILLAMINE IN BLOOD AND URINE

A. Introduction

In spite of the wide use of penicillamine in medicine, very little is known about its pharmacology. The known metabolites of penicillamine in man are penicillamine disulfide (PSSP), the penicillamine-cysteine mixed disulfide (PSSC), and S-methylpenicillamine (177, 178). In a recent study of the metabolism of penicillamine, these metabolites could account for only some 50% of the penicillamine administered for the treatment of cystinuria (178).

In this chapter, a cation-exchange procedure with electrochemical detection is described for the determination of reduced penicillamine (PSH) and the total penicillamine (reduced plus oxidized disulfide metabolites) in the nonprotein fraction of plasma, erythrocytes, whole blood, and urine. The total penicillamine is determined after electroreduction at a mercury pool electrode. Results for blood and urine from rheumatoid arthritis patients on penicillamine therapy are presented and discussed. Only the D-isomer of penicillamine was used in this work.

B. Chromatographic Conditions

A phosphate-citrate buffer of pH 3.0 and ionic strength of 0.04 was chosen as eluent. The 0.2 x 30-cm Zipax SCX strong cation-exchange column was used for the separation step. For some separations, a 0.2 x 3-cm precolumn also packed with Zipax SCX was added. It was added primarily for the urine samples, which, after repeated injections, tended to contaminate the exchanger which first came into contact with the sample. A flow rate of 0.7 mL/min. was used, and the electrochemical detector was operated at +0.1 V vs. SCE.

Without the precolumn, a capacity factor of about 2.2, depending slightly on the packing procedure, was obtained for penicillamine with the above conditions. Both cysteine and glutathione gave k' values of less than 0.5 and appeared as part of the unretained charging-discharging peak.

C. Results

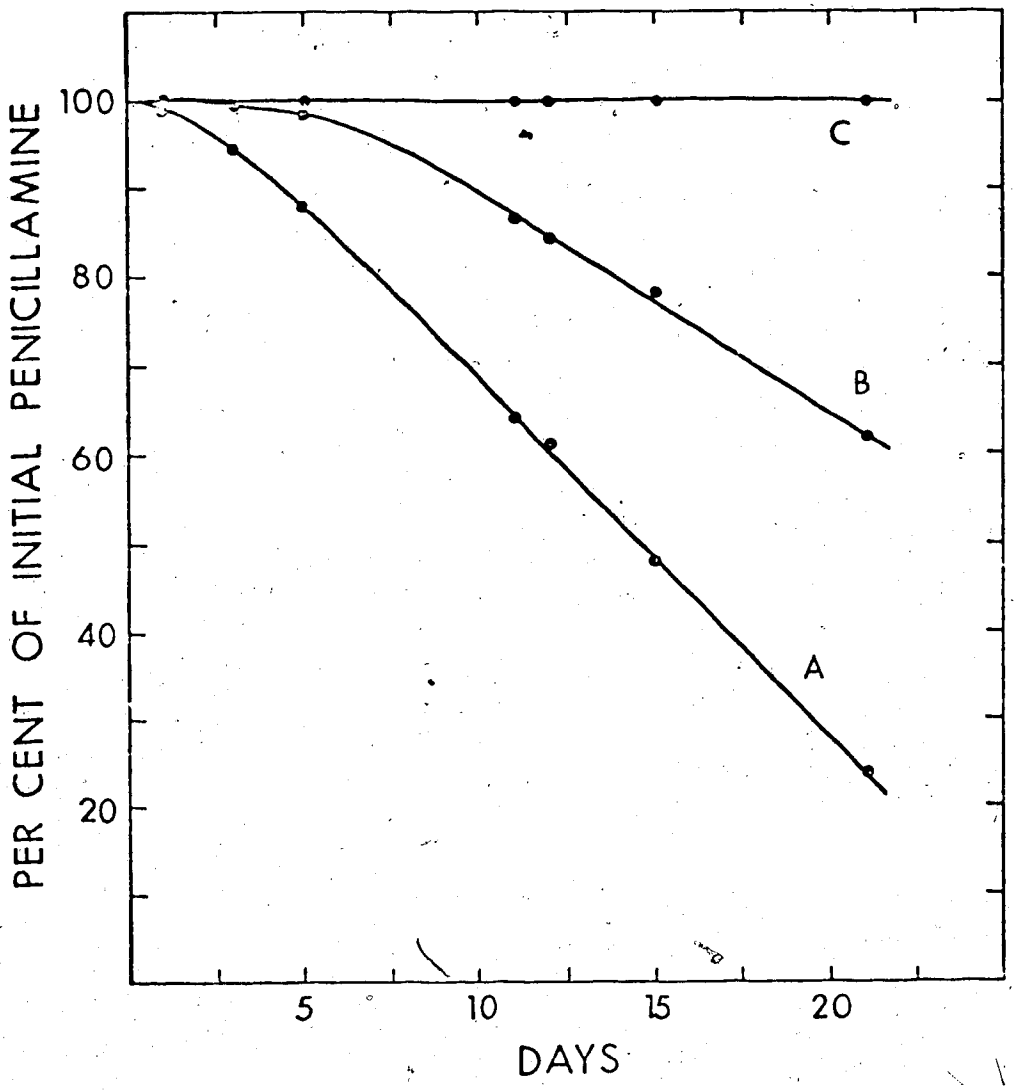
Stability of Penicillamine Solutions: Since thiols are readily oxidized (179), the stability of reduced penicillamine solutions was studied to find stable conditions for standards and samples. Figure 25 shows the stability of 1.00×10^{-3} M PSH in distilled water (solution pH 6.2) stored at room temperature (Curve A) and 4° C (Curve B), and of 1.00×10^{-3} M PSH in distilled water containing 1 g/L $Na_2H_2EDTA \cdot 2H_2O$ at room

Figure 25. Time course for the stability of 1.00×10^{-3} M PSH

(A) in distilled water (solution pH 6.2) stored at room temperature (25° C)

(B) in distilled water stored at 4° C

(C) in distilled water containing 1 g/L $Na_2H_2EDTA \cdot 2H_2O$ stored at room temperature.


PER CENT OF INITIAL PENICILLAMINE
100
80
60
40
20
5
10
15
20
DAYS
C
B
A

temperature (Curve C). A curve identical to C was obtained for a penicillamine-EDTA solution stored at 4° C. EDTA apparently stabilizes the PSH solutions by complexing trace metals which catalyze thiol oxidation (179). The data in Figure 25 were obtained by comparing the peak height obtained chromatographically for a stored solution to that of a freshly prepared PSH standard. Similar experiments with PSH in 0.2 M HCl solution, in normal urine to which 2 M HCl was added to give a final HCl concentration of 0.2 M, and in 50 g/L metaphosphoric acid solution indicated that PSH in these solutions is stable for at least a day at room temperature.

The stability of reduced penicillamine in urine was also studied to determine how soon after delivery the urine has to be acidified to avoid significant loss of PSH by oxidation. The results in Figure 26 indicate that urine samples should be acidified immediately after delivery. The results were obtained by adding 0.1 mL of a 1.00×10^{-3} M PSH solution to 0.8 mL of normal urine. After a given time period, the mixture was acidified with 0.1 mL of 500 g/L metaphosphoric acid. The peak from 10-μL injections was then compared to that of a urine solution to which metaphosphoric acid had been added before the PSH. From similar experiments, it was determined that the PSH concentration in plasma is not decreased significantly during the

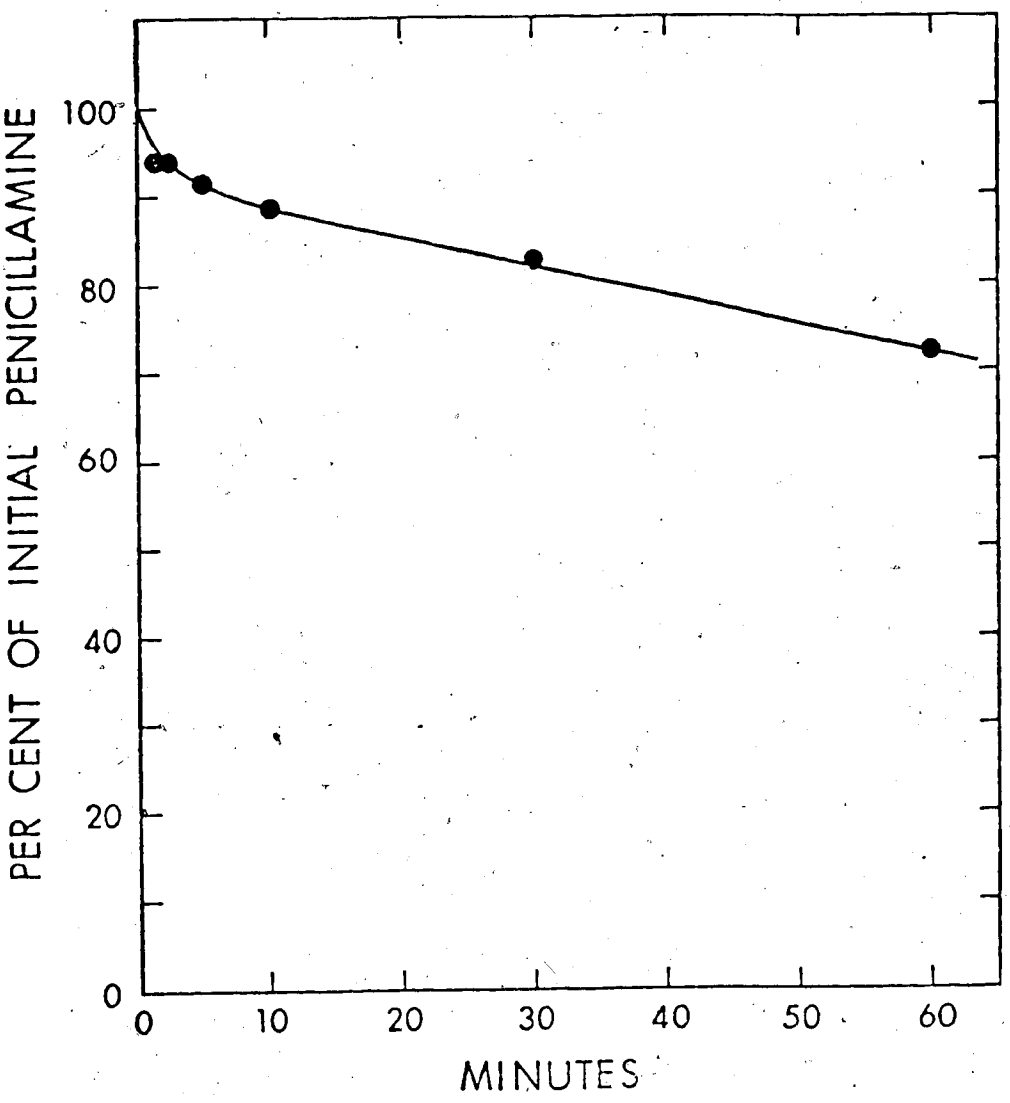


Figure 26. Time course for the stability of 1.1×10^{-4} M PSH in normal urine at room temperature.

time required for centrifugation and analysis, possibly because the EDTA present initially in the Vacutainer complexes trace metals which catalyze thiol oxidation.

Standard Curves: Standard curves were obtained by plotting peak height (nA) vs. the PSH concentration of standard solutions. The calibration curve for plasma was prepared from standard PSH solutions containing 50 g/L metaphosphoric acid. Calibration curves for reduced penicillamine in urine and for total PSH in plasma, erythrocytes, whole blood, and urine were prepared from standard PSH solutions in 0.2 M HCl. Calibration curves were linear from 5×10^{-7} M to 1×10^{-3} M PSH. At 5×10^{-7} M the signal-to-noise ratio was 5:1, where S/N = average signal amplitude/RMS noise with RMS noise = average peak-to-peak noise/2.5. To give some indication of the sensitivity of this method, 10 µL of 5×10^{-7} M PSH corresponds to 0.75 ng of PSH.

Disulfide Exchange Reactions: Thiols and disulfides undergo disulfide exchange reactions (180), the rates of which are pH dependent, decreasing rapidly as the pH decreases because the deprotonated form of the thiol is the reactive form (181). In the procedure for the determination of PSH in whole blood, the sample is first hemolyzed with a dilute EDTA solution at near neutral pH. Hemolysis releases the reduced and oxidized penicillamine as well as the other thiols and disulfides of the erythrocyte. After about 1 min.,

metaphosphoric acid is added which lowers the pH, thus essentially stopping any disulfide exchange, and precipitates the proteins. To determine if a significant amount of PSH is formed or lost by disulfide exchange with oxidized or reduced glutathione, GSSG and GSH, respectively, the forward rates for the disulfide exchange reactions represented by Equations 13, 14, and 15 were measured.

$$PSSP + GSH \rightleftharpoons PSSG + PSH \qquad (13)$$

$$PSSC + GSH \rightleftharpoons GSSC + PSH \qquad (14)$$

$$GSSG + PSH \rightleftharpoons PSSG + GSH \qquad (15)$$

Penicillamine-cysteine mixed disulfide (PSSC) was tested in addition to PSSP since both have been found to be major metabolites in patients treated for rheumatoid arthritis with D-penicillamine (177,178). The experiments were performed at 25° C in a phosphate-citrate buffer of pH 7.5 and ionic strength of 0.04. All solutions were thoroughly deaerated before mixing, and oxygen-scrubbed nitrogen was bubbled through the solutions during the reaction to provide effective mixing. In the study of Reactions 13 and 14, the PSH concentration was measured as a function of time by determining

the PSH in aliquots. The reaction was stopped by making the aliquots 0.2 M in HCl. For reaction times of less than 1 min., the whole test solution, rather than an aliquot, was acidified by rapidly adding enough 2 M HCl with a tuberculin syringe to make the final solution 0.2 M in HCl. In the study of Reaction 15, the GSH concentration was measured as a function of time by HPLC (Chapter V). The initial change in PSH concentration is too small to permit precise values for the forward rate constant k_f to be obtained when the decrease in PSH is measured.

The time course for formation of PSH by disulfide exchange in a solution initially containing 1.00×10^{-3} M PSSP and 1.00×10^{-3} M GSH is shown in Figure 27. From the initial rate of the reaction which is linear with time up to about 3 min. as shown in Figure 28, the forward rate constant (defined by Equation 16) for Reaction 13 was found to be 2.8×10^{-2} L/mol·s.

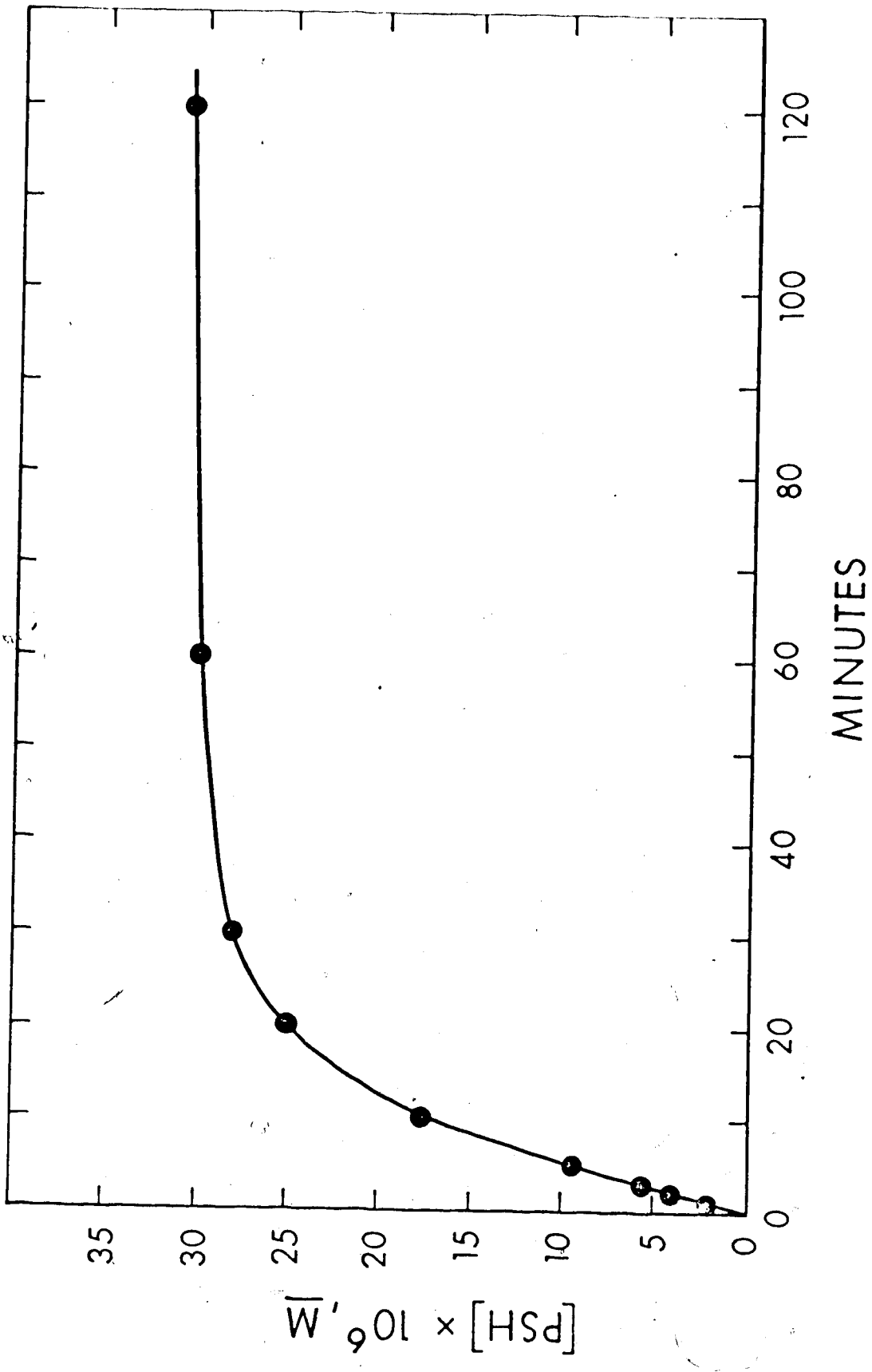
$$-\frac{d[PSH]}{dt} = k_f[PSSP][GSH] \qquad (16)$$

Changing either the initial PSSP or the GSH concentration by a factor of 2 did not alter the observed rate constant. The forward rate constants for Reactions 14

Figure 27. Time course for the formation of PSH by the disulfide exchange reaction

$$PSSP + GSH \rightleftharpoons PSSG + PSH$$

Solution conditions were phosphate-citrate buffer of pH 7.5 and ionic strength of 0.04, 25° C. The solution initially contained 1.00×10^{-3} M PSSP and 1.00×10^{-3} M GSH.


[PSH] x 10⁶, M
0
5
10
15
20
25
30
35
0
20
40
60
80
100
120
MINUTES

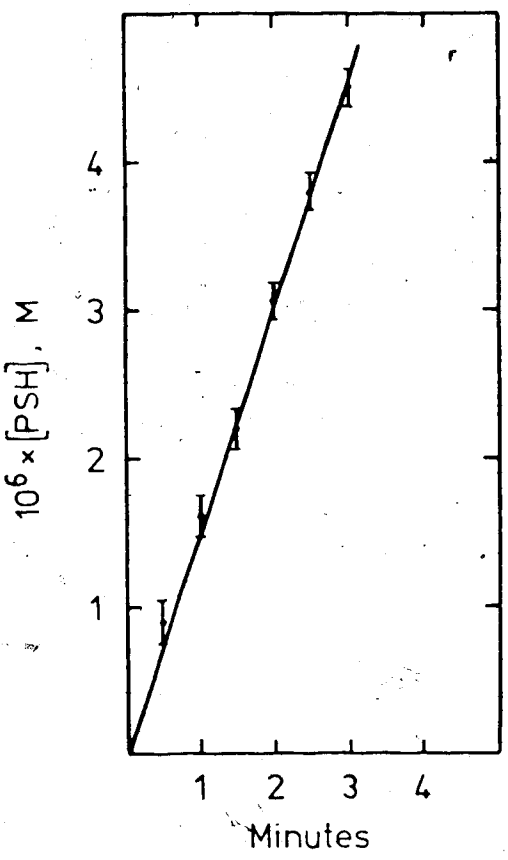


Figure 28. Time course for the initial 3 min. of the reaction for which the results are shown in Figure 27.

and 15 at identical conditions were 0.73 L/mol·s and 0.43 L/mol·s, respectively. These rate constants coupled with the small concentrations of GSH and GSSG in the hemolyzed solutions indicate that essentially no PSH is lost or formed by disulfide exchange during the hemolysis step.

Reduced Penicillamine in Blood and Urine: Figures 29 and 30 show typical chromatograms obtained for a urine and a whole blood sample from a rheumatoid arthritis patient on penicillamine therapy. The sharp, unretained peaks at ca. 1 min. after sample injection are due to a combination of double layer capacitance effects, which result from the sample medium being different from the mobile phase, and detector response to chloride, traces of cysteine and, in the case of whole blood and erythrocyte samples, reduced glutathione in the sample. Both cysteine and reduced glutathione are essentially unretained with the chromatographic conditions used. The peak appearing between the PSH peak at about 3 min. and the unretained peak in Figure 30 is possibly due to hydrolysis products of GSH such as L-cysteinylglycine or γ-L-glutamyl-L-cysteine since it did not appear in plasma samples.

The reduced penicillamine levels of the urine and blood from outpatients at the University of Alberta Hospital who were being treated with 750 mg of D-penicillamine per day for rheumatoid arthritis are listed

Figure 29. Representative chromatogram obtained for a urine sample from a patient on D-penicillamine therapy for rheumatoid arthritis. Peak height corresponds to 5.7×10^{-5} M PSH.

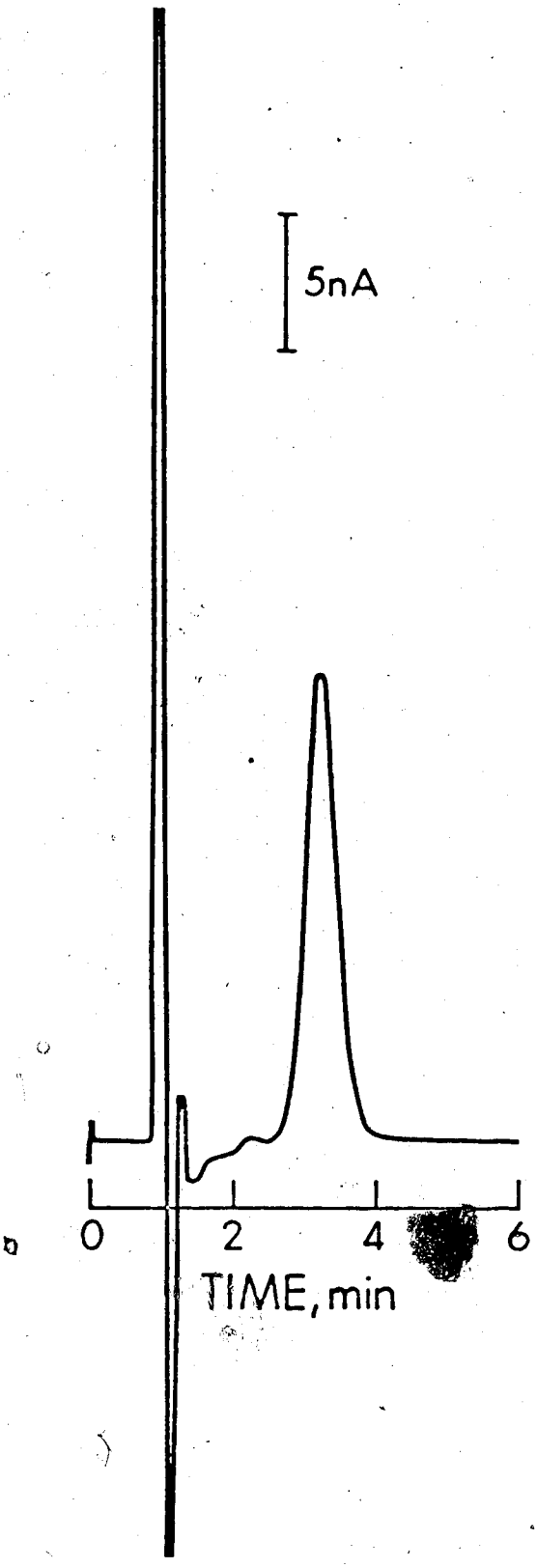
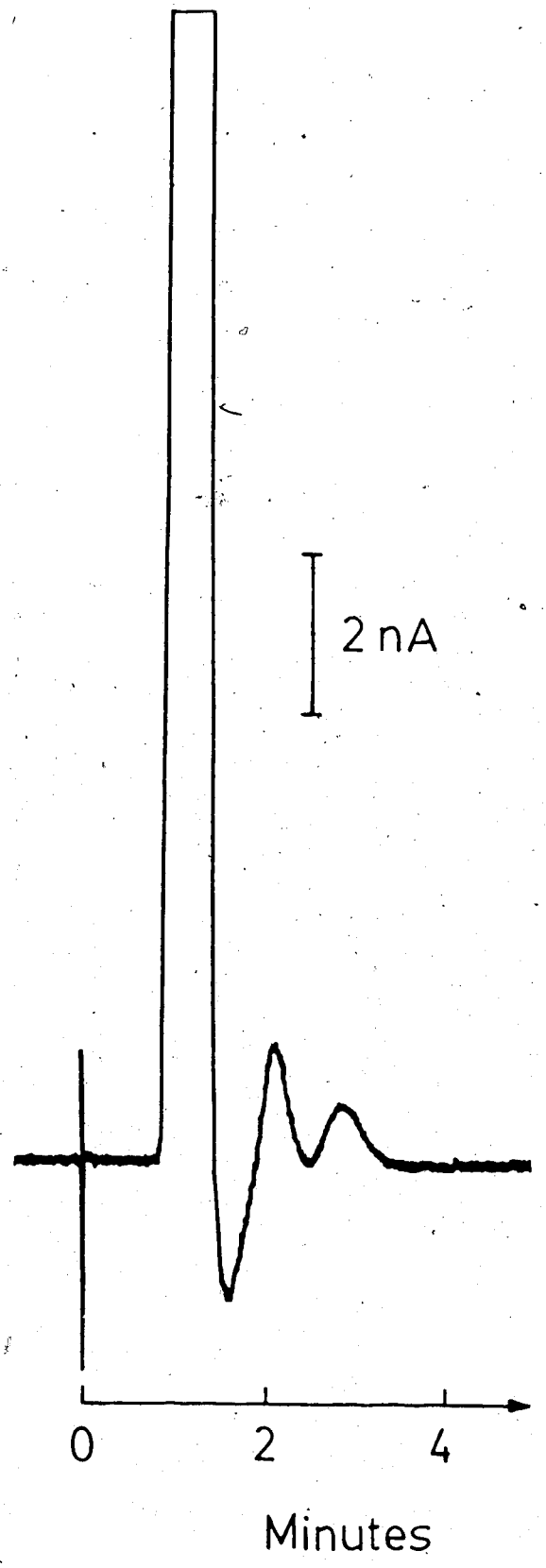

5nA
0
2
4
6
TIME, min

Figure 30. Representative chromatogram obtained for a whole blood sample from a patient on D-penicillamine therapy for rheumatoid arthritis. The penicillamine peak occurs at about 3 min. Peak height corresponds to 2.2×10^{-6} M PSH.


2 nA
0
2
4
Minutes

in Table 3. The penicillamine was taken orally in doses of 250 mg. Approximate time intervals after the last tablet as indicated by the patients are also listed in Table 3. The average relative standard deviations for the determination of reduced penicillamine in the various sample media, calculated from the results of 3-5 injections for each sample, are also given in Table 3. The relative standard deviations for the packed erythrocytes and whole blood results are generally larger than those for urine and plasma analysis because, after sample preparation, the PSH levels of the packed erythrocytes and whole blood samples encountered in this work approach the detection limit of the detector. Recoveries of 98 ± 3% and 99 ± 3% were obtained for PSH added to two separate plasma samples immediately before acidification. The amount of PSH added was of the same order as that originally present.

Besides plasma, several serum samples from patients treated with D-penicillamine for rheumatoid arthritis were assayed for PSH. The serum which was treated the same way as plasma, gave considerably lower results than those obtained for plasma. When exposed to air without having been acidified, the samples showed a rapid decrease in PSH concentration. The difficulties encountered with serum as compared to plasma are therefore most probably due to the absence of EDTA in the case of serum which leaves heavy metal ions free to

TABLE 3

Penicillamine Concentrations (mg/L) of Blood and Urine from Patients on D-Penicillamine Therapy for Rheumatoid Arthritis

Patient	Approx. Interval since last Tabl. (hours)	Urine		Plasma		Packed Erythrocytes		Whole Blood[a]		Hematocrit %	Whole Blood[b]	
		[PSH]	$[PSH]^{c}_{TOT}$	[PSH]	$[PSH]^{c}_{TOT}$	[PSH]	$[PSH]^{c}_{TOT}$	[PSH]	$[PSH]^{c}_{TOT}$		[PSH]	$[PSH]^{c}_{TOT}$
1	1.5	29.4	86	2.80	6.9	0.8	1.0	1.8	3.7	45	1.90	4.2
2	4.0[d]	0.94	91	0.90	3.4	0.5	0.55	0.6	2.2	40	0.74	2.3
3	3.0	13.2	143	1.12	4.6	0.4	0.4	0.7	3.2	40	0.82	2.9
4	1.0[d]	-	254	2.60	8.1	0.7	0.75	1.7	4.2	45	1.74	4.8
5	5.5[d]	47	223	1.91	5.2	0.9	1.0	1.6	3.5	40	1.50	3.5
6	-	25.4	374	2.03	6.2	0.5	0.6	1.2	4.0	35	1.48	4.2
7	-	-	-	1.08	4.2	0.6	0.8	1.1	3.3	33	0.92	3.1
8	6.0[d]	235	922	0.93	3.0	-	-	0.5	1.8	-	-	-
9	3.5	14.6	79	0.47	2.0	-	-	0.3	1.0	-	-	-
10	1.0	194	795	1.28	4.2	-	-	1.1	2.5	-	-	-
11	4.0	203	1360	0.71	3.3	-	-	0.7	2.4	-	-	-
12	0.5	2.5	89	0.90	2.7	-	-	0.7	1.8	-	-	-
13	1.5	53	440	1.24	4.5	-	-	1.0	2.7	-	-	-
Av. rel. std. dev.[e]		2.3%	1.7%	3.9%	2.2%	16%	13%	11%	5.0%			

a) Observed results.

b) Calculated from the observed results for plasma and packed erythrocytes and the hematocrit.

c) The total of reduced penicillamine and the oxidized penicillamine in the nonprotein fractions.

d) 500 mg of D-penicillamine taken.

e) Average relative standard deviation calculated from the results of 3-5 injections for each sample.

catalyze air oxidation of PSH.

Total Penicillamine in Blood and Urine: The procedure for total penicillamine gives the sum of the reduced penicillamine and the oxidized penicillamine (PSSP and PSSC) (178,182) in the nonprotein fraction. The procedure is based on the quantitative reduction of the oxidized forms, followed by determination by HPLC of the total reduced penicillamine. Several methods of disulfide reduction were studied in addition to the electrolysis method.

A chemical reduction of PSSP with $NaBH_4$ at room temperature was attempted. To 5 mL of 1.0×10^{-3} M PSSP, deaerated with oxygen-scrubbed nitrogen, was added 100 mg of solid $NaBH_4$. After 30 min., the reaction was stopped by dropwise addition of 30% H_3PO_4. This gave a yield of less than 5%, in agreement with reported results (42). Addition of Cu^{2+} as catalyst increased the yield to 66%. Other metal ions, e.g. Fe^{3+}, Zn^{2+}, and Hg^{2+}, as catalysts gave lower and irreproducible yields. All metal ions were complexed with excess EDTA at the completion of the reaction. Besides giving less than quantitative reduction, procedures with $NaBH_4$ were tedious and required complete deaeration of samples, which caused excessive foaming of urine samples. Reduction with sulfite was also attempted, but without success. Some attempts based on reduction by disulfide exchange with sulfhydryl-containing molecules gave low

yields. An additional consideration is that the thiol added in excess should preferably be unretained with the chromatographic conditions used so as to avoid interference with the PSH determination or saturation of the ion exchanger.

Electroreduction at a Hg pool cathode was found to be quantitative for both PSSP and PSSC, was complete within 10-15 min. at an electrolysis current of 6.43 mA, and did not require deaeration of the sample solution either before or during electrolysis. The recovery of PSSP (1.00×10^{-5} M) added to the urine of several rheumatoid arthritis patients on penicillamine therapy was 100 ± 3%, 98 ± 2%, and 97.5 ± 2%, and 98.2 ± 1.3% recovery was obtained for PSSP added to 8 normal urine samples. Penicillamine-cysteine mixed disulfide gave recoveries of 99 ± 2% and 98 ± 2% when added to normal urine to give a 1.00×10^{-5} M PSSC solution. All urines were diluted at least 2:25 with 0.2 M HCl because it was observed that a yellowish film formed on the Hg surface and gave poor recoveries for several less diluted urine samples. Recoveries of 103 ± 3% and 97 ± 3% were obtained for PSSP added to the plasma of rheumatoid arthritis patients. For PSSC, the recoveries were 98.5 ± 3% and 99 ± 3%.

The total penicillamine levels in the urine and blood from patients treated with 750 mg of D-penicillamine per day for rheumatoid arthritis are given in

Table 3. The average relative standard deviations for the determinations, calculated as described above for reduced penicillamine, are also given in Table 3.

To determine if the results listed in Table 3 for reduced and total penicillamine in plasma, packed erythrocytes, and whole blood are internally consistent, the reduced and total penicillamine concentrations of whole blood were predicted from the levels found for plasma and packed erythrocytes and the estimated hematocrit. The calculated and observed concentrations show good agreement.

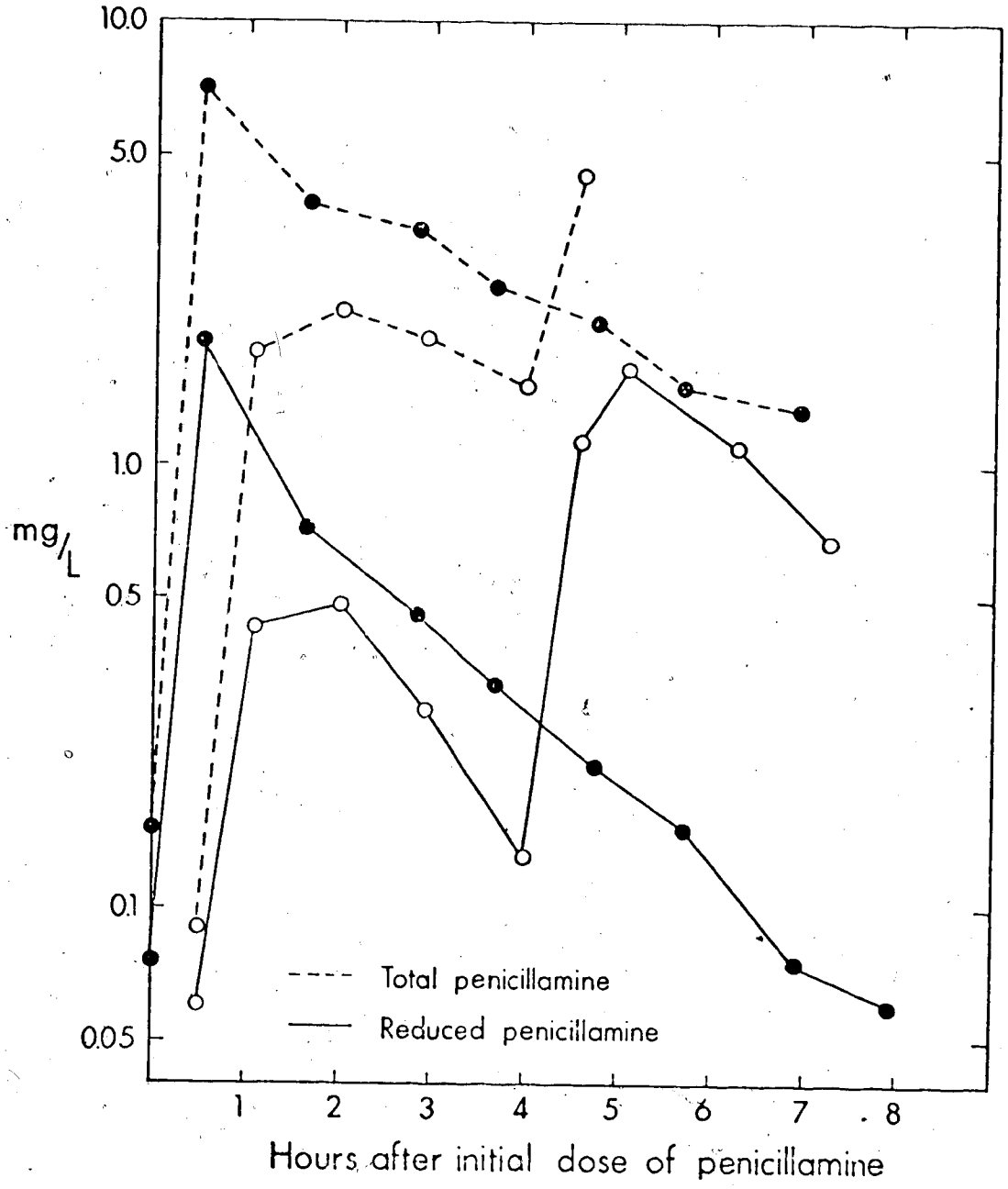
Direct detection of oxidized penicillamine with the electrochemical detector operated at -1.2 V vs. SCE was also attempted. The 0.2 x 30-cm Zipax SCX column was used with a phosphate-citrate buffer of pH 4.0 (ionic strength of 0.1). Under these conditions, the detection limit was approximately 5×10^{-4} M PSSP, which is too high for direct detection to be applicable to the samples encountered in the present work.

Plasma Clearance Test: To determine whether the assay developed for penicillamine would be of practical value in more extensive pharmacokinetic and bioavailability studies in patients receiving D-penicillamine for rheumatoid arthritis, a PSH clearance test was performed in two volunteers in collaboration with the Rheumatic Disease Unit at the University of Alberta. Figure 31 shows the plasma levels obtained after ingestion of

Figure 31. Time course for plasma penicillamine concentrations in two subjects after ingestion of 750 mg of D-penicillamine.

o——o——o : Subject A. Initial dose of 250 mg and then 500 mg D-penicillamine 4 hours later.

●——●——● : Subject B. Single 750 mg dose of D-penicillamine.


10.0
5.0
1.0
0.5
0.1
0.05
mg/L
Total penicillamine
Reduced penicillamine
1
2
3
4
5
6
7
8
Hours after initial dose of penicillamine

750 mg of D-penicillamine in the two volunteers. Subject A, a normal male adult, had not received any previous penicillamine and ingested initially 250 mg, then 4 hours later a further 500 mg. Subject B, a female adult with rheumatoid arthritis, had received 250 mg of D-penicillamine on the two preceding days and on the morning of the test, immediately after the initial blood sample, ingested 750 mg D-penicillamine in a single dose. Urine was collected in HCl-containing vessels over the next 8 hours in two 4-hour periods. The results of this are shown in Table 4.

D. Discussion

The very low penicillamine detection limits of the procedure described in this chapter result from the high sensitivity of the Hg pool electrode for the sulfhydryl group. Selectivity is achieved by coupling the electrode with a liquid chromatographic separation. Because the detector responds only to chloride and the sulfhydryl-containing amino acids and peptides in the sample when operated at a potential of +0.1 V vs. SCE, it is only necessary that the chromatographic step separate penicillamine from the other sulfhydryl-containing molecules. This is easily and quickly done; with the mobile phase and chromatographic column used, penicillamine is eluted with a retention time of ca. 3.3 min. whereas cysteine and glutathione are essenti-

TABLE 4

Reduced and Total Penicillamine (moles/L) Excretion in the Urine of Two Subjects who Ingested 750 mg of D-Penicillamine

	Subject A[a]			Subject B[b]		
	[PSH]	$[PSH]^{c}_{TOT}$	Total PSH mg/4hr	[PSH]	$[PSH]^{c}_{TOT}$	Total PSH mg/4hr
1st 4hr	1.6×10^{-4}	2.06×10^{-3}	43	1.05×10^{-3}	4.6×10^{-3}	65
2nd 4hr	1.0×10^{-3}	4.5×10^{-3}	92	1.7×10^{-3}	1.46×10^{-3}	34

a) Ingested 250 mg D-penicillamine at outset and 500 mg D-penicillamine after 4 hours.

b) Ingested 750 mg D-penicillamine at outset.

c) The total of the reduced penicillamine and the oxidized penicillamine.

ally unretained.

In contrast, the chromatographic step in auto analyzer procedures with ninhydrin detection must resolve the penicillamine from all other ninhydrin-positive components of the sample. Because of the complexity of biological fluids, this can require long elution times. For example, in a recent study of penicillamine metabolism, elution times on the order of 8 hours were necessary to elute penicillamine disulfide (178). It is of interest that with the auto analyzer method no reduced penicillamine could be detected in fresh urine from patients on D-penicillamine therapy for the treatment of rheumatoid arthritis or cystinuria (178), which is considerably different from the findings of the present study (Table 3). The failure to detect reduced penicillamine in the previous study could be due to its rapid oxidation since no mention is made of precautions to exclude oxygen from reagents or mobile phase, or to incomplete resolution of reduced penicillamine from other ninhydrin-positive components, perhaps glycine (109).

Reduced penicillamine in urine and plasma generally is determined colorimetrically as its Fe(III) complex (108), although this method has been reported to lack precision (109). Oxidized penicillamine generally is determined by auto analyzer methods with ninhydrin detection. Other methods in the literature

include a gas chromatographic determination of the esterified forms of reduced and oxidized penicillamine as their thiazolidine or Schiff base derivatives, respectively (132), and an auto analyzer procedure for total penicillamine which is based on its quantitative conversion to penicillaminic acid by performic acid oxidation, with ninhydrin detection (109). An immunological assay also has been described (183). It would seem that the methods described in this chapter offer advantages over the methods mentioned above in terms of simplicity, analysis time, and the ease with which this method can determine both the reduced and oxidized penicillamine.

For the patients encountered in this work, both the free and the oxidized penicillamine in the blood was distributed unevenly between the plasma and erythrocytes, with the reduced penicillamine concentration some 2-4 times higher and the total penicillamine some 5-10 times higher in the plasma. It also is of interest that the distribution between the reduced and oxidized forms is considerably different for plasma and red blood cells, as indicated by the ratio of total penicillamine to reduced penicillamine concentrations (Table 5). Most significant, perhaps, is the finding that most of the penicillamine in the red blood cells is in the reduced form, since the reduced form is the active form in the treatment of methylmercury (184) and

TABLE 5

Ratio of Total Penicillamine to Reduced Penicillamine[a]

Patient	Urine	Plasma	Packed Erythrocytes	Whole Blood
1	2.9	2.5	1.2	2.1
2	97	3.8	1.1	3.7
3	11	4.1	1.0	4.6
4	-	3.1	1.1	2.5
5	[illegible]	2.7	1.1	2.2
6	15	3.0	1.2	3.3
7	-	3.9	1.3	3.0
8	3.9	3.2	-	3.6
9	5.4	4.3	-	3.[illegible]
10	4.1	3.3	-	2.3
11	6.7	4.6	-	3.4
12	36	3.0	-	2.6
13	8.3	3.6	-	2.7

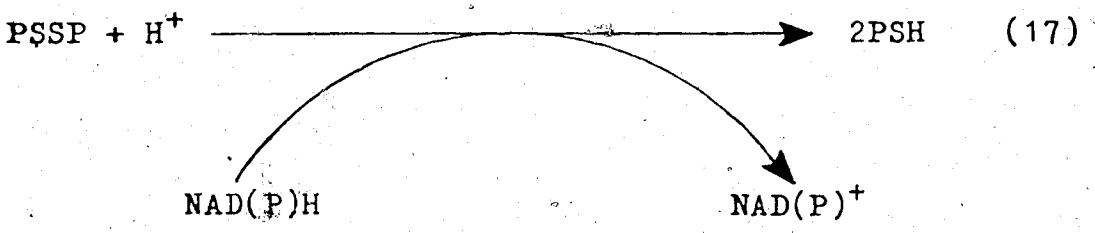
a) Calculated from the results in Table 3.

lead (185) poisoning by penicillamine, and in the postulated mechanism of action at the molecular level in the treatment of cystinuria (186). Considering the intracellular reducing power of the erythrocytes, it is to be expected that most of the penicillamine in the red blood cells will be present in the reduced form. Oxidized penicillamine, once inside the cell, is reduced in any of three ways (187).

a) Enzymatically catalyzed reduction by disulfide exchange with the excess glutathione present in the cell.

b) Reduction by disulfide exchange as in system a), but without enzymatic catalysis.

c) Direct enzymatic reduction of the penicillamine disulfide bond represented by Reaction 17.



The oxidized glutathione produced by processes a) and b) is rapidly reduced with a reduced pyridine nucleotide in the presence of glutathione reductase, thus maintaining the GSH-GSSG status of the cell.

The wide range of penicillamine concentrations

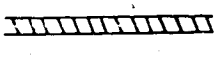
seen in Table 3 correlate poorly with the intervals after ingestion. As these were estimates given by the patients, they are very approximate values, but this may also reflect differences in the pharmacokinetics of the drug in different individuals. Penicillamine appears to be rapidly excreted through the kidney as demonstrated by the relatively high concentration of penicillamine found in the urine of patients when compared to the concentrations seen in plasma. In the two subjects studied in more detail, the plasma levels appeared to peak between one and two hours after ingestion of the drug (Figure 31) and then fell rapidly with significant urinary excretion, not only of oxidized, but also of reduced penicillamine. It is of interest that the ratio of total penicillamine to reduced penicillamine (Figure 32) in the two subjects rapidly increases above the normal range obtained from Table 3 for patients treated with D-penicillamine over an extended time period. This indicates that the oxidizing power of the plasma towards reduced penicillamine decreases after prolonged treatment with this drug, probably due to extensive formation of serum protein-penicillamine mixed disulfides (PrSSP) and reduction of serum protein-cysteine mixed disulfides (PrSSC) according to Equations 18 and 19.

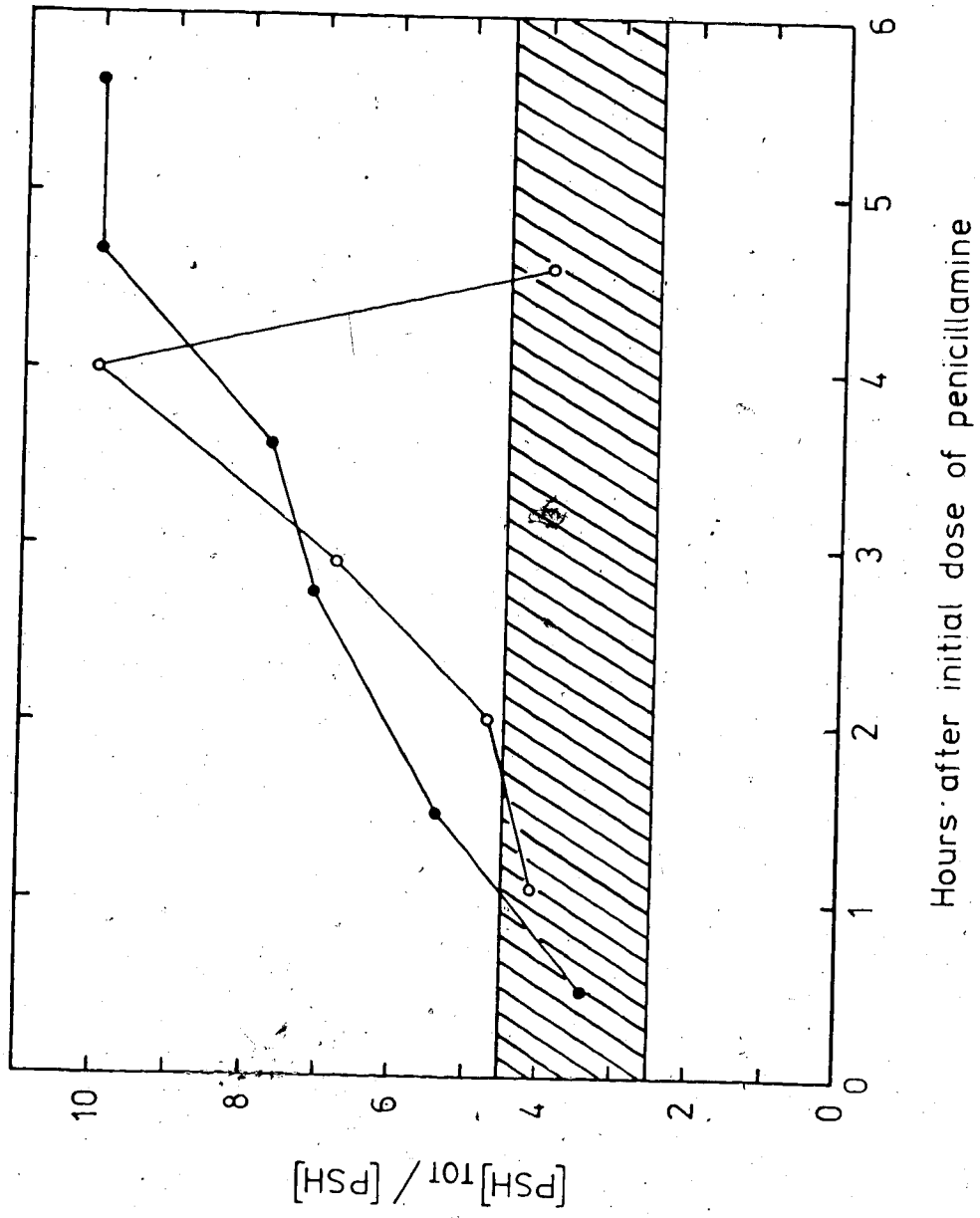
$$PrSSC + PSH \rightleftharpoons PrSSP + CSH \qquad (18)$$

Figure 32. Time course for the ratio of total penicillamine to reduced penicillamine calculated from the data in Figure 31.

o——o——o : Subject A.

•——•——• : Subject B.

 : Normal range according to Table 3 for patients treated with D-penicillamine over an extended time period.


[PSH]tot / [PSH]
0
2
4
6
8
10
0
1
2
3
4
5
6
Hours after initial dose of penicillamine

$$PrSSC + PSH \rightleftharpoons PrSH + PSSC \qquad (19)$$

If the reduced penicillamine is the active form of the drug in the treatment of rheumatoid arthritis, the above postulation could partly explain why most patients need to be treated for several months before a significant improvement is observed.

No attempts were made to try to fit a pharmacokinetic model to the data in Figure 31 since a single dose experiment rarely is enough for reliable kinetic results. In addition, the drug was taken orally rather than intravenously which complicates the situation to such an extent that often two completely different models can be fitted to the data from a single concentration-time curve (188). The experiment, however, suggests that the described method may provide a useful technique for a more detailed study on the bioavailability and pharmacokinetics of D-penicillamine.

CHAPTER VII

DETERMINATION OF CYSTEINE IN URINE AND PLASMA AND OF HOMOCYSTEINE IN PLASMA

A. Introduction

The current methods of choice for the determination of cysteine, homocysteine, and their oxidized forms cystine, homocystine, and the cysteine-homocysteine mixed disulfide seem to be automated amino acid analyses with ninhydrin detection (20,30,32) requiring 6-13 hours per run. Cysteine and homocysteine are usually converted to their S-carboxymethyl derivatives by reaction with iodoacetate before the separation step (30).

In this chapter, a simple and sensitive cation-exchange procedure with electrochemical detection is described for the determination of reduced cysteine and total cysteine in urine and plasma and of total homocysteine in plasma. The totals of cysteine and homocysteine are determined after electroreduction at a mercury pool electrode. Results are presented which suggest that the disulfide exchange reactions between cysteine, cystine, and plasma albumin and its cysteine mixed disulfides are a source of error in the iodoacetate method (30) for the determination of cysteine in plasma.

B. Chromatographic Conditions

For the urine samples, a 1% H_3PO_4 solution was used as eluent, and for the plasma samples, a pH 2.5 phosphate-citrate buffer of ionic strength of 0.03 was used. The separations were performed on the 0.2 x 50-cm column dry-packed with Zipax SCX strong cation exchanger. A flow rate of 0.5 mL/min. was used, and the electrochemical detector was operated at +0.1 V vs. SCE. Under these conditions, the capacity factor for cysteine was 1.4 and the capacity factor for homocysteine was 3.2.

C. Results

Cysteine in Urine: The reduced and total cysteine levels found in the urine of four normal adult males are listed in Table 6. Also listed in Table 6 are the results of recovery studies for cystine. In the recovery studies, 1.0 mL of a 5.00×10^{-5} M cystine solution was added to 4.0 mL of urine diluted 2:25 with 0.2 M HCl. Figure 33 shows a representative chromatogram of reduced cysteine in a urine sample. The unretained peak appearing at about 1.5 min. is due to double layer capacitance effects as well as detector response to chloride in the sample.

A standard addition procedure was used for quantitation of cysteine in urine rather than a calibration curve because the retention time of the cysteine

TABLE 6

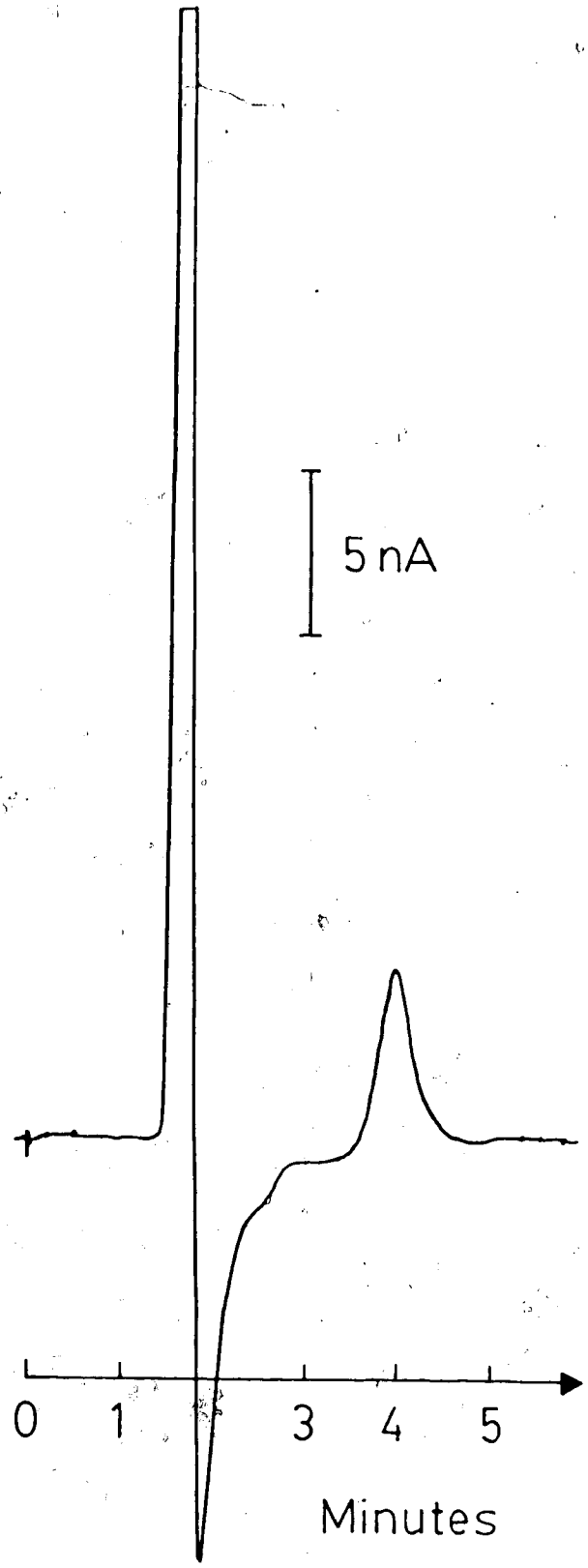
Cysteine (CSH) and Total Cysteine in Urine of Four Male Adults[a]

Sample	$[CSH]^b$	$[CSH]^b_{TOT}$	$\frac{[CSH]_{TOT}}{[CSH]}$	Recovery of cystine, %
1	3.5 ± 0.1	27.6 ± 0.6	7.9	97 ± 4
2	3.6 ± 0.1	37.0 ± 0.8	10.3	101 ± 5
3	2.1 ± 0.1	20.8 ± 0.6	9.9	99 ± 4
4	3.8 ±	22.6 ± 0.4	6.0	100 ± 4

a) Uncertainties are standard deviations for replicate analyses.

b) 10^5 x moles/L.

Figure 33. Representative chromatogram from the determination of cysteine in a urine sample. The sharp unretained peak at about 1.5 min. is due to changes in double layer capacitance at the mercury surface as well as to the detector response to chloride present in the sample. Peak height corresponds to 1.6×10^{-5} M cysteine.


5 nA
0
1
3
4
5
Minutes

is affected by the urine matrix, probably due to the high concentration of potassium ions which tend to saturate the resin. The standard addition was performed by adding 0.9 mL of diluted urine to 0.1 mL of a 1.5×10^{-4} M or a 3.0×10^{-4} M cysteine solution in 0.2 M HCl. The detector showed linear response for cysteine concentrations in the range of 10^{-3} M to 10^{-6} M, which is about the limit of detection.

Cysteine and Homocysteine in Plasma: The procedures developed for plasma samples permit the determination of the levels of reduced cysteine, total cysteine which includes cystine and any other nonprotein disulfide forms of cysteine, and total homocysteine which has been reported to be present in normal plasma in the mixed disulfide form with cysteine (20). A representative chromatogram of an electrolyzed plasma sample is shown in Figure 34 where the cysteine peak appears at 4 min. and the homocysteine peak at about 6.5 min. The peaks in Figure 34 were identified by comparison with standard solutions containing 40 g/L metaphosphoric acid and 0.2 M HCl. Under these conditions, ergothioneine has a k' of 2.4. The results obtained for the plasma from five normal adults are presented in Table 7.

During the development of the procedure, the contents of one Vacutainer were left exposed to air for about 5 min. after centrifugation to determine the

Figure 34. Representative chromatogram from the detection of cysteine and homocysteine in an electrolyzed plasma sample. The sharp unretained peak at about 1.5 min. is due to changes in double layer capacitance at the mercury surface as well as to the detector response to chloride present in the sample. The cysteine peak appears at 4 min. and the homocysteine peak at about 6.5 min. Between 1/2 min. and 2 min., recorder sensitivity was changed to keep the unretained peak on scale. Peak heights correspond to 6.0×10^{-5} M cysteine and 6.1×10^{-6} M homocysteine.

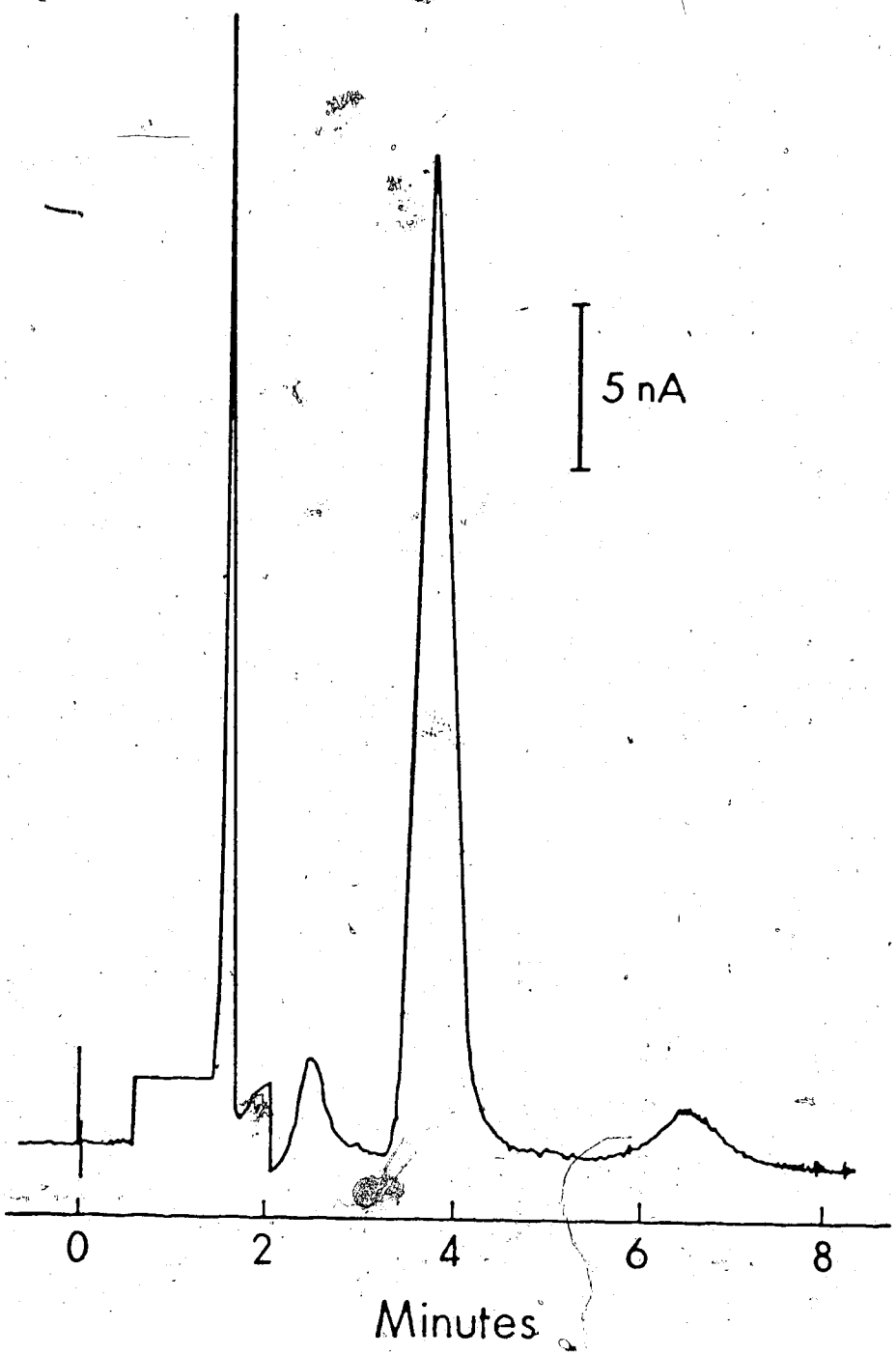

5 nA
0
2
4
6
8
Minutes

TABLE 7

Cysteine (CSH), Total Cysteine, and Total Homocysteine in Plasma of Five Adults[a]

Sample	$[CSH]^b$	$[CSH]^b_{TOT}$	$\frac{[CSH]_{TOT}}{[CSH]}$	$[Homocysteine]^b_{TOT}$
1	1.09 ± 0.04	12.9 ± 0.3	11.8	1.5
2	0.90 ± 0.03	10.3 ± 0.2	11.3	1.1
3	1.08 ± 0.04	13.2 ± 0.3	12.2	1.8
4	1.09 ± 0.03	15.3 ± 0.4	14.0	1.2
5	0.76 ± 0.03	10.6 ± 0.2	14.0	0.9

a) Uncertainties are standard deviations for replicate analyses.

b) 10^5 x moles/L.

extent to which cysteine is lost by oxidation before the sample can be acidified. A decrease of approximately 5% from the initial cysteine concentration was observed, indicating this not to be a significant source of error.

Recovery studies were done for cysteine and cystine. For cystine, recoveries of 96 ± 4% and 99 ± 5% were obtained by adding 0.5 mL of 5.0×10^{-4} M cystine to 4.5 mL of treated plasma. Several different recovery studies were done for cysteine. In one study, cysteine was added to the metaphosphoric acid solution used to precipitate the plasma proteins. One aliquot of the plasma was then acidified with metaphosphoric acid solution and another with the metaphosphoric acid to which had been added cysteine. Two separate experiments gave recoveries of 97% and 98%, which validates the procedures used.

In another cysteine recovery study, the Vacutainers were opened before blood collection, deaerated with O_2-scrubbed nitrogen, and 0.10 mL of a deaerated standard cysteine solution in 0.9% NaCl was added. The Vacutainers were then evacuated on a vacuum line. Blood was then collected, acidified, centrifuged, and analyzed as before. As indicated in Table 8, recoveries of approximately 15% were obtained for additions of cysteine calculated to increase the plasma cysteine

TABLE 8

Recovery Studies of Cysteine Added to Plasma

Sample	[Cysteine], 10^5 x moles/L			Recovery %	$[Cysteine]^a_{TOT}$, 10^5 x moles/L		Recovery[b] %	K[c]	
	Initial	Calculated After Addition	Found After Addition		Initial	Found After Addition		Initial	After Addition
A	1.10 ± 0.02	1.82	1.20 ± 0.02	14	13.3 ± 0.2	14.7 ± 0.3	233 ± 83	9.2	9.7
A	1.10 ± 0.02	2.03	1.25 ± 0.03	16	13.3 ± 0.2	15.0 ± 0.3	218 ± 64	9.2	9.6
B	1.16 ± 0.02	2.56	1.41 ± 0.03	18	11.4 ± 0.2	13.5 ± 0.3	183 ± 44	7.4	7.5

a) Sample electrolytically reduced prior to HPLC analysis.

b) % of added cysteine not recovered in reduced form.

c) Independent values calculated from the [cysteine] and $[cysteine]_{TOT}$ found before addition and found after addition. See text for details.

concentration by 0.72×10^{-5} M, 0.93×10^{-5} M, and 1.40×10^{-5} M. In these studies, the volume of blood collected was obtained by the difference in weight of the tube before and after collection, and the plasma volume was calculated from the measured hematocrit. In the calculations, it was assumed that a negligible amount of cysteine enters the blood cells. To determine if the amount of EDTA in the Vacutainer has any effect on the recovery, a recovery study was done using a standard solution of cysteine which also contained 1 g/L of $Na_2H_2EDTA \cdot 2H_2O$. Again, the recovery was about 15%. Finally, the plasma samples to which standard cysteine solutions had been added were electrolyzed and the total cysteine determined. As can be seen from the results presented in Table 8, after electrolysis, the recovered amount was twice that added (i.e. 200%).

D. Discussion

The levels obtained for reduced cysteine in urine (Table 6) agree well with those reported by Brigham et al (30), but are considerably lower than the normal range of $5.8 \times 10^{-5} - 9.0 \times 10^{-5}$ M obtained with the DTNB method by Rootwelt (43). The total cysteine levels in Table 6, however, are in good agreement with the normal range reported by Rootwelt (43), who measured total cysteine after reduction of cystine with Thiolated Sephadex. The sum of cysteine and cystine

measured chromatographically by Brigham et al (30), on the other hand, is considerably lower than the total cysteine levels presented in Table 6. It has been shown that acid hydrolysates of urine give considerably higher cystine levels than nonhydrolyzed urine (189) due to the presence of conjugated cystine. In the above procedure, partial hydrolysis of the conjugate would be expected as well as total reduction of liberated and conjugated cystine during electrolysis. This, in addition to the contribution from small amounts of mixed disulfides, may explain the high cystine values as compared to those reported by Brigham et al.

The levels obtained for reduced cysteine in plasma (Table 7) lie in a rather narrow concentration range, which is considerably lower than the range reported by others (30,122,123), all of whom used iodoacetate to make the S-carboxymethyl derivative of cysteine. However, the total cysteine levels are all higher than the sum of the reported normal cysteine and cystine levels (30,190). The results from the second set of plasma cysteine recovery studies (Table 8) suggest that these differences result from relatively fast disulfide exchange equilibria in the plasma, which affect the results obtained by methods which employ S-carboxymethyl or other S-methyl cysteine derivatives (see later).

Table 7 also shows the concentrations of total

plasma homocysteine. These concentrations are several times higher than would be expected if homocysteine were present primarily in the cysteine-homocysteine mixed disulfide form (20). Unless some unidentified sulfhydryl-containing molecule interferes with the above homocysteine determination, the difference must be accounted for by the presence of homocystine.

Taken together, the results of the various plasma recovery studies indicate that the added cysteine is not lost by oxidation due to the presence of oxygen. If so, the recovery after electrolysis would be 100% rather than the 200% observed. Instead, they are consistent with a rather fast disulfide exchange equilibrium between cysteine (CSH), cystine (CSSC), protein-sulfhydryl groups (PrSH), and protein-cysteine mixed disulfides (PrSSC) as represented by Equation 20.

$$CSH + PrSSC \rightleftharpoons CSSC + PrSH \qquad (20)$$

Evidence for protein-cysteine mixed disulfides in plasma was first observed by King (191), and a normal concentration of about 300 micromolar has been indicated (192). The serum protein sulfhydryl content of normal persons is quite stable, and rarely fluctuates below the 400-600 micromolar range (193). Using these values and the measured concentrations of cysteine and

cystine, an equilibrium constant as defined by Equation 21

$$K = \frac{[CSSC][PrSH]}{[CSH][PrSSC]} \qquad (21)$$

was estimated for the above reaction. As can be seen from Table 8, the calculated values for K from results obtained with and without the addition of cysteine are approximately the same, which indicates that the disulfide exchange equilibrium is established within the time scale of these experiments (approximately 10 min.). The presence of a rather fast equilibrium between serum albumin and thiols or disulfides has also been observed by others (194,195).

In those methods which use excess iodoacetate to make the S-carboxymethyl derivative of cysteine, the protein-SH groups are also converted into the S-carboxymethyl derivative form. Depending on the rate constants for the reaction between iodoacetate and cysteine and for the reaction between iodoacetate and the protein-SH groups, the equilibrium represented by Equation 20 will shift to the left or to the right during derivatization. Since the methods employing the S-carboxymethyl derivative of cysteine give high results for cysteine and low results for cystine when compared with the results

obtained by the HPLC method with electrochemical detection, it appears that iodoacetate reacts faster with cysteine than with the protein-SH groups. This causes the equilibrium represented by Equation 20 to shift to the left resulting in a production of more cysteine which gives high values for cysteine and low values for cystine. In the HPLC method with electrochemical detection, the position of the equilibrium is frozen upon acidification of the plasma. These considerations suggest that the results in Table 7 more nearly represent normal plasma cysteine and cystine levels than do the currently accepted values (30,122,123,190).

The described HPLC methods are fast and sensitive, and both the reduced and total cysteine levels and the total homocysteine level can be determined. Since the thiols are the only components, apart from chloride, of plasma and urine which are electroactive at the applied potential of +0.1 V vs. SCE, this reduces considerably the requirements and the time of the chromatographic step as compared to amino acid analyzer procedures using ninhydrin detection.

CHAPTER VIII

DETERMINATION OF CYSTEINE AND GLUTATHIONE IN SOME FRUITS

A. Introduction

The main nonprotein thiols in plants are cysteine and glutathione. Exceptions include the mung bean, which contains homoglutathione (196). Nonprotein thiols are thought to be involved in various aspects of plant physiology, including such processes as protoplasmic streaming (197), electron transport and phosphorylation during photosynthesis (198), cell division (199), and keeping ascorbic acid in its reduced form (80). They also are thought to be involved in frost hardiness (200,201), and it has been demonstrated that thiol compounds and thiol reagents can regulate the ripening of Bartlett pears (202). Knowledge about these processes at the molecular level, which at present is limited, requires selective and sensitive methods of analysis. There are few reports in the literature on the determination of nonprotein thiols in fruits and vegetables.

In this chapter, the approach of liquid chromatography with selective electrochemical detection to determine the concentrations of cysteine and glutathione in some fruits is described. Since the technique is applicable to thiols in general, it should be easily

extendable to the determination of cysteine and other thiol compounds in plant protein hydrolysates and other food products.

B. Chromatographic Conditions

A 0.5% H_3PO_4 solution was used as eluent. Separation of cysteine and glutathione was performed on the 0.2 x 50-cm Zipax SCX strong cation-exchange column. A flow rate of 0.5 mL/min. was used, and the electrochemical detector was operated at +0.1 V vs. SCE. Under these conditions, the capacity factor for glutathione was 0.9 and for cysteine 1.8.

C. Results

Figure 35 shows a typical chromatogram obtained for juice from a ripe tomato treated as described at the end of Chapter II. The unretained peak at about 1.5 min. after sample injection is due to double layer capacitance effects combined with detector response to chloride in the sample. The peaks at ca. 3 min. and 4.5 min. are due to glutathione and cysteine, respectively. The total elution time is about 6 min. The limits of detection for the described conditions are ca. 2×10^{-6} M for glutathione and ca. 3×10^{-6} M for cysteine. The levels of glutathione and cysteine in various fruits investigated are listed in Table 9. Standard mixtures of glutathione and cysteine were

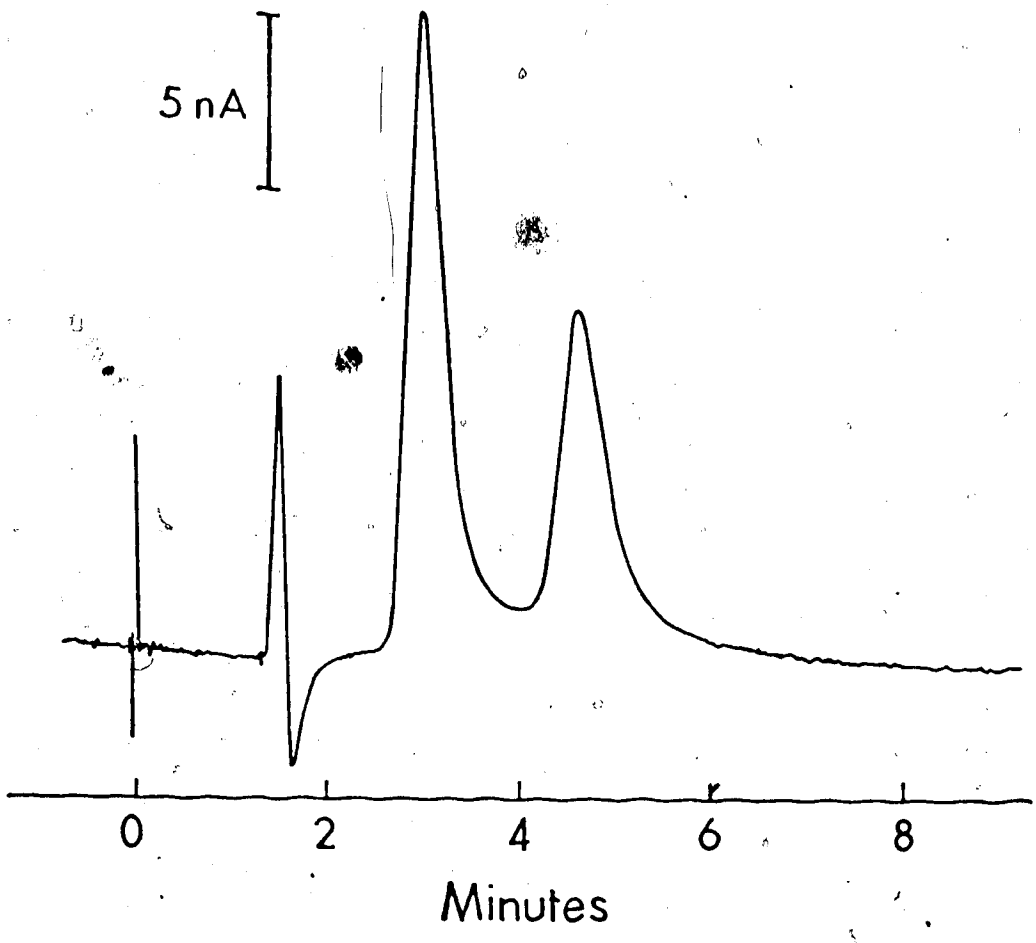


Figure 35. Representative chromatogram obtained from juice from a ripe tomato. Peak heights correspond to 6.8×10^{-5} M glutathione and 4.6×10^{-5} M cysteine.

TABLE 9

Concentrations of Cysteine (CSH) and Glutathione (GSH) in the Juice of Various Fruits[a]

Sample	[GSH][b]	[CSH][b]
Tomato 1	16.5	17.1
Tomato 2	6.6	10.6
Tomato 3	13.6	9.2
Tomato juice (Libby's)	2.2	2.1
Orange 1	11.6	0.9
Orange 2	7.6	0.8
Green grapes	18.5	0.9
Lemon	1.7	0.3
Kiwi-fruit	22.5	1.7
Cantaloupe	6.9	0.4

a) The concentrations given are the result of replicate analyses with average relative standard deviations of ± 4.0%.

b) 10^5 x moles/L.

prepared in 1% H_3PO_4 and calibration curves were prepared in terms of the height of the chromatographic peak vs. thiol concentration.

The stability of cysteine and glutathione to oxidation in some of the juices was also studied. The juices were left exposed to the air at room temperature for varying periods of time before acidification with H_3PO_4. Typical results are presented in Table 10. In the juice from the tomatoes, glutathione appears to be oxidized faster than cysteine, which is surprising in view of the report that glutathione is the more stable towards oxidation (203). In general, it is to be expected that the stability of the thiols will depend on the pH of the juice, and as discussed in Chapter II, Section D, once the sample is acidified, both cysteine and glutathione are stable on the time scale of these determinations.

D. Discussion

The described HPLC method is fast and sensitive, and with this method both the cysteine and glutathione levels can be determined simultaneously. Since the thiol compounds are the only components of fruit which are electroactive at the applied potential of +0.1 V vs. SCE, the only requirement of the chromatographic step is that it separate cysteine from glutathione. To test for potential interference from ascorbic acid, a

TABLE 10

Stability of Cysteine (CSH) and Glutathione (GSH) in Fruit Juices to Oxidation

Time[a]	Tomato Juice		Orange Juice	
	[GSH][b]	[CSH][b]	[GSH][b]	[CSH][b]
0	16.5	17.1	11.6	0.9
½ hr	16.9	16.3	11.8	0.9
1 hr	14.2	16.4	11.4	0.9
12 hr	11.9	18.0		

a) The length of time the fruit juice was exposed to air before acidification.

b) 10^5 x moles/L.

1.0×10^{-3} M solution of this vitamin was injected onto the column. This gave no response apart from the unretained peak at about 1.5 min.

The present study focused on the development of a method for the determination of the reduced forms of cysteine and glutathione. With a pre-electrolysis of the sample at a mercury pool electrode as described in the previous chapters for blood and urine analyses, it should be possible to determine the totals of the cysteine and of the glutathione in the reduced and the nonprotein disulfide forms by this method.

Other methods which have been used for the determination of the nonprotein thiol content of fruits and other plant materials include an argentimetric-amperometric titration (200,204,205), polarography (80), colorimetry based on reaction with Ellman's reagent (206), a p-chloromercurybenzoate titration (207), and filter paper chromatography (208). In comparison to the HPLC method, the titration, polarographic, and colorimetric methods provide only the total nonprotein thiol content. The filter paper chromatography method is selective but considerably more involved. Jansen and Jang (207) achieved selectivity by an elaborate procedure which required some 60 liters of juice.

It is of interest that, of the various fruits investigated, only the tomatoes contained a significant

amount of cysteine. This result is in disagreement with the results of Zuman (80) who concluded from polarographic measurements that his tomato samples contained only glutathione. It may be that the different kinds of tomatoes studied contain different relative amounts of cysteine and glutathione. The two oranges tested contained less glutathione and cysteine than found by Miller and Rockland (208). Again, this may be due to different varieties or to the oxidation of thiol during storage of the oranges. The highest glutathione concentrations were found in kiwi-fruit and green grapes, whereas the lowest level appeared in the lemon.

BIBLIOGRAPHY

1. P.C. Jocelyn, "Biochemistry of the SH Group", Academic Press, New York, 1972, p. XV.
2. B.L. Larson and G.W. Salisbury, J. Biol. Chem., 201, 601 (1953).
3. L. Young and G.A. Maw, "The Metabolism of Sulphur Compounds", Methuen & Co Ltd, London, 1958, p. 11.
4. W.H. Wollaston, Phil. Trans. Roy. Soc., 223 (1810).
5. K.A.H. Mörner, Hoppe-Seyler's Z. Physiol. Chem., 28, 595 (1899).
6. C. Neuberg, Ber., 35, 3161 (1902).
7. A. Heffter, Med. Naturwiss. Arch., 1, 81 (1907).
8. F.G. Hopkins, Biochem. J., 15, 286 (1921).
9. Ref. 1, p. 10.
10. H. [illegible]berg and S. Rapoport, Acta Biol. Med. Ger., 21, 559 (1968).
11. G.C. [illegible]ls, J. Biol. Chem., 229, 189 (1957).
12. E. Racker, J. Biol. Chem., 217, 867 (1955).
13. S. Nagai and S. Black, J. Biol. Chem., 243, 1942 (1968).
14. H. Modig, Biochem. Pharmacol., 17, 177 (1968).
15. L.F. Chasseaud, Drug Metab. Rev., 2, 185 (1973).
16. A. Meister, in "Glutathione: Metabolism and Function", I.M. Arias and W.B. Jacoby, Eds., Raven Press, New York, 1976, p. 35.

17. Ref. 1, pp. 206-211.

18. Ref. 1, p. 4.

19. L. Laster, S.H. Mudd, J.D. Finkelstein, and F. Irreverre, J. Clin. Invest., 44, 1708 (1965).

20. J.A. Schneider, K.H. Bradley, and J.E. Seegmiller, J. Lab. Clin. Med., 71, 122 (1968).

21. W.E. Knox, Am. J. Hum. Genet., 10, 3 (1958).

22. S.H. Mudd, in "Inherited Disorders of Sulphur Metabolism", N.A.J. Carson and D.N. Raine, Eds., Churchill Livingston, London, 1971, p. 204.

23. A.D. Patrick and B.D. Lake, J. Clin. Path., 21, 571 (1968).

24. J.M. Walshe, Am. J. Med., 21, 487 (1956).

25. L. Magos and T. Stoytchev, Br. J. Pharmacol., 35, 121 (1969).

26. Multicenter Trial Group, Lancet, 257 (1973).

27. P.J. Hissin and H. Russell, Anal. Biochem., 74, 214 (1976).

28. W.H. Hadley, W.F. Bousquet, and T.S. Miya, J. Pharm. Sci., 63, 57 (1974).

29. W.H. Stein, J. Biol. Chem., 201, 45 (1953).

30. M.P. Brigham, W.H. Stein, and S. Moore, J. Clin. Invest., 39, 1633 (1960).

31. G.W. Frimpter and A. Bass, J. Chromatogr., 7, 427 (1962)

32. J.C. Crawhall, C.J. Thompson, and K.H. Bradley, Anal. Biochem., 14, 405 (1966).

33. J.W. Purdie, R.A. Gravelle, and D.E. Hanafi, J. Chromatogr., 38, 346 (1968).

34. J.O. Jeppsson and I.M. Karlsson, J. Chromatogr., 72, 93 (1972).

35. B. Fowler and A.J. Robins, J. Chromatogr., 72, 105 (1972).

36. J.S. Dohan and G.E. Woodward, J. Biol. Chem., 129, 393 (1939).

37. H.B. Vickery and A. White, J. Biol. Chem., 99, 701 (1933).

38. S. Graft, E. Maculla, and A.M. Graff, J. Biol. Chem., 121, 11 (1937).

39. H.T. Clarke, J. Biol. Chem., 97, 235 (1932).

40. O. Gawron, S. Mahboob, and J. Fernando, J. Am. Chem. Soc., 86, 2283 (1964).

41. W.W. Cleland, Biochemistry, 3, 480 (1964).

42. K. Bir, J.C. Crawhall, and D. Mauldin, Clin. Chim. Acta, 30, 183 (1970).

43. K. Rootwelt, Scand. J. Clin. Lab. Invest., 19, 325 (1967).

44. R. Benesch and R.E. Benesch, Arch. Biochem., 19, 35 (1948).

45. R.E. Benesch and R. Benesch, Arch. Biochem., 28, 43 (1950).

46. L.A.Ae. Sluyterman, Biochim. Biophys. Acta, 25, 402 (1957)

47. L.A.Ae. Sluyterman, Anal. Biochem., 14, 317 (1966).

48. A.J. Grimes, Nature (London), 205, 94 (1965).

49. K. Aibara, E.O. Herreid, and H.K. Wilson, J. Dairy Sci., 43, 1736 (1960).

50. Kh.F. Schol'ts, Biochemistry, 29, 495 (1964).

51. R. Cecil and J.R. McPhee, Biochem. J., 59, 234 (1955).

52. J.H. Ladenson and W.C. Purdy, Clin. Chem., (Winston-Salem, N.C.), 17, 908 (1971).

53. P. Sen and K. Bahadur, Talanta, 21, 968 (1974).

54. I.M. Kolthoff, W. Stricks, and L. Morren, Anal. Chem., 26, 366 (1954).

55. R. Cecil, Biochim. Biophys. Acta, 18, 154 (1955).

56. E.P. Przbylowicz and L.B. Rogers, Anal. Chim. Acta, 18, 596 (1958).

57. G. Calcutt, Biochim. Biophys. Acta, 44, 364 (1960).

58. D.A. Doornbos, Pharm. Weekbl., 102, 1095 (1967).

59. M. Wronski, Analyst (London), 90, 697 (1965).

60. H.E. Tunncliffe, Biochem. J., 19, 194 (1925).

61. J.W. Thompson and C. Voegtlin, J. Biol. Chem., 70, 793 (1926).

62. W.A. Perlzweig and G. Delrue, Biochem. J., 21, 1416 (1927).

63. H.L. Mason, J. Biol. Chem., 86, 623 (1930).

64. E.J. King and C.C. Lucas, Biochem. Z., 235, 66 (1931).

65. J. Kühnau, Biochem. Z., 230, 353 (1931).

66. N. Gavrilescu, Biochem. J., 25, 1190 (1931).

67. C.F. Bickford and R.E. Schoetzow, J. Am. Pharm. Assoc., 26, 409 (1937).

68. A.P. Kreshkov and L.B. Oganesyan, J. Anal. Chem. (U.S.S.R.), 26, 534 (1971).

69. G.E. Woodward and E.G. Fry, J. Biol. Chem., 97, 465 (1932).

70. I.A. Divin, V.P. Grechanovskii, and Ya.I. Malusis, Lab Prakt. (U.S.S.R.), 1, 25 (1937).

71. A. Fujita and I. Numata, Biochem. Z., 299, 246 (1938).

72. P. Seeligman, Arch. Bioquim. Quim. Farm. Tucuman, 10, 105 (1962); Chem. Abs., 60, 2256.

73. G. Weller, Rev. Franc. Etudes Clin. Biol., 10, 547 (1965).

74. D.M. Coulson, W.R. Crowell, and S.L. Friess, Anal. Chem., 22, 525 (1950).

75. A.P. Kreshkov and L.B. Oganesyan, J. Anal. Chem. (U.S.S.R.), 28, 2012 (1973).

76. R.J. Thibert and M. Sarwar, Microchim. Acta, 259 (1969)

77. I.M. Kolthoff and W. Stricks, Anal. Chem., 23, 763 (1951).

78. I.M. Kolthoff and C. Barnum, J. Am. Chem. Soc., 62, 3061 (1940).

79. W. Stricks and I.M. Kolthoff, J. Am. Chem. Soc., 74, 4646 (1952).

80. P. Zuman, Coll. Czech. Chem. Comm., 16, 510 (1951).

81. S.J. Leach, Austr. J. Chem., 13, 520 (1960).

82. P.D.J. Weitzman and H.J. Tyler, Anal. Biochem., 43, 321 (1971).

83. H. Berge and D. Jeroschewski, Z. Anal. Chem., 212, 278 (1965).

84. J. Pradac, J. Pradacova, and J. Koryta, Biochim. Biophys. Acta, 237, 450 (1971).

85. J. Fischl, I. Sason, and S. Segal, Clin. Chem. (Winston-Salem, N.C.), 7, 674 (1961).

86. A.F. Rosenthal and A. Yaseen, Clin. Chim. Acta, 26, 363 (1969).

87. R. Bierich and A. Rosenbohm, Hoppe-Seyler's Z. Physiol. Chem., 215, 151 (1933)

88. K. Uhlenbroock, Hoppe-Seyler's Z. Physiol. Chem., 236, 192 (1935)

89. H. Herrmann and S.G. Moses, J. Biol. Chem., 158, 33 (1945).

90. E. Beutler, O.S. Duron, and B.K. Kelly, J. Lab. Clin. Med., 61, 882 (1963).

91. C.W.I. Owens and R.V. Belcher, Biochem. J., 94, 705 (1965).

92. F. Tietze, Anal. Biochem., 27, 502 (1969).

93. P.L. Wendell, Biochem. J., 117, 661 (1970).

94. J. Roberts and N.S. Agar, Clin. Chim. Acta, 34, 475 (1971).

95. T.D. Stevenson, B.L. McDonald, and S. Roston, J. Lab. Clin. Med., 56, 157 (1960).

96. W.W. Kay and K.C. Murfitt, Biochem. J., 74, 203 (1960).

97. P.C. Jocelyn, Anal. Biochem., 18, 493 (1967).

98. K.T. Lee and I.K. Tan, Clin. Chim. Acta, 53, 153 (1974)

99. V.H. Cohn and J. Lyle, Anal. Biochem., 14, 434 (1966).

100. P.C. Jocelyn and A. Kamminga, Anal. Biochem., 37, 417 (1970).

101. A.A. Fernandez and R.J. Henry, Anal. Biochem., 11, 190 (1965).

102. B. Vassel, J. Biol. Chem., 140, 323 (1941).

103. D.K. Mecham, J. Biol. Chem., 151, 643 (1943).

104. M.K. Gaitonde, Biochem. J., 104, 627 (1967).

105. B. States and S. Segal, Clin. Chim. Acta, 43, 49 (1973).

106. S. Roston, Anal. Biochem., 6, 486 (1963).

107. J.A. Schneider, K.H. Bradley, and J.E. Seegmiller, Anal. Biochem., 23, 129 (1968).

108. P.R, Pal, J. Biol. Chem., 234, 618 (1959).

109. K. Borner, Hoppe-Seyler's Z. Physiol. Chem., 341, 264 (1965).

110. E. Bernt and H.U. Bergmeyer, in "Methods of Enzymatic Analysis", H.U. Bergmeyer, Ed., Academic Press, Inc., New York, 1974, Vol. pp 1643-1647.

111. G.E. Woodward, J. Biol. Chem., 109, 1 (1935).

112. M. Koivusalo and L. Uotila, Anal. Biochem., 59, 34 (1974).

113. L. Lack and M. Smith, Anal. Biochem., 8, 217 (1964)

114. E.F. Schroeder and G.E. Woodward, J. Biol. Chem., 129, 283 (1939).

115. B.E. Davidson and F.J. Hird, Biochem. J., 93, 232 (1964)

116. T. Wieland, K. Dose, and G. Pfeiderer, Biochem. Z., 326, 442 (1955)

117. A.H. Gordon, Biochem. J., 45, 99 (1949).

118. E.L. Smith and E.F. Tuller, Arch. Biochem. Biophys., 54, 114 (1955).

119. B.J. White and R.G. Wolfe, J. Chromatogr., 7, 516 (1962).

120. H. Wernze and W. Koch, Klin. Wochschr., 43, 454 (1962).

121. R.F. Bayfield and E.R. Cole, J. Chromatogr., 40, 470 (1969).

122. D.R. London and T.H. Foley, Clin. Sci., 29, 129 (1965).

123. L.E. Rosenberg, J.L. Dumont, and J.M. Holland, New England J. Med., 273, 1239 (1965).

124. W.D. Block, M.E. Markovs, and B.F. Steele, Am. J. Clin. Nutr., 22, 33 (1969).

125. C.W. Tabor and H. Tabor, Anal. Biochem., 78, 543 (1977).

126. S. Moore, J. Biol. Chem., 238, 235 (1963).

127. H.J. Miles, E.R. Stadtman, and W.W. Kielley, J. Am. Chem. Soc., 76, 4041 (1954).

128. M.J. Smith and R. Rodnight, Biochem. J., 72, 1P (1957).

129. R. Muenze, Wiss. Z. Karl-Marx Univ. Leipzig, Math.-Naturw. Reihe, 18, 621 (1969).

130. J.M.L. Mee, J. Chromatogr., 87, 258 (1973).

131. R.F. McGregor, G.M. Brittin, and M.S. Sharon, Clin. Chim. Acta, 48, 65 (1973).

132. E. Jellum, V.A. Bacon, W. Patton, W. Pereira, and B. Halpern, Anal. Biochem., 31, 339 (1969).

133. W.J. Blaedel and J.W. Todd, Anal. Chem., 33, 205 (1961).

134. J.G. Koen, J.F.K. Huber, H. Poppe, and G. den Boef, J. Chromatogr. Sci., 8, 192 (1970)

135. P.L. Joynes and R.J. Maggs, J. Chromatogr. Sci., 8, 427 (1970)

136. P.T. Kissinger, C. Refshauge, R. Dreiling, and R.N. Adams, Anal. Lett., 6, 465 (1973)

137. D.C. Johnson and J. Larochelle, Talanta, 20, 959 (1973)

138. Y. Takata and G. Muto, Anal. Chem., 45, 1864 (1973).

139. P.T. Kissinger, L.J. Felice, R.M. Riggin, L.A. Pachla, and D.C. Wenke, Clin. Chem. (Winston-Salem, N.C.), 20, 992 (1974)

140. B. Fleet and C.J. Little, J. Chromatogr. Sci., 12, 747 (1974)

141. L.R. Taylor and D.C. Johnson, Anal. Chem., 46, 262 (1974)

142. E. Pungor, K. Toth, Z. Geher, G. Nagy, and M. Varadi, Anal. Lett., 8, ix (1975)

143. T. Wasa and S. Musha, Bull. Chem. Soc. Jpn., 48, 2176 (1975)

144. C.L. Blank, J. Chromatogr., 117, 35 (1976)

145. R.C. Buchta and L.J. Papa, J. Chromatogr. Sci., 14, 213 (1976)

146. J.E. Lankelma and L.J. Papa, J. Chromatogr., 125, 357 (1976).

147. P.T. Kissinger, Anal. Chem., 49, 447A (1977).

148. J.A. Cox and A. Przyjazny, Anal. Lett., 10, 869 (1977).

149. B.G. Julin, H.W. Vandenborn, and J.J. Kirkland, J. Chromatogr., 112, 443 (1975).

150. R. Benesch and R.E. Benesch, Biochim. Biophys. Acta, 23, 643 (1957).

151. J.C. Crawhall, E.F. Scowen, and R.W.E. Watts, Br. Med. J., 1, 1411 (1964).

152. D.D. Perrin and B. Dempsey, "Buffers for pH and Metal Ion Control", Chapman and Hall Ltd., London, 1974, p. 147 and p. 153.

153. L. Meites, Anal. Chim. Acta, 18, 364 (1958).

154. W. Kemula, Rocz. Chem., 26, 281 (1952).

155. V.G. Levich, "Physicochemical Hydrodynamics", Prentice Hall, New York, N.Y., 1962.

156. Z. Feher, G. Nagy, K. Toth, and E. Pungor, Analyst (London), 99, 699 (1974).

157. W.B. Schaap and P.S. McKinney, Anal. Chem., 36, 1251 (1964).

158. W.L. Belew, D.J. Fisher, and M.T. Kelley, Chem. Instr., 2, 297 (1970)

159. J. Yamada and H. Matsuda, Electroanal. Chem. and Interfac. Electrochem., 44, 189 (1973).

160. K.J. Vetter, "Electrochemical Kinetics", Academic Press, New York, 1967, p. 73.

161. B.L. Karger, L.R. Snyder, and C. Horvath, "An Introduction to Separation Science", John Wiley & Sons, Inc., 1973, pp. 159-161

162. C. Mairess-Ducarmois, G. Patriarche, and J. Vandenbalck, Anal. Chim. Acta, 71, 165 (1974)

163. C. Mairess-Ducarmois, G. Patriarche, and J. Vandenbalck, Anal. Chim. Acta, 76, 299 (1975)

164. F. Helfferich, "Ion Exchange", McGraw-Hill, New York, 1962

165. D.L. Rabenstein, J. Am. Chem. Soc., 95, 2797 (1973)

166. E.J. Kuchinskas and Y. Rosen, Arch. Biochem. Biophys., 97, 370 (1962)

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

168. H.A. Laitinen and W.E. Harris, "Chemical Analysis", McGraw-Hill, Sec. Ed., 1975, pp. 41-42.

169. Ref. 161, pp. 353-356.

170. Ref. 161, pp. 30-31.

171. Ref. 161, pp. 356-359.

172. Ref. 161, pp. 344-345.

173. C. Horvath, B. Preiss, and S.R. Lipsky, Anal. Chem., 39, 1422 (1967).

174. J.J. Kirkland, J. Chromatogr. Sci., 8, 72 (1970).

175. R.E. Leitch and J.J. DeStefano, J. Chromatogr. Sci., 11, 105 (1973).

176. Ref. 161, p. 137.

177. K. Gibbs and J.M. Walshe, Q. J. Med., 40, 275 (1971).

178. D. Perrett, W. Sneddon, and A.D. Stephens, Biochem. Pharm., 25, 259 (1976).

179. Ref. 1, pp. 94-110.

180. Ref. 1, p. 121.

181. I.M. Kolthoff, W. Stricks and R.C. Kapoor, J. Am. Chem. Soc., 77, 4733 (1955)

182. J.C. Crawhall, E.F. Scowen, and R.W.E. Watts, Br. Med. J., 1, 1270 (1973).

183. E.S.K. Assem and M.R. Vickers, Postgrad. Med. J., Suppl., 50, 10 (1974).

184. F. Bakir, S.F. Damluji, L. Amin-Zaki, M. Murtadha, A. Khalidi, N. Al-Rawi, S. Tikriti, H.I. Chahir, T.W. Clarkson, J.C. Smith, and R.A. Doherty, Science, 181, 230 (1973).

185. S. Selander, K. Cramer, and L. Hallberg, Br. J. Indust. Med., 23, 282 (1966).

186. J.C. Crawhall, E.F. Scowen, and R.W.E. Watts, Br. Med. J., 1, 588 (1963).

187. Ref. 1, pp. 165-166.

188. J.G. Wagner, J. Pharmakokin. Biopharm., 1, 363 (1973).

189. A. Neuberger, Ann. Rev. Biochem., 18, 243 (1949).

190. J.C. Crawhall, P.S. Lietman, J.A. Schneider, and J.E. Seegmiller, Am. J. Med., 44, 330 (1968).

191. T.P. King, J. Biol. Chem., 236, PC5 (1961).

192. Ref. 1, p. 253.

193. A. Lorber, C.M. Pearson, W.L. Meredith, and L.E. Gautz-Mandell, Ann. Int. Med., 61, 423 (1964).

194. T.E. Isles and P.C. Jocelyn, Biochem. J., 88, 84 (1963).

195. A. Lorber, C.C. Chang, D. Masuoka, and J. Meacham, Biochem. Pharm., 19, 1551 (1970).

196. P.R. Carnegie, Biochem. J., 89, 471 (1963).

197. N. Kamiya, Ann. Rev. Pl. Physiol., 11, 323 (1960).

198. Ref. 1, pp. 281-283.

199. T. Stonier and H.M. Yang, Plant Physiol., 51, 391 (1973).

200. J. Levitt, C.Y. Sullivan, N.O. Johansson, and R.M. Pettit, Plant Physiol., 36, 611 (1961).

201. H. Kohn and J. Levitt, Plant Physiol., 41, 792 (1966).

202. C. Frenkel, Bot. Gaz., 137, 154 (1976).

203. P.C. Jocelyn, Clin. Chim. Acta, 3, 401 (1958).

204. I.M. Kolthoff and W.E. Harris, Anal. Chem., 18, 161 (1946).

205. N. Weissman, E.G. Schoenback, and E.B. Armistead, J. Biol. Chem., 187, 153 (1950).

206. G.L. Ellman, Arch. Biochem. Biophys., 82, 70 (1959).

207. E.F. Jansen and R. Jang, Arch. Biochem. Biophys., 40, 358 (1952).

208. J.M. Miller and L.B. Rockland, Arch. Biochem. Biophys., 40, 416 (1952).