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# Interaction of Selenite and Arsenite on Amino Acids and Related Compounds

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# INTERACTIONS OF SELENITE AND ARSENITE ON AMINO ACIDS AND RELATED COMPOUNDS

BY

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# INTERACTIONS OF SELENITE AND ARSENITE ON AMINO ACIDS AND RELATED COMPOUNDS

This thesis is approved as a creditable, independent investigation by a candidate for the degree, Master of Science, and acceptable as meeting the thesis requirements for this degree; but without implying that the conclusions reached by the candidate are necessarily the conclusions of the major department.

Thesis Advisor

Hĕad of the Major Department

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Also the generous help of Mrs. H. L. Klug with matters of construction and grammar is gratefully acknowledged.

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## LIST OF ABBREVIATIONS

GSH - reduced glutathione

CYS - cystine

MET - methionine

HIS - histidine

HOM - homocysteine

TAU - taurine

CYST - cysteine

SER - serine

ISO - isoleucine

LEU - leucine

LYS - lysine

$AsO_2$ - sodium arsenite

$SeO_3$ - sodium selenite

Se - selenium

PHY - phenylalanine

PRO - proline

THR - threonine

TRP - tryptophane

TYR - tyrosine

VAL - valine

ALA - alanine

ARG - arginine

GLU - glutamic acid

ASP - aspartic acid

GLY - glycine

$AsO_3$ - sodium arsenate

As - arsenic

M - molar or moles

# INTRODUCTION

It has been repeatedly verified that selenium, when fed as sodium selenite or sodium selenate, or injected intravenously or intraperitoneally, is chemically bound to the protein in various tissues (1). Further, it has been shown that selenium can be incorporated into protein in such organisms as yeast and bacteria, or even more complex organisms such as rats, dogs, and mice (2, 3, 4).

It is the intent of this author to explore the role of a non-enzymatic reaction between the amino acids and selenium. Reports by previous investigators (5) indicate that further work in this area is needed. Rosenfeld and Beath (6), and a number of other workers (3, 4), reported finding selenium compounds in the various tissues of rats and dogs. Tests in the South Dakota State College Chemistry laboratories indicate that selenium as selenite can be reduced to elemental selenium in an alkaline solution. Other workers have reported the formation of seleno-glutathione (7) and seleno-cysteine (8) from a non-enzymatic reaction of the amino acid or the tripeptide and sodium selenite. Thus it seemed that investigations along this line would prove fruitful.

# HISTORY

Investigations which led to the discovery of selenium as the causative agent for the "alkali disease" in animals were directed largely by Dr. Kurt Franke of the South Dakota Agricultural Experiment Station (9). Franke observed the symptoms of chronic selenium poisoning, then known as "alkali disease", of farm animals and collected samples of the forage and grain grown in the involved regions (9). When the forage and grain were fed to normal laboratory animals, similar symptoms developed. This led to the discovery by Robinson (10) that selenium was actually the toxic agent. Chemical analysis of all the samples obtained by Franke (9) then showed them to contain toxic amounts of selenium. The protein of the samples contained most of the selenium (9), and selenium was detected in the soil of all known regions where toxic grain and forage grew (10).

The first biological evidence to indicate that selenium was the sole causative agent of "alkali disease" was the development, after the addition of sodium selenate or selenite to a normal diet, of signs in the rat which appeared identical with those produced by the natural toxicant (11). The general signs of "alkali disease" as described by Trelease and Beath (12) are dullness and lack of vitality of the animal, emaciation, anemia, stiffness of joints, lameness, roughened coat, loss of hair, and hoof lesions and deformities. These signs are in contrast to those for acute selenium poisoning which Trelease and Beath (12) describe as a breath with a garlicky odor, signs of nervousness, and fear. These primary signs are followed by quietness and somnolence.

Respiration becomes difficult. The reflexes are decreased, but the heart action remains normal. The labored breathing is followed by opisthotonus, tetanic spasms in the muscles of the extremities, clonic spasms, and death. A marked sign of acute poisoning is the gradual fall in blood pressure within fifteen to twenty minutes which continues until death occurs. The primary action of selenium appears to be on the central nervous system, and death is due to respiratory failure (12).

Ever since the attempt by Cameron (13) in 1880 to supply selenate to plants and thereby replace the sulfur of the plant proteins with selenium, many investigators have attempted to explain the toxicity of selenium compounds on the basis of such an exchange. Because the two compounds are so similar chemically it has seemed plausible to assume that most organisms are unable to distinguish between them. It would follow, then, that selenium compounds would appear as psuedo-sulfur compounds and disrupt the normal metabolic pathways for sulfur.

In 1942 Stekol (8) reported the formation of seleno-tetracysteine from the reaction of aqueous $1x10^{-2}$ molar cysteine hydrochloride with $2.5x10^{-3}$ molar sodium selenite. This reaction mixture gave a white granular substance with a selenium to nitrogen ratio of one to four respectively. Investigations by Klug and Petersen (14) established the stoichiometry of the reaction between selenious acid and cysteine to be one mole of selenious acid to four moles of cysteine giving as products cystine and seleno-dicysteine.

Lampson and Klug (7) reported the preparation of selenium tetraglutathione by mixing aqueous solutions of $1x10^{-2}$ molar reduced glutathione

with $2.5 \times 10^{-3}$ molar selenious acid. The reaction product was precipitated with absolute ethanol. An elemental analysis of the compound formed showed a four to one ratio of sulfur to selenium respectively (7). Tests were conducted to show that the compound formed was not merely a mechanical mixture of the two reactants. Iodometric titration of an aqueous solution of the compound showed no free sulfhydryl groups to be present. No free selenious acid could be titrated with sodium thiosulfate solution (7).

It is noteworthy that the seleno-tetraglutathione prepared by Lampson and Klug (7), when fed to laboratory animals was markedly less toxic than selenium fed as sodium selenite. This fact would seem to indicate that the reaction of selenium with sulfhydryl compounds converts it to a less toxic form. Subsequently Petersen (15) reported the reaction products of reduced glutathione and selenite to be oxidized glutathione, and seleno-diglutathione.

Investigations carried out by Tsen and Tappel (16) showed that selenium as selenite seemed to function best as a catalyst for the oxidation of reduced glutathione in a mole ratio of one mole of selenite to 100 moles of reduced glutathione. These authors also reported the oxidation of other sulfhydryl compounds by selenite and found it to be a better catalyst than copper (II) ion. Further, Tsen and Tappel (16) showed that tellurite and arsenite were good inhibitors of selenite catalysis. These authors suggested that a compound of arsenite and glutathione might be the active inhibitor of selenite catalysis.

The lessening of the toxicity of orally ingested selenium by

# EXPERIMENTAL PROCEDURE

## Materials

Albino rats, Sprague-Dawley strain, raised on the South Dakota State College campus were used for the enzymatic studies. They were fed Purina Laboratory Chow ad libitum. Cytochrome c was obtained from Nutritional Biochemicals Corporation, Cleveland, Ohio, and standardized on a Beckman DU spectrophotometer (24). Sodium selenite and sodium selenate were obtained as chemically pure reagents from Fisher Scientific Company, Chicago, Illinois. The amino acids cystine, methionine, aspartic acid, glycine, glutamic acid, and leucine, as well as taurine, were obtained from Mann Fine Chemicals, New York, New York, as D-L mixtures. Homocysteine, serine, cysteine, alanine, arginine, isoleucine, lysine, phenylalanine, threonine, tryptophane, tyrosine, and valine and also proline, glutathione, and ninhydrin were purchased from Nutritional Biochemicals Corporation as D-L mixtures. The paper used for the chromatographic studies was Whatman, grade number one, secured from W. R. Balston, Ltd., London. All other solutions were made up from chemically pure laboratory chemicals. Two times glass-distilled water was used for preparing all solutions.

## Methods

### Manometric techniques

The enzymatic as well as the non-enzymatic studies were conducted using a Lardy-Warburg Respirometer manufactured by Gilson Medical

Electronics, Madison, Wisconsin. The succinoxidase system studied was that outlined by Schneider and Potter (24, 25). The system was prepared as follows:

(A) Preparation of reactants:

1. 0.1 molar phosphate buffer was made up by adding 50 milliliters of one molar sodium dihydrogen phosphate to 21 milliliters of 2 molar sodium hydroxide, diluting the mixture to 500 milliliters and adjusting the pH to 7.4 if necessary.

2. 0.5 molar sodium succinate was prepared by dissolving 5.4 grams in water and diluting to 40 milliliters.

3. $1 \times 10^{-4}$ molar cytochrome c was prepared by adding 16.5 milligrams to 10 milliliters of water and standardizing on a Beckman DU spectrophotometer.

4. $1.2 \times 10^{-3}$ molar calcium and aluminum mixture was prepared by diluting 1.2 milliliters each of 0.1 molar solutions of calcium chloride and aluminum chloride to a final volume of 10 milliliters.

5. Directions for the preparation of liver homogenates are given below; however, to prepare 10 milligram assay, 0.1 milliliter of a 10 percent homogenate must be used, or 0.2 milliliter of a 5 percent homogenate.

(B) Preparation of flask for study:

1. Phosphate buffer, 0.1 molar . . . . . 1.0 ml
2. Sodium succinate, 0.5 molar . . . . . 0.3 ml
3. Cytochrome c, $1 \times 10^{-4}$ molar . . . . . 0.4 ml
4. Calcium and aluminum mixture, $1.2 \times 10^{-2}$ molar . . . 0.1 ml
5. Rat liver homogenate, 5 percent . . . . . 0.2 ml
6. Sodium hydroxide, 2 molar, in center cups . . . . . 0.2 ml
7. Water . . . . . 1.0 ml
8. Papers were added to the center cups.

When other material such as sodium selenite was added, the amount of water was decreased accordingly to maintain a final volume of 3.2 milliliters. All studies were made at 37 degrees centigrade. Sodium arsenite and glutathione were prepared prior to each run.

The rats were killed by decapitation, and the liver tissue quickly excised and placed on a watch glass surrounded by ice. The chilled tissue was weighed quickly and accurately on a Roller-Smith torsion balance of 500 milligram capacity. Usually 400 to 500 milligrams of liver tissue were used in the preparation of the homogenate. As soon as the tissue was weighed it was transferred to the homogenizer tube which contained a known amount of distilled water. For the preparation of a 10 percent homogenate, the weight of the tissue was multiplied by nine, and this weight of water minus the known amount in the tube was added, assuming 1.0 milliliter of water weighed 1.0 gram.

The Potter-Elvejhem (24, 26) homogenate technique was employed in preparing the tissue for study. The homogenizer functions by tearing the tissue with the cutting teeth and then grinding it between the walls of the test tube and the sides of the pestle. The preparation is considered to be relatively cell-free (27) but often contains shreds of connective tissue. Since the connective tissue tended to clog ordinary pipettes, Mohr pipettes with slightly enlarged openings were used. Also, the pipettes were drawn out at the tips in such a manner that they would fit into the side arm of the Warburg flask or drain against the wall of the main well of the flask.

When the homogenate and all other reactants had been added to the

Warburg flasks, the flasks were attached to the greased manometer joints using spring clips. The Warburg flasks were then placed in a water bath which had been previously adjusted to 37 degrees centigrade. On occasion, when the reaction of amino acids with selenite or arsenite was being studied, the flasks were flushed with oxygen prior to the run.

At the time the flasks were placed in the water bath a regular time interval for all subsequent manipulations of each flask was established, for example, 20 seconds per flask. After all of the flasks had been placed in the water bath, a warm-up period of 6 to 8 minutes was allowed for the flasks and their contents to become equilibrated to the temperature of the bath. During the warm-up period the fluid in the manometer arms was adjusted to the 150 millimeter mark. At the end of the warm-up period the stopcocks on the manometers were closed, the 20 second time interval was observed for each flask, and a zero reading was taken. Subsequent readings were taken at 10 minute or other timed intervals.

Readings were obtained on the manometers by adjusting the fluid in the manometer to the original 150 millimeter mark on the right column of the manometer and reading the level of the fluid in the left column of the manometer. All readings were checked against a flask containing a volume of distilled water equal to the reaction volume. This flask served as a thermobarometer and corrected for changes in temperature and barometric pressure during the course of the study.

In the enzymatic studies, the enzyme, the substrate, and the inhibitor were incubated in various combinations by using the main well

and side arm of the flasks as reservoirs. Thus, it was possible to incubate the enzyme with the substrate, the enzyme with the inhibitor, or the substrate with the inhibitor, and to add the third variable or variables at an arbitrarily determined time.

The amino acid, taurine, and glutathione solutions used in this study were prepared as $1x10^{-2}$ molar solutions in a 0.1 molar phosphate buffer of pH 7.25 and stored in the refrigerator. It was found subsequently that best results were obtained when glutathione was freshly prepared prior to each study.

When studying the reaction of amino acids with the sodium selenite or sodium arsenite, the selenite or arsenite was usually put in the side arm and, on occasion, the flasks were gassed with oxygen. Also by using Warburg flasks with two side arms, it was possible to study the inhibition by sodium arsenite of selenite catalysis of the oxidation of sulfhydryl compounds. Studies were made by incubating the selenite with the sulfhydryl compound and adding the arsenite subsequently or by incubating the arsenite with the sulfhydryl compound and adding the selenite subsequently. At the end of each manometric study of reactions of sodium selenite or sodium arsenite with the added compounds, samples from the contents of each flask were chromatographed.

Chromatographic techniques

Descending paper chromatograms were run in a chromatography cabinet manufactured by Research Specialties Company, Berkeley, California. Also, ascending spot chromatograms were run in glass cylindrical chambers purchased from Microchemical Specialties Company, Berkeley, California.

Several solvent systems were tried so as to achieve maximum separation of amino acids, and products, if any were formed. A solvent of tertiary butyl alcohol, formic acid, and water in a ratio of 70:15:15 by volume was used most often for descending work. Normal-butyl alcohol, acetic acid, and water in a ratio of 60:10:20 by volume, gave best results for ascending chromatography. All chromatograms were run at room temperature.

Each glass solvent trough was cradled in a stainless steel trough support placed in the top of the cabinet. The glass anti-siphon rods were placed lengthwise above the edge of the glass trough with ends resting in the notches of the cradle. The anchor rod was placed in the bottom of the solvent trough. This rod anchored the edge of the chromatogram after it had been placed in the trough. See Figure I. The hanger rods, or anti-siphon rods, act as siphon breakers. Without these rods the chromatograms would tend to lie too close to the inside surface of the trough. This would then cause the solvent to move up between the paper and the glass and siphon over the edge in amounts that would exceed the solvent-carrying capacity of the paper.

Whatman number one paper was used for the chromatography of the amino acid mixtures. With a graphite pencil a line of origin was marked three inches from the edge of the paper. Two folds were then made parallel to the origin line at one-half and two and one-half inches below the line of origin. The folds were made so that the paper would hang as indicated in Figure II. The first fold acted as an "anchor hook" and lay along the bottom of the trough "hooking" under the anchor rod. The second fold fell along the anti-siphon rod and allowed the paper to hang

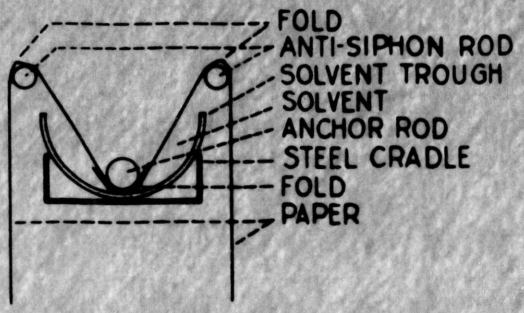



Figure I. Solvent Trough

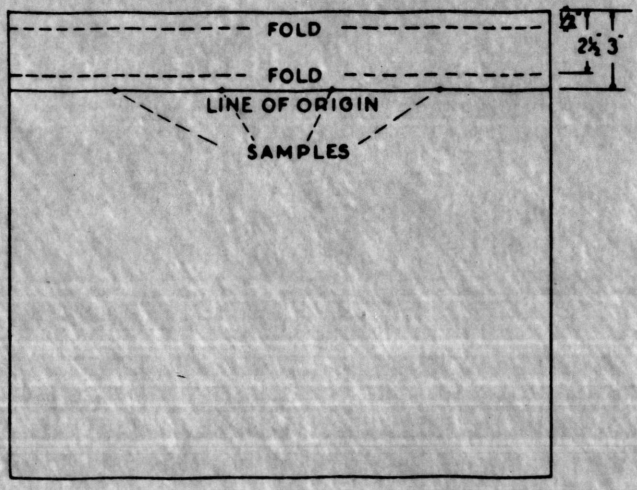



Figure II. One-Dimensional Layout

vertically. See Figure I.

After the papers were marked and folded, they were placed on a smooth table top for application of the samples to be chromatographed. The samples were applied with the penciled origin line facing upward. The folds held the paper beneath the line of origin away from the table and prevented the wet sample from transferring to the table surface.

Many one-dimensional chromatograms were run simultaneously on the same paper by applying samples at three centimeter intervals along the line of origin. The points of application were marked and labelled with a graphite pencil.

The diameter of the origin spots was one centimeter or less. The concentration of the solution to be chromatographed was not critical, since large quantities of dilute solutions could be applied by repeated applications. This process can be hastened considerably by the use of a hair dryer to evaporate the spots. Also, all spots were applied with capillary tubes which were firepolished to prevent puncturing the paper from repeated applications.

Initially the solvent assemblies were placed in the cabinet. Before the papers were placed in the assembly, a check was made to see that the assemblies were properly spaced so that adjacent papers would not touch. Then the papers were hung over the anti-siphon rods and "anchored" in place with anchor rods. When all chromatograms were in place, the lid of the cabinet was secured and 75 to 100 milliliters of solvent were added to each trough. This was added through the lid at one end of the trough so as not to be applied directly on the papers. The

time required to develop the descending chromatograms varied from 12 to 36 hours, depending on the solvent used, approximately 4 hours being required for the smaller ascending spot chromatograms. Progress of the moving solvent could be observed through the window of the cabinet or the glass wall of the cylinders. The paper was removed when the solvent front had descended to within 2 inches of the edge of the paper. For the ascending papers the solvent was allowed to travel 15 to 20 centimeters up the paper. The solvent front was then marked with a graphite pencil and the chromatograms developed with the ninhydrin spray reagent and/or stannous chloride solution.

Ninhydrin, 0.25 percent in water saturated butanol, was used to detect the amino acids, taurine, and glutathione. Stannous chloride, 0.1 molar acidified with hydrochloric acid, was used to detect selenite, by the appearance of a brown spot, and arsenite, by the appearance of a black spot, on the chromatogram at the same position as a ninhydrin positive spot occurs.

## RESULTS AND DISCUSSION

### Enzyme studies

The succinoxidase enzyme system was chosen for this study because (a) it was relatively easy to establish a functional system, (b) it is known to require sulfhydryl groups for its activity (12), (c) preliminary investigations have revealed that it is inhibited by sodium selenite (12), and (d) the reaction could be followed employing manometric techniques. The succinoxidase system involves the following series of reactions:

(a) Succinic acid $\longrightarrow$ Fumaric acid plus two hydrogen atoms
(in presence of the enzyme succinic dehydrogenase)

(b) Two hydrogen atoms $\longrightarrow$ two hydrogen ions plus two electrons

(c) Two cytochrome c (oxidized) plus two electrons $\rightarrow$ two cytochrome c (reduced)

(d) Two cytochrome c (reduced) plus one-half molecule oxygen $\rightarrow$ oxide ion plus two cytochrome c (oxidized)
(in presence of cytochrome oxidase enzyme)

(e) Oxide ion plus two hydrogen ions $\longrightarrow$ one molecule water

Thus it is possible to measure the rate of the reaction from the moles of oxygen consumed. This is measured as microliters on the manometer. The volume in microliters divided by 22.4 yields a value for micro-moles of oxygen. For these reasons the succinoxidase system was selected in an effort to relate an enzymatic, selenite inhibited, reaction to a non-enzymatic, selenite catalyzed, reaction. Each system involves sulfhydryl groups.

Studies were conducted using rat liver homogenates. The liver

tissue was chosen (a) because it is readily accessible from the animal, (b) because it is relatively simple and easy to prepare a homogenate and (c) because it has been shown that selenium damages the liver first and usually in the greatest amount when compared with other tissues (12).

Experiments were run, utilizing a rat liver homogenate as a source of the enzyme, to determine the inhibitory effect of sodium selenite on an enzymatic reaction requiring sulfhydryl groups for the activity of the enzyme. Initial studies were made to determine the concentration of sodium selenite which would cause 50 percent inhibition of the enzyme activity. It was determined that $2 \times 10^{-3}$ molar aqueous sodium selenite, when added to a functioning system, would cause 50 percent inhibition during a 50 minute period. A more concentrated solution of selenite, $2 \times 10^{-2}$ molar, gave approximately 80 percent inhibition in 30 minutes. Further, a more dilute solution of selenite, $1 \times 10^{-3}$ molar, only inhibited the activity about 15 percent during the course of a 60 minute study. Results of these studies are shown in Figure III.

Since these results did not reveal whether the enzyme was being inhibited directly or whether the selenite was interfering with the substrate, further investigations were carried out to clarify the exact nature of the inhibition by selenite. An attempt to answer this was made by incubating the enzyme system with the selenite for definite periods of time and then adding the substrate. As a result, it was shown that there was a definite loss in the total activity of the enzyme system when the selenite was incubated with the enzyme preparation. This, then, would seem to indicate that the selenite is inactivating

Figure III. Effect of Selenite Concentration On Inhibition of Rat Liver Succinoxidase

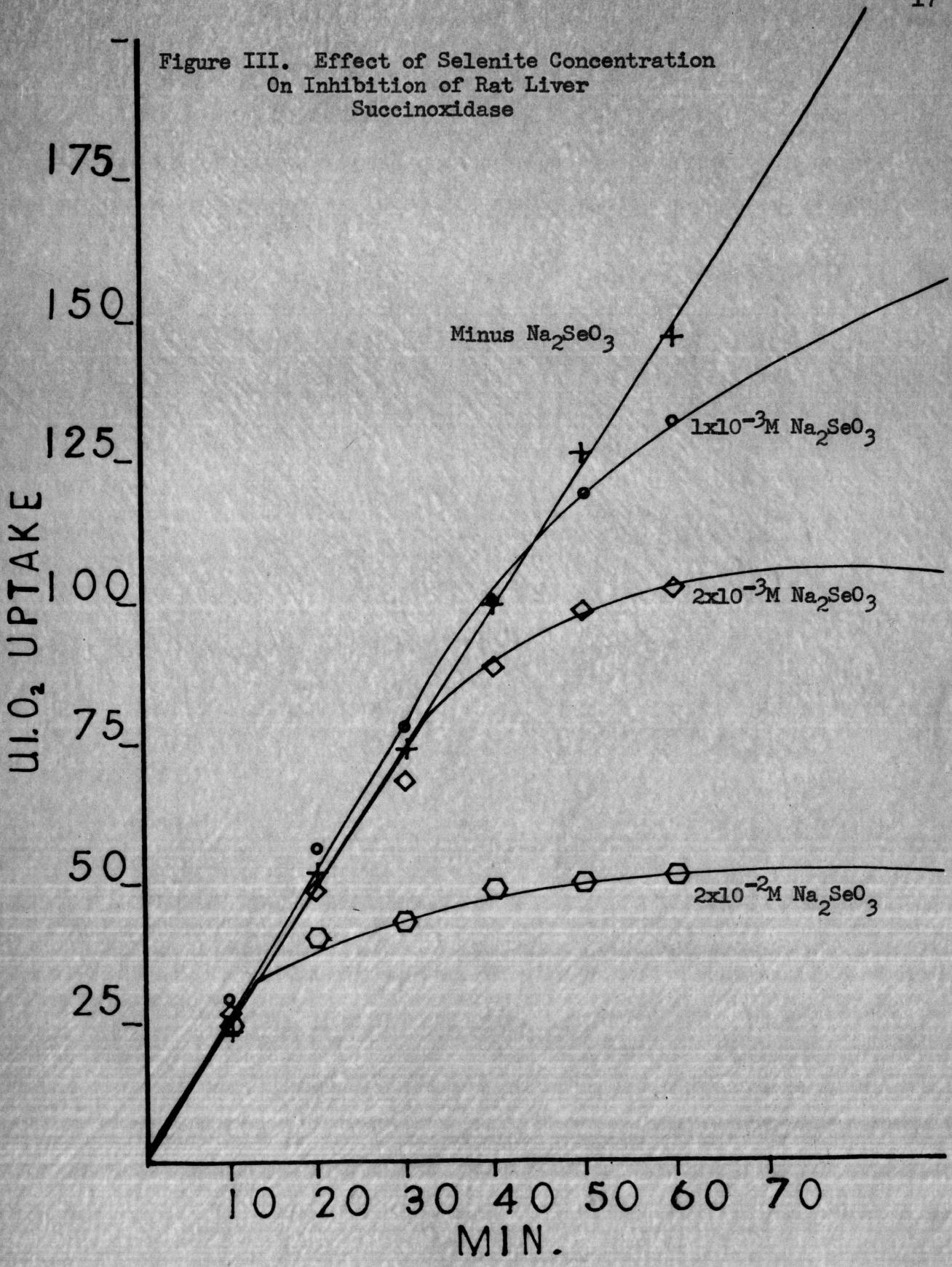
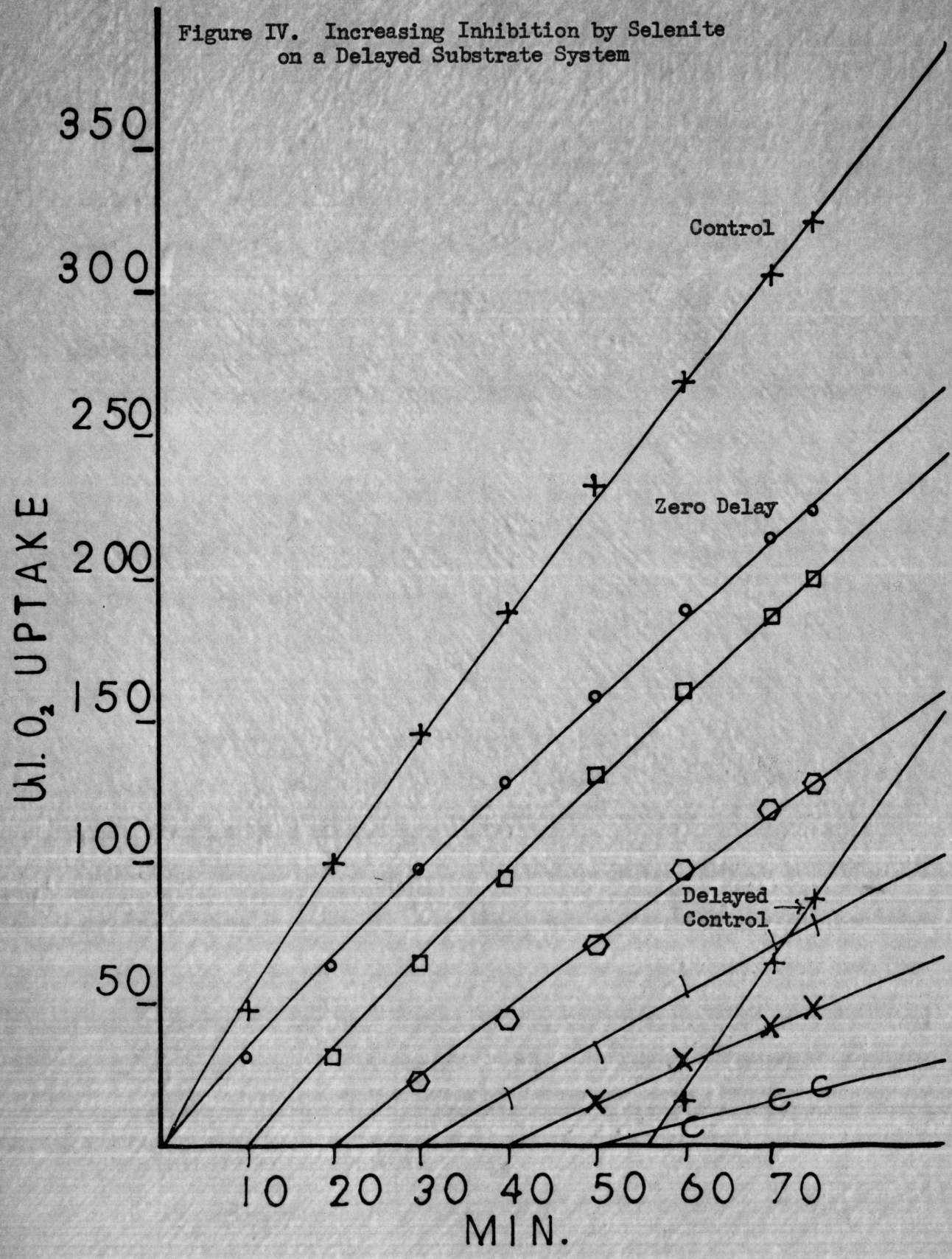


Figure IV. Increasing Inhibition by Selenite on a Delayed Substrate System



the enzyme system and not tying up the substrate, as is shown in Figure IV.

A delayed control reaction was employed in the latter series of experiments to verify that the loss of activity of the enzyme was really due to an inhibition by selenite and not a result of denaturation, the time elapsed, or some other factor. The result of this study is also shown in Figure IV.

It was also revealed that, by incubating the selenite with the enzyme and delaying the addition of the substrate, selenite was 100 times more effective as an inhibitor of the reaction. This is verified by the fact that $2x10^{-5}$ molar sodium selenite caused 50 percent inhibition in these studies as compared with a concentration of $2x10^{-3}$ molar selenite on the previous studies when the substrate was not delayed. The results are shown in Figure V and clearly point out this difference.

Up to this point it was impossible to tell whether or not the inhibition of the enzyme system by selenite was irreversible. Experiments were designed to show what effect an increased concentration of the substrate, sodium succinate, would have on the inhibitory action of selenite. It was revealed, as a result of these experiments, that even as much as a four-fold increase in substrate concentration was ineffective in releasing the inhibition of the enzyme by selenite. This fact is shown more clearly in Figure VI. Thus it would appear that selenite irreversibly inhibits the activity of the succinoxidase system by reacting directly with the enzyme.

It is rather striking that, when inhibition of the succinoxidase

Figure V. Inhibition Effect of Selenite When Incubated With Enzyme

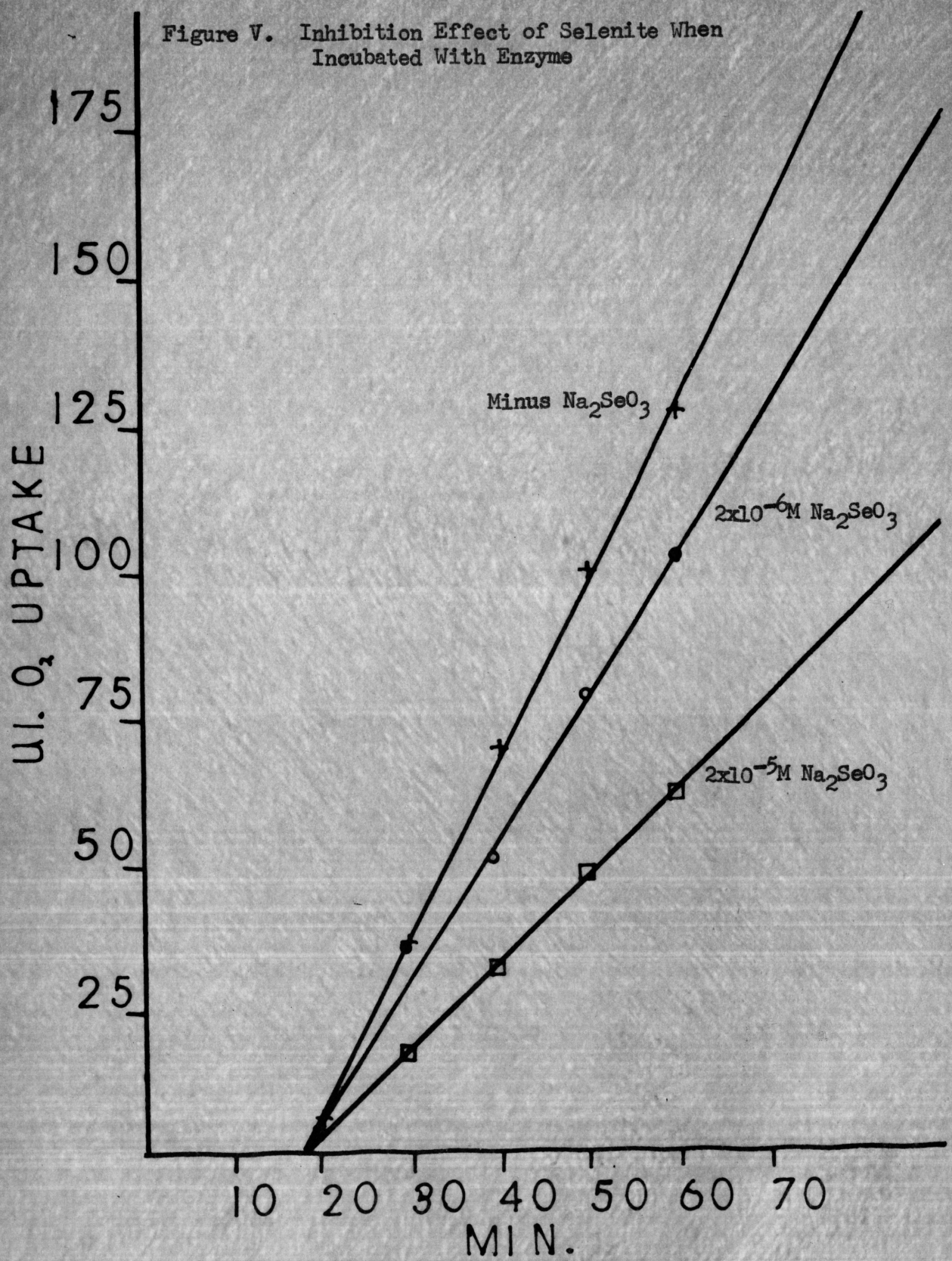
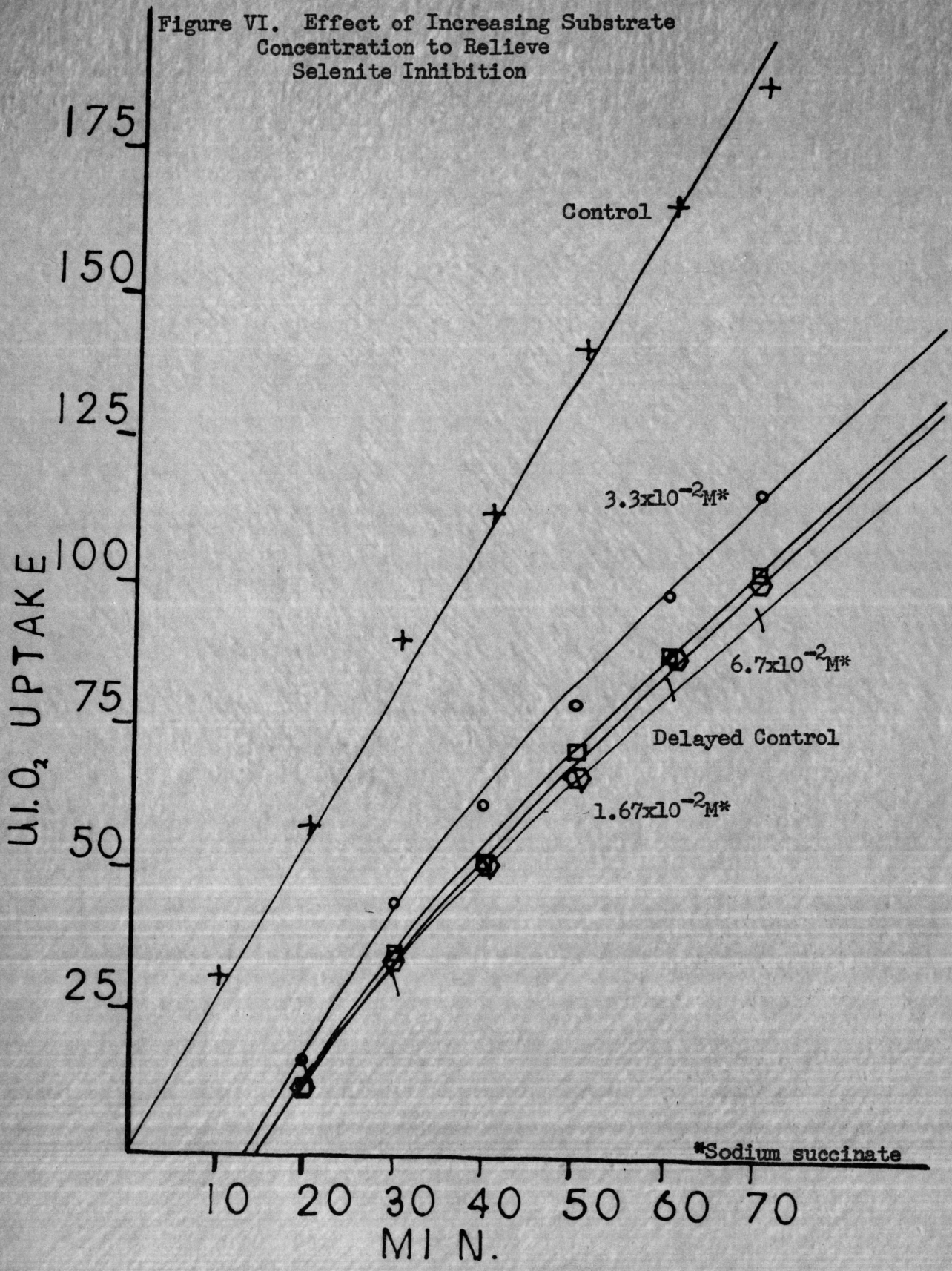


Figure VI. Effect of Increasing Substrate Concentration to Relieve Selenite Inhibition



system by selenite is studied, there should appear to be an initial increase in the rate of the reaction, as measured by microliters of oxygen uptake per unit time. An examination of the initial rates of all reactions shows that those involving selenite and the enzyme have an initial increase in the rate of the reaction that is not shown by the control reactions. This initial increase in the rate of reaction is apparent even in the results of the studies of the delayed substrate system. When the initial rates of reaction, observed in the delayed substrate system, are compared with subsequent readings and the readings of the control, it is found that the rate drops after an initial increase in the inhibited systems. This would seem to indicate an initial catalytic effect by selenite on the enzyme system. See Table 1.

Table 1. Initial Catalytic Effect of Selenite on the Delayed Succinoxidase System

| Sample | Initial $O_2$ uptake (ul.) | Second reading | Third reading | Fourth reading |
|---|---|---|---|---|
| Control | 66.7 | 64.5 | 61.5 | 47.4 |
| Zero delay | 48.0 | 68.0* | 65.0* | 49.0 |
| 10 min. delay | 38.2 | 49.5* | 62.5* | 51.6 |
| 20 min. delay | 6.2 | 24.8* | 1.5 | — |
| 30 min. delay | 20.2 | 40.4* | 44.7* | 28.8 |
| 40 min. delay | 22.3 | 38.7* | 23.8 | — |

*Shows an initial increase in rate of reaction

An attempt to explain this initial catalytic effect by selenite on the succinoxidase system led this author to investigate the action of sodium selenite on a number of sulfhydryl compounds and amino acids. It would be rewarding, as a result of these studies, to correlate the activity of selenite as a catalyst for the oxidation of sulfhydryl compounds, to the initial catalytic effect on the enzyme system. Both of which involve sulfhydryl groups.

Non-enzymatic studies

Investigations were made on the interaction of sodium selenite and 20 amino acids, taurine, and glutathione. It was shown, as a result of manometric and chromatographic investigations, that sodium selenite reacted with cysteine, homocysteine, and glutathione. Further investigations are reported on herein to substantiate these findings. Phenylalanine and threonine, when studied manometrically, gave total oxygen uptakes of 35.2 microliters and 40.3 microliters respectively. However, chromatography of samples of the reaction mixtures did not reveal that any change had occurred or that any new product had been formed as a result of the oxygen uptake. Taurine was included in the study to see whether selenite would react with a sulfonic group. No evidence of a reaction was detected between selenite and taurine. Results of these studies are shown in Figures VII, VIII, and IX.

A series of experiments established an optimum pH for the ensuing reactions, and a mole ratio of the sulfhydryl compound to selenite. As a result of these experiments, it was found that maximum oxygen uptake was obtained at a pH of 7.25 and a mole ratio of glutathione or cysteine

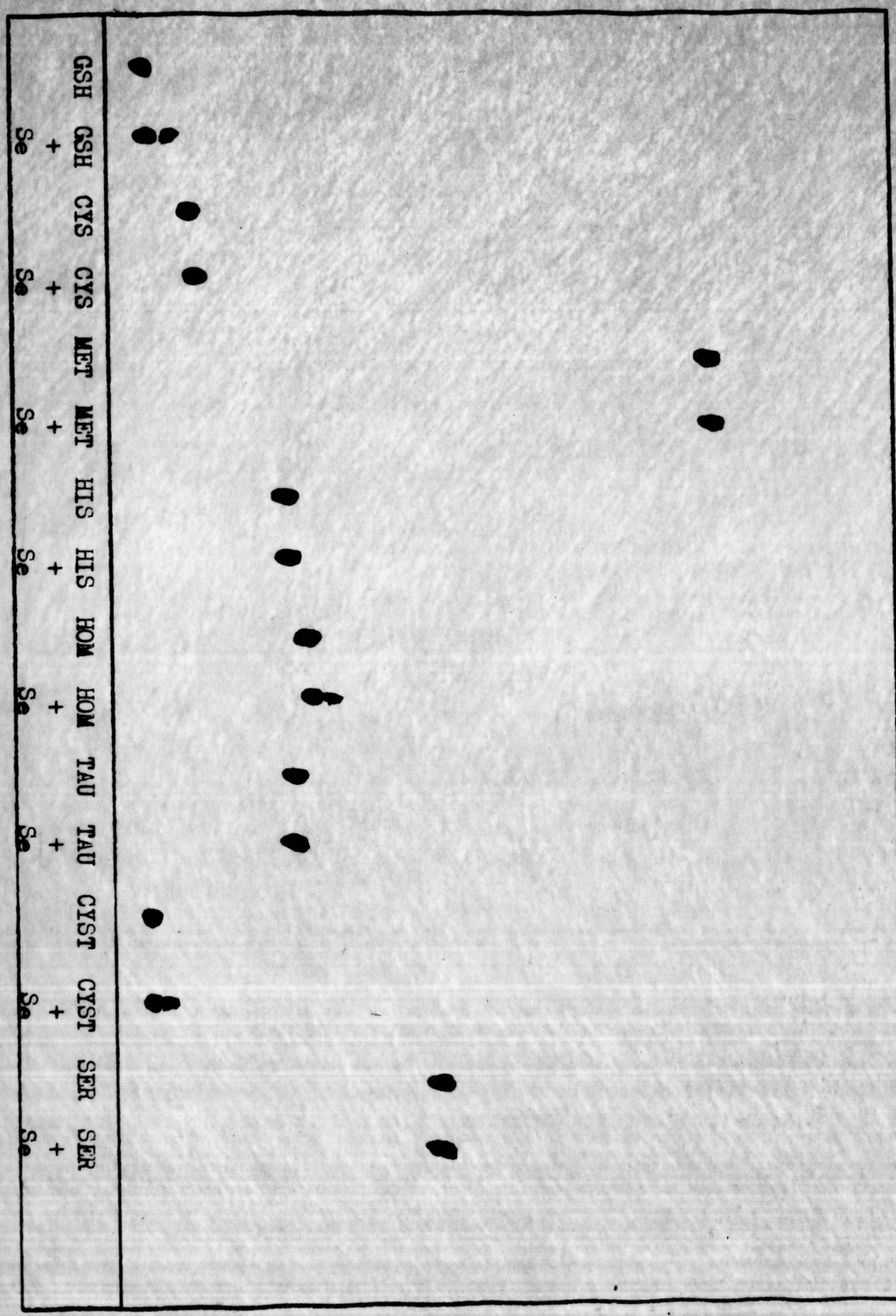



Figure VII. Chromatogram of Selenite with Amino Acids, Glutathione, and Taurine; descending in tertiary butyl alcohol, formic acid, and water, 70:15:15 by volume

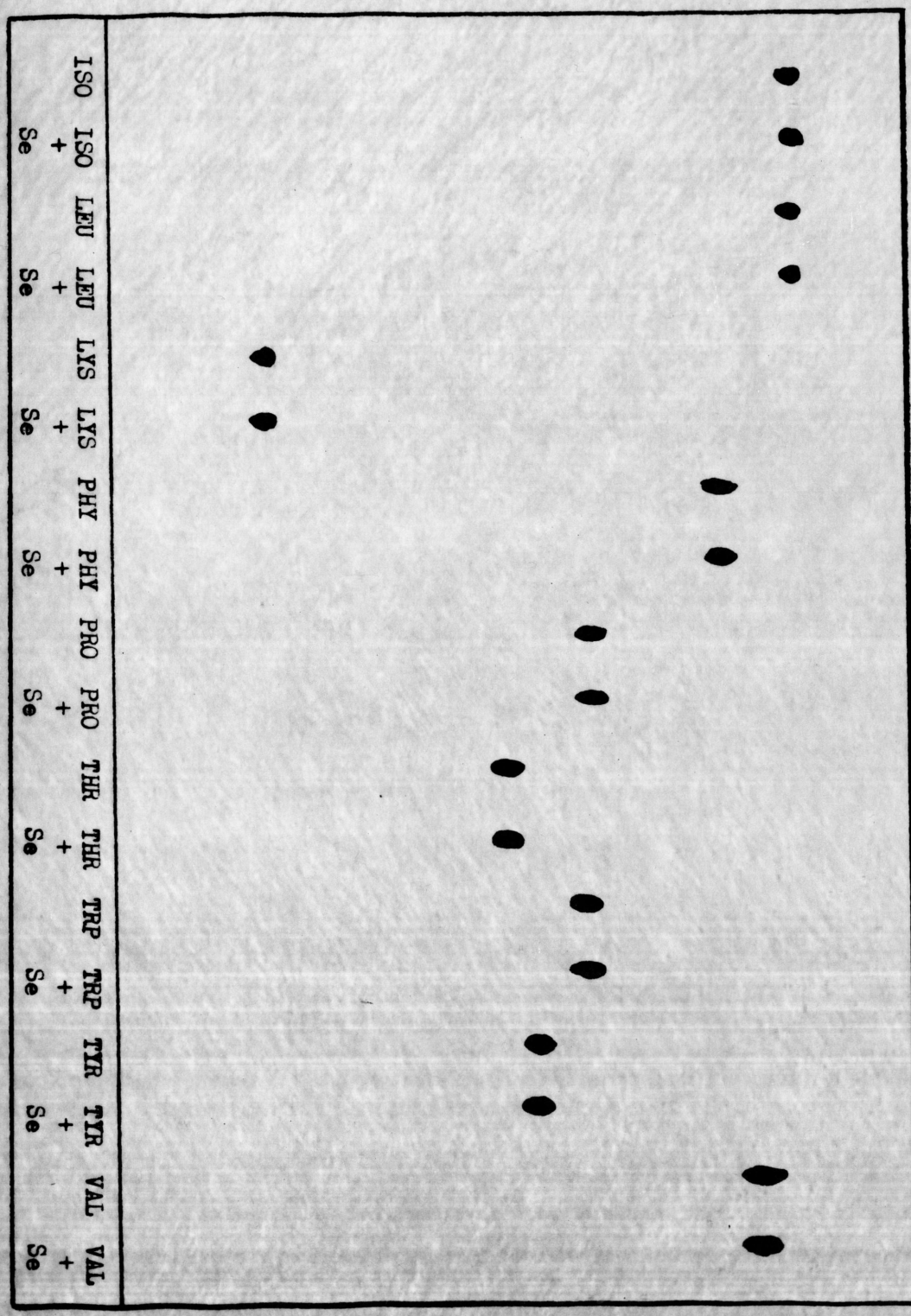



Figure VIII. Chromatogram of Selenite with Amino Acids; descending in tertiary butyl alcohol, formic acid, and water, 70:15:15 by volume

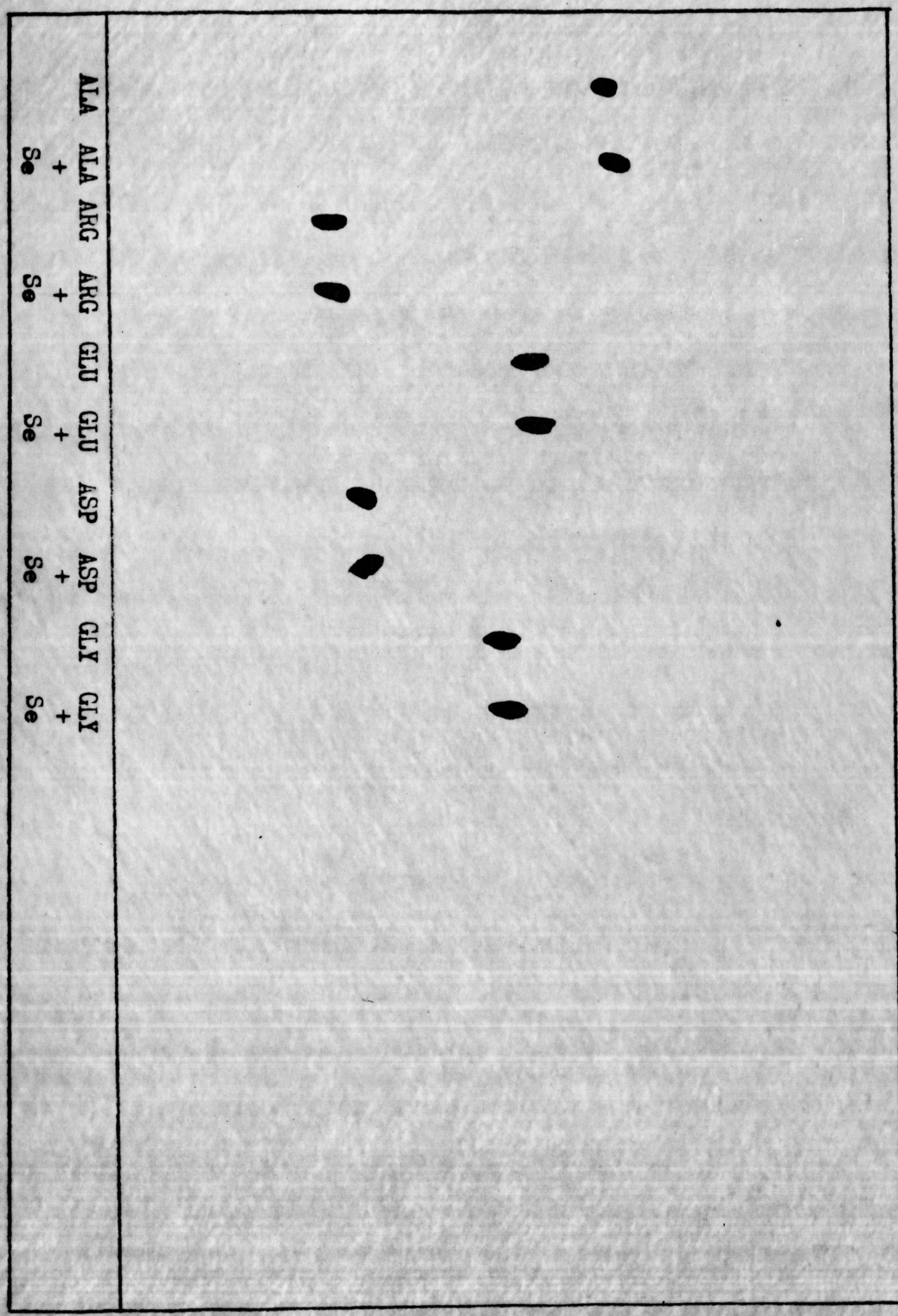



Figure IX. Chromatogram of Selenite with Amino Acids; descending in tertiary butyl alcohol, formic acid, and water, 70:15:15 by volume

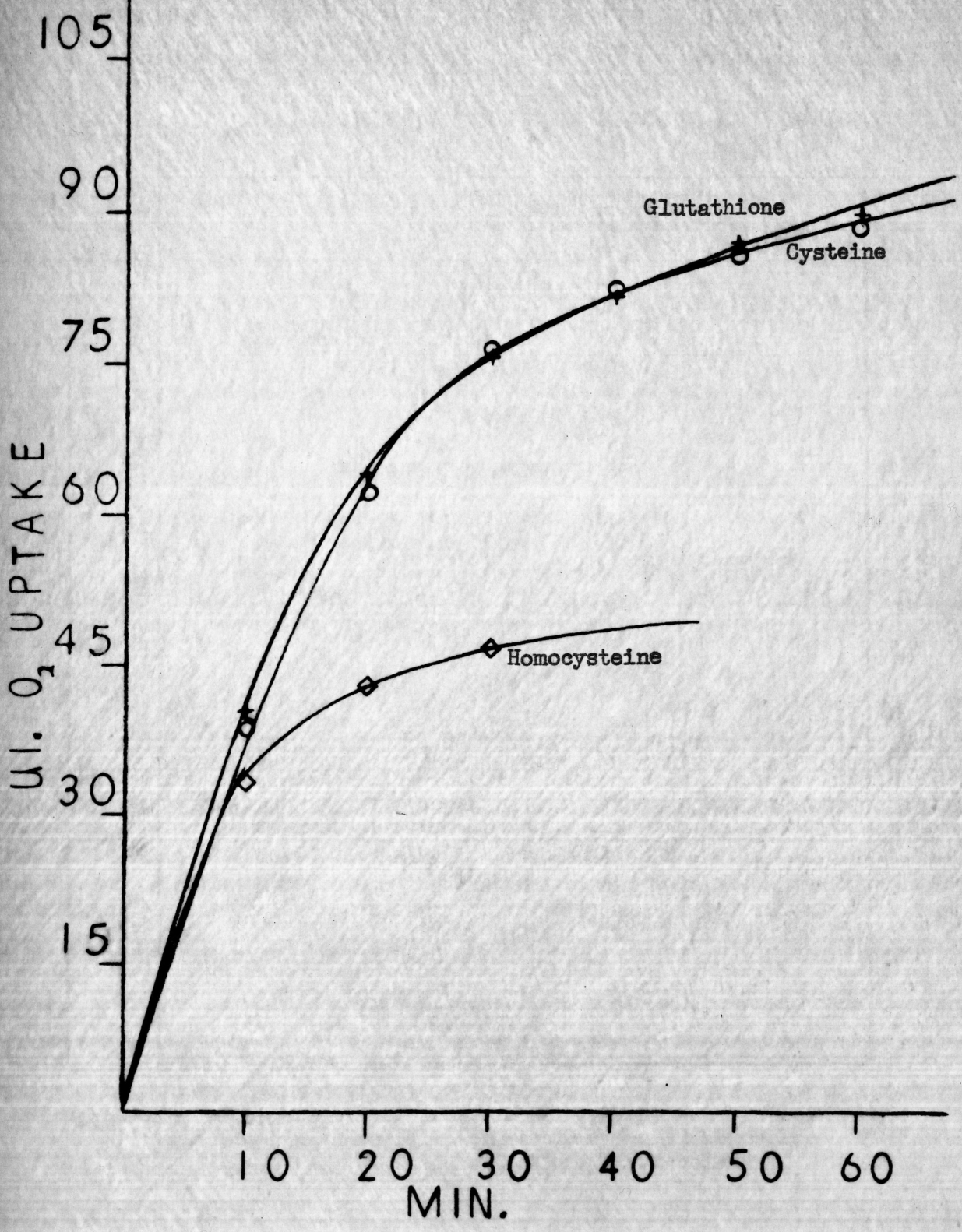
to selenite of 100 to 1. The final concentrations for both glutathione and cysteine was $1x10^{-2}$ molar, and for sodium selenite it was $1x10^{-4}$ molar. Homocysteine, being less soluble, was used as a saturated solution. Results of the selenite catalysis of these compounds are shown in Figure X. It should also be mentioned that the rates of reaction could be increased to some extent by gassing the flasks with oxygen.

Tsen and Tappel (18) reported that arsenite inhibited the catalytic oxidation of glutathione by selenite and suggested that a compound of glutathione and arsenite might be the active inhibitor. Therefore, the interaction of arsenite with the amino acids, taurine, and glutathione were studied. Results of these studies are shown in Figures XI, XII, and XIII. The manometric studies of the compounds gave no evidence of a reaction with arsenite with the exception of glutathione. Glutathione showed some evidence of being oxidized by arsenite which was more noticeable when the flasks were initially gassed with oxygen.

A study of the inhibition of selenite catalysis by arsenite showed an optimum final concentration of arsenite to be $1.5x10^{-4}$ molar for the system of $1x10^{-2}$ molar glutathione and $1x10^{-4}$ molar sodium selenite. Results of the inhibition by arsenite are shown in Figure XIV. It should also be noted that it was necessary to prepare arsenite freshly for each series of investigations.

Also noted in this study was the occurrence of reaction products formed from arsenite and arsenate with glutathione. Evidence of these products is shown in Figure XV, along with the reaction products of selenite with glutathione and cysteine. The following $R_f$ values were

Figure X. Catalytic Effect of Selenite on Oxidation of Homocysteine, Cysteine, and Glutathione



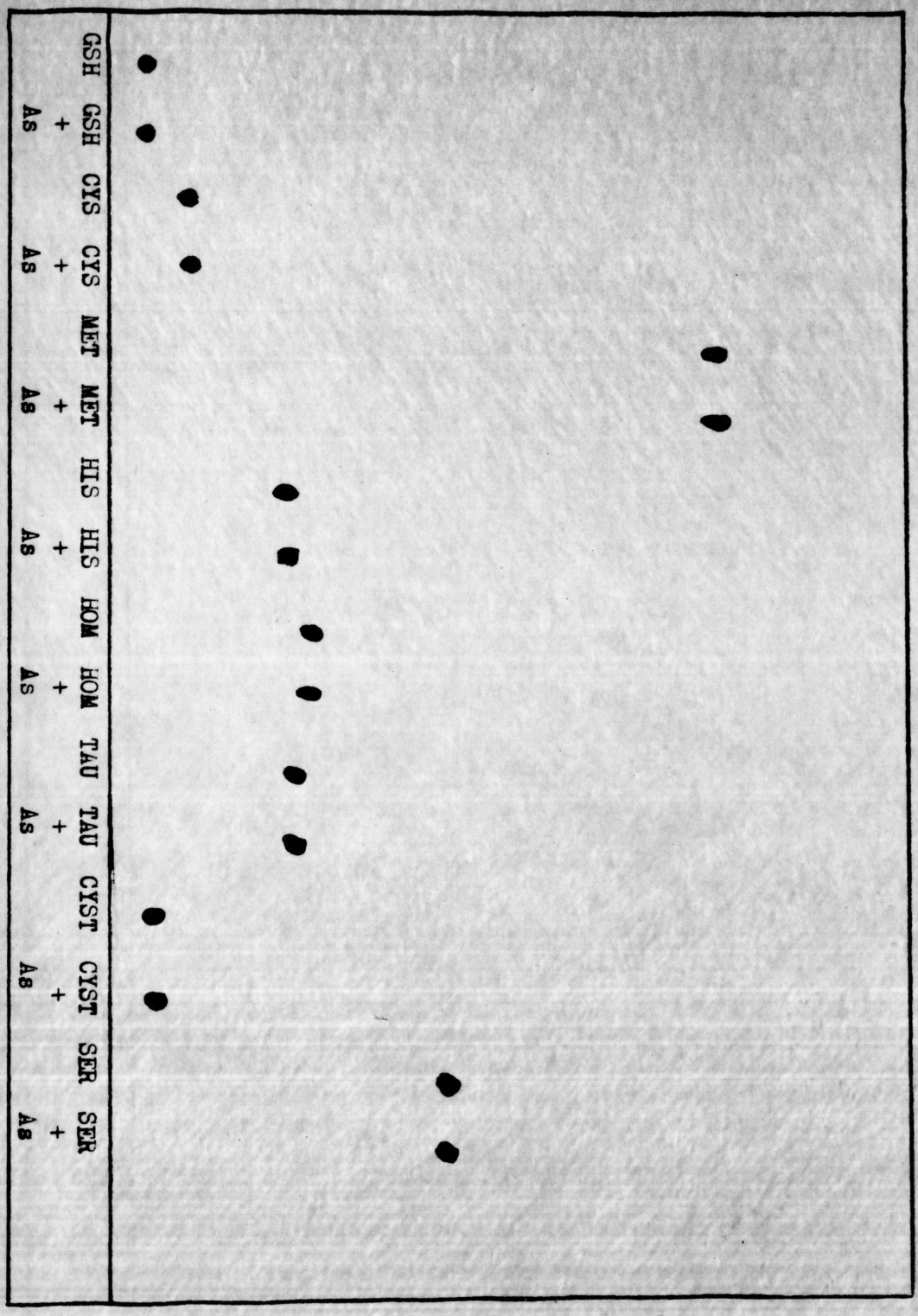



Figure XI. Chromatogram of Arsenite with Amino Acids, Glutathione and Taurine; descending in tertiary butyl alcohol, formic acid, and water, 70:15:15 by volume

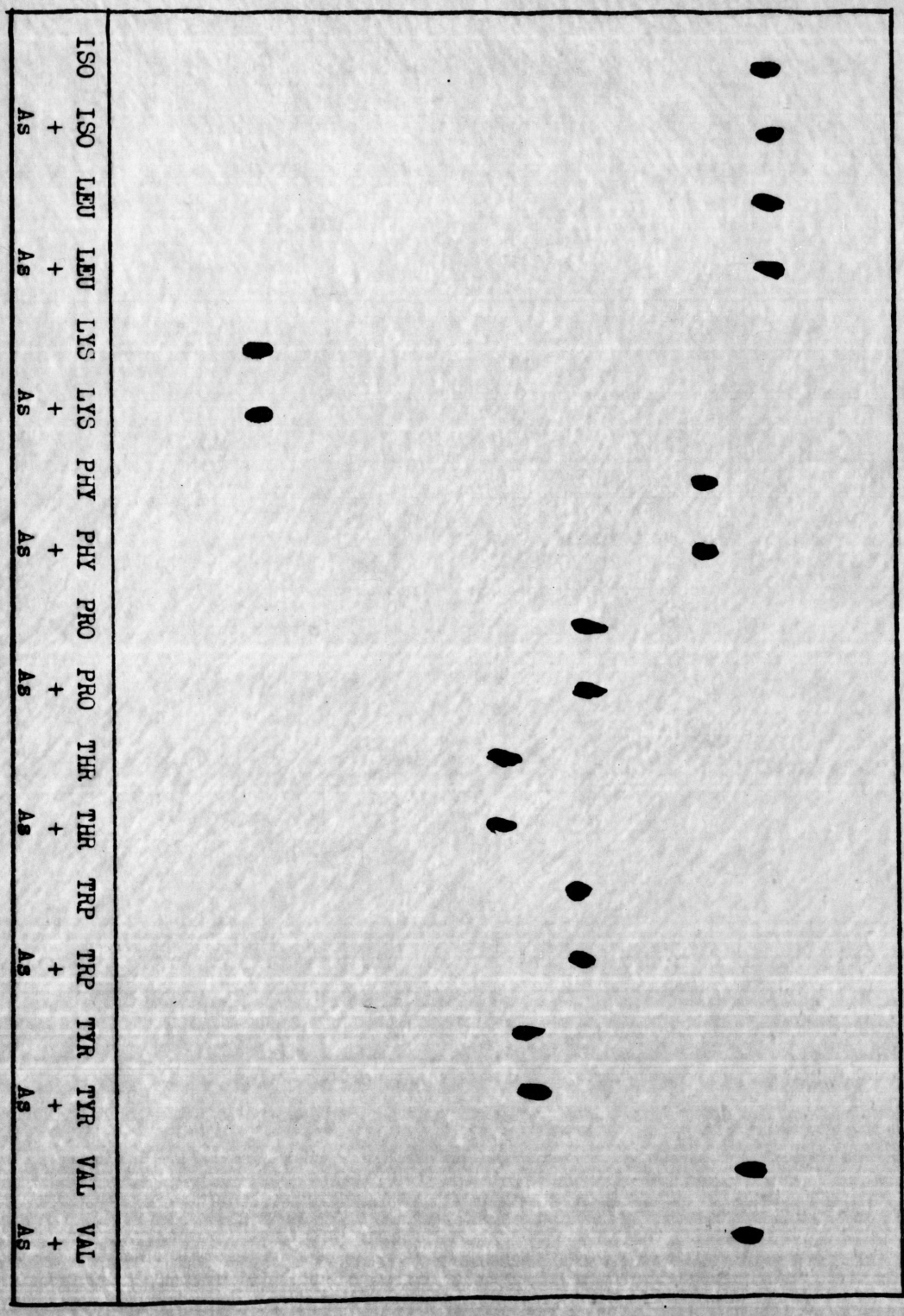



Figure XII. Chromatogram of Arsenite with Amino Acids; descending in tertiary butyl alcohol, formic acid, and water, 70:15:15 by volume

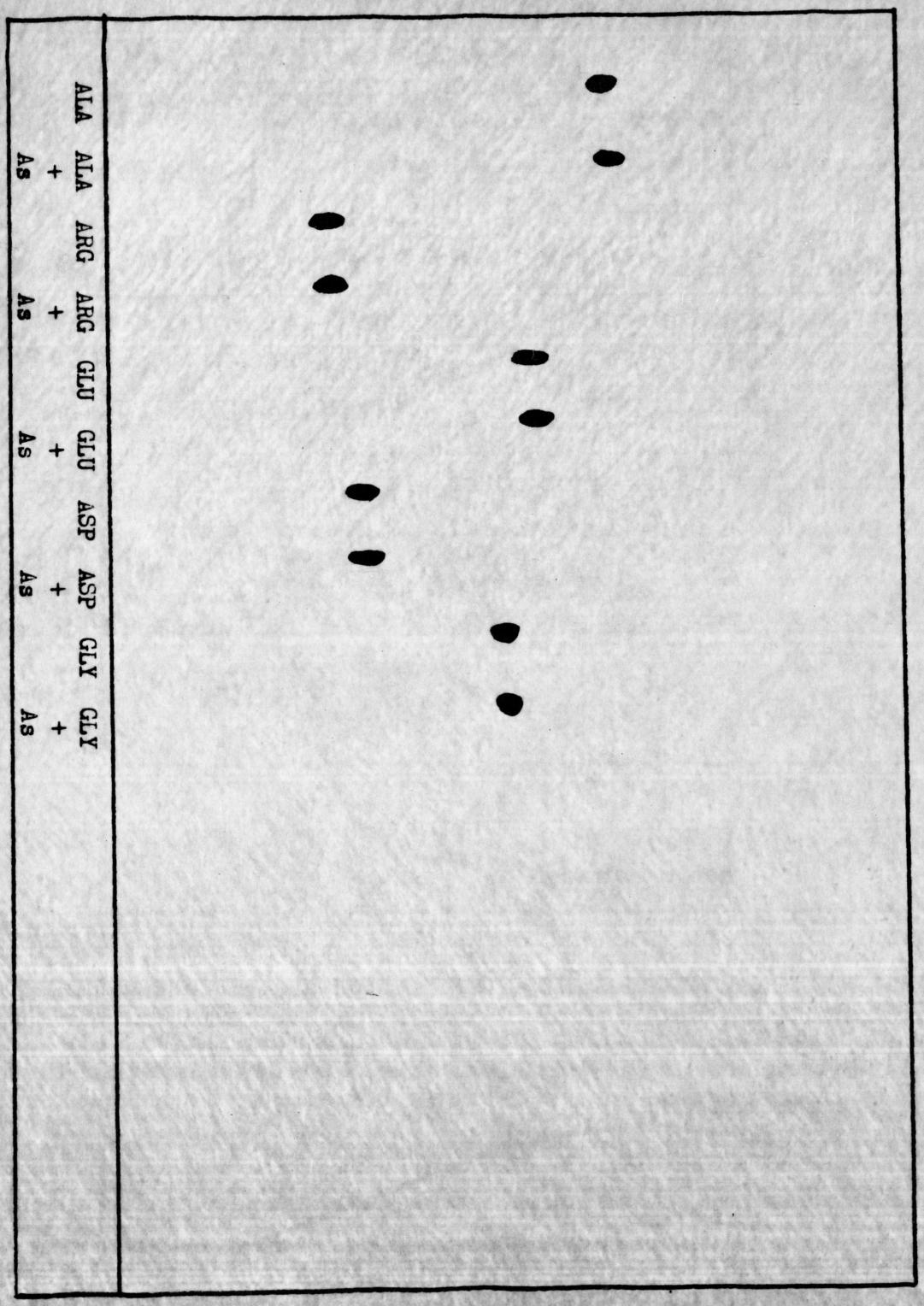
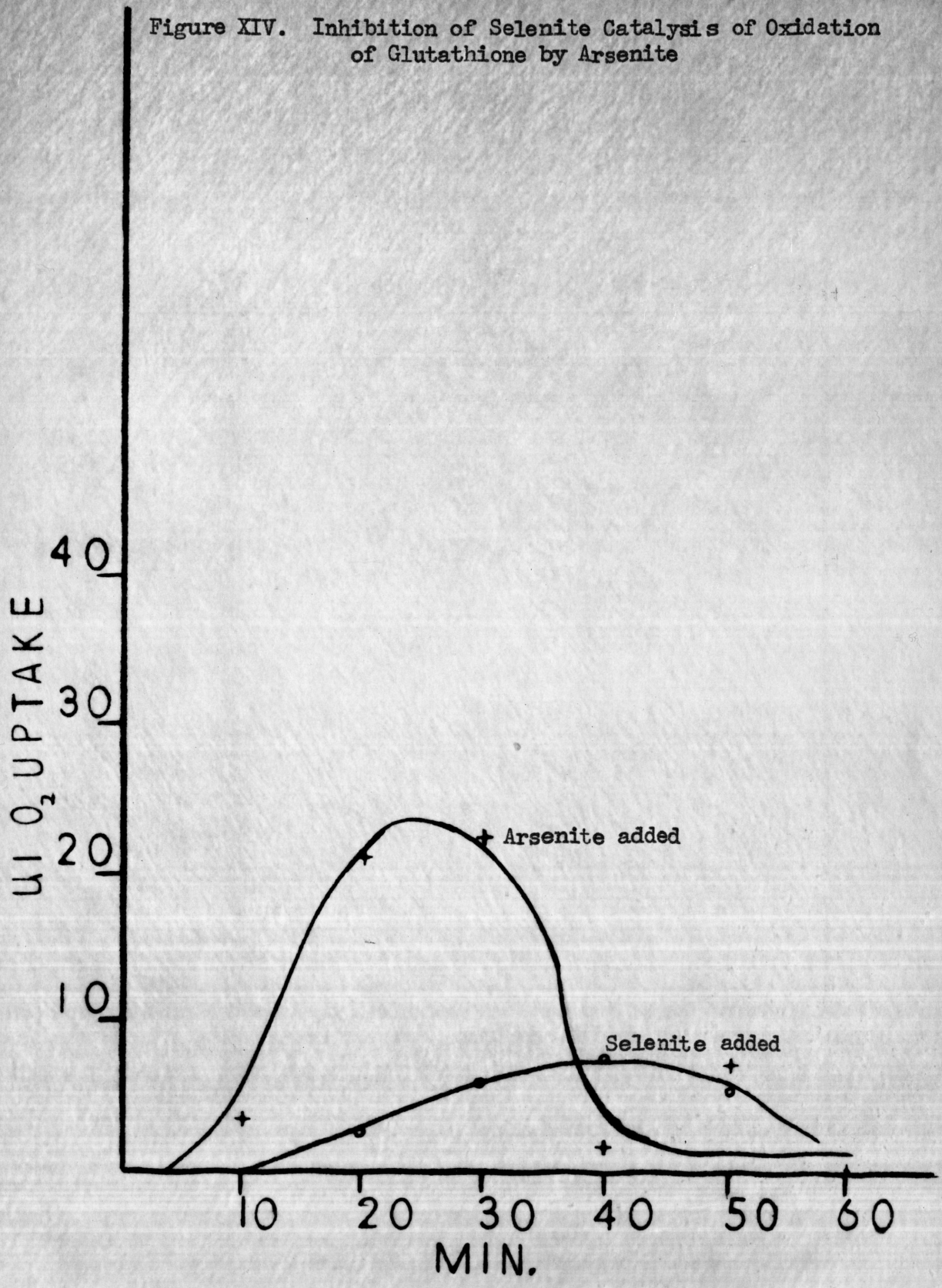



Figure XIII. Chromatogram of Arsenite with Amino Acids; descending in tertiary butyl alcohol, formic acid, and water, 70:15:15 by volume

Figure XIV. Inhibition of Selenite Catalysis of Oxidation of Glutathione by Arsenite



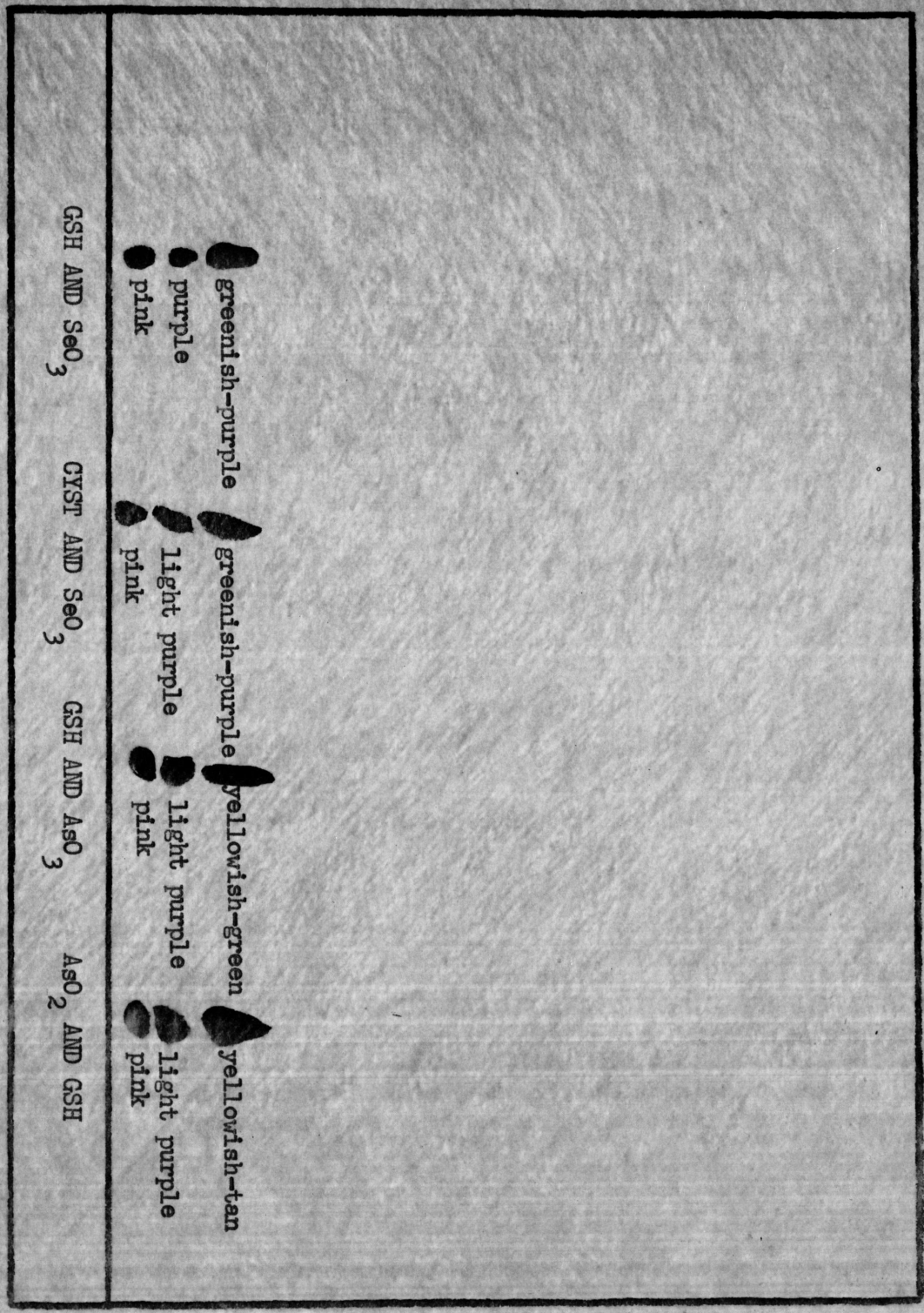



Figure XV. Chromatogram of Reaction Products of Glutathione and Cysteine with Arsenite, Arsenate, and Selenite; ascending in butanol, acetic acid, and water, 60:10:20 by volume

obtained for the compounds shown in Figure XV:

(a) for the spots from glutathione and selenite, the $R_f$ values were 0.065, 0.121, and 0.212 increasing up the chromatogram in that order;

(b) for cysteine and selenite, the $R_f$ values were 0.033, 0.133, and 0.233 respectively;

(c) for arsenite and glutathione, the $R_f$ values were 0.057, 0.114, and 0.200 respectively;

(d) for glutathione and arsenate, the $R_f$ values were 0.065, 0.121, and 0.212 respectively.

An $R_f$ value is the ratio of the distance a given substance will travel in relation to the distance the solvent travels on the paper chromatogram. Thus all $R_f$ values must lie between zero and one. To obtain the $R_f$ values reported above the chromatograms were run in an ascending manner to a height of about 16 centimeters in a solvent of normal-butanol, acetic acid, and water in a ratio of 60:10:20 respectively, at room temperature in glass cylinders described earlier.

To the knowledge of this author this is the first evidence of an actual product formed from the reaction of glutathione with arsenite or arsenate. Tsen and Tappel (16) suggested the occurrence of a product of arsenite and glutathione, however, they gave no evidence in their work that one actually existed. It is possible that the compounds formed could be an arseno-triglutathione or an arseno-pentaglutathione respectively.

It is significant that the endogenous study of arsenite and selenite gave no indication of a reaction. This lends support to the idea that arsenite forms a complex with the sulfhydryl compound to prevent oxidation by selenite. This was also shown by adding selenite to a

mixture of arsenite and glutathione and not getting the characteristic catalytic oxidation of the sulfhydryl group by selenite.

It would be interesting to see what effect the compound, reported in this paper, formed from the reaction of arsenite and glutathione would have on the catalytic activity of selenite. It has been shown by Tsen and Tappel (16) that the seleno-glutathione is a good inhibitor of selenite catalysis of the oxidation of glutathione. Could one also expect the arseno-glutathione to prevent this catalytic effect of selenite on sulfhydryl groups? If so, perhaps the compound would have some pharmaceutical value because it prevents the activity of selenite at a concentration of $1.5 \times 10^{-4}$ moles and does not cause oxidation of the glutathione itself. It is possible that this is how arsenite is able to alleviate the toxicity of selenite in animals.

An analysis of the chromatograms of the interaction of arsenite with glycine, lysine, glutamic acid, and aspartic acid showed signs of a change occurring. When the chromatograms were sprayed with ninhydrin, to detect the amino acids, and subsequently with stannous chloride, to detect arsenite, the amino acids showed up as a brown spot under an ultraviolet light source. This brown spot did not occur on the control group, the group minus the arsenite, treated with the same spray reagents. Further work is needed to prove the interaction, if any.

Investigations also revealed that, when the compound formed between glutathione and selenite is eluted from the chromatogram and subjected to an aklaline treatment of hot 2 molar sodium hydroxide for 15 minutes and rechromatographed, the intensity of the brown color with stannous

chloride, which is used for the detection of selenite, is greatly increased. This would seem to indicate that selenite, when in combination with a sulfhydryl compound in an organic form, is much less reactive. This is in agreement with results by Lampson and Klug (7) concerning the reduced toxicity of selenite when it is converted to an organic form.

Further studies were carried out to check such factors as ionic strength, type of buffer, and endogenous effects. A series of reactions were devised to determine the extent of an ion concentration effect, if any. This was done by substituting solutions, of comparable ionic strength to sodium selenite, in the reaction flasks for the selenite normally added. Solutions of sodium sulfite, sodium hydrogen sulfite, sodium sulfate, and sodium hydrogen sulfate were tried. In no instance did the ion effect of the above solutions exceed 20 percent of the total uptake. As a result of these studies it was found that, although there was some ionic effect, it could not account for the catalytic effect due to selenite. A tris (trihydroxymethyl amino methane) buffer was employed with equally valid results as compared with the phosphate buffer used for the bulk of the investigations. When selenite and arsenite were mixed together in a respirometer flask no evidence of a reaction was detected on the manometer. All of these factors substantiate the fact that selenite can act as a catalyst for a non-enzymatic reaction and the reaction can be inhibited by arsenite.

It is of interest that arsenite is used to alleviate selenite toxicity in animals. Perhaps arsenite functions in much the same manner in the animal as it does in a non-enzymatic reaction. If arsenite would

protect the free sulfhydryl groups in the organism from interacting with inorganic selenite, which converts the selenite to an organic form, this would explain in part its beneficial effect. It is logical to assume that the reaction of selenite with sulfhydryl groups in the organism is much like that of the non-enzymatic reaction studied here. It has been demonstrated that glutathione when fed with selenite to rats protects them from the harmful effects normally produced by selenite (12). However, if glutathione is added after the selenite has been administered, it is ineffective in relieving the toxic effects of selenium poisoning. It is the feeling of this author that a greater understanding of the non-enzymatic reactions will lend a good deal of insight into what happens to the metabolism of an organism when subjected to doses of inorganic selenium.

Is the interaction of selenite with sulfhydryl groups a detoxifying effort by the organism, or is it a nonselective random type non-enzymatic reaction converting inorganic selenite to an organic form? This question and many others remain to be answered before a complete understanding of the selenium problem is achieved.

## SUMMARY

It has been determined, as a result of the enzymatic studies with selenite, that under these conditions (a) selenite acts as an inhibitor of the succinoxidase system by irreversibly inhibiting the activity of the enzyme; (b) selenite is 100 times more effective, on a 10 percent rat liver homogenate, as an inhibitor when incubated with the enzyme prior to adding the substrate; and (c) selenite shows an initial catalytic effect on the succinoxidase system.

As a result of the non-enzymatic interactions of selenite it has been shown that (a) selenite catalyzes the oxidation of cysteine, homocysteine, and glutathione to give chromatographically distinct products; (b) a molar ratio of 100 to 1, for sulfhydryl compound to selenite, and a pH of 7.25 gave optimum catalytic activity; and (c) selenite did not give chromatographic or manometric evidence of having reacted with any of the other compounds used in this study.

Investigation revealed that arsenite would inhibit the catalytic role of selenite in the oxidation of sulfhydryl compounds. The concentration of arsenite employed in this study inhibited selenite activity but did not itself cause oxidation. Chromatographically distinct products of the reaction of arsenite and glutathione and of arsenate and glutathione were detected for the first time.

It was also determined that selenite is less sensitive to detection by stannous chloride in an organic form than when the selenite is in an inorganic state.

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