1949

Separation of the Selenium Compounds in Seleniferous Plant Protein Hydrolysates by Paper Partition Chromatography

Arnold L. Smith

SEPARATION OF THE SELENIUM COMPOUNDS IN SELENIFEROUS PLANT PROTEIN HYDROLYSATES BY PAPER PARTITION CHROMATOGRAPHY

by

Arnold L. Smith

Submitted to the Graduate Faculty

of

South Dakota State College of Agriculture and Mechanic Arts

in Partial Fulfillment of the Requirements for

the Degree of Master of Science

August 1949

This is to certify that, in accordance with the requirements of South Dakota State College for the Master of Science Degree, Mr. Arnold L. Smith has presented to this committee three bound copies of an acceptable thesis, done in the major field; and has satisfactorily passed a two-hour oral examination on the thesis, the major field, Chemistry, and the minor field, Mathematics.

Advisor

August 25, 1949
Date

Head of Major Department

Head of Minor Department

Rep. of Graduate Committee

ACKNOWLEDGEMENTS

This work was done as part of the regular research studies in the Experiment Station Chemistry Department. I wish to express my appreciation for permission to report it as a thesis. I wish to thank Dr. A. L. Moxon for his many helpful suggestions. Mr. Richard Harshfield assisted greatly in the selenium analysis work, and the photographic work was done by Mr. E. I. Whitehead, Miss Frances Moyer, and Mr. Robert Pengra. To them I extend my thanks.

The author.

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INTRODUCTION

In 1934 Franke (1) reported that the protein of "alkalied" or "toxic" wheat carried the toxicant, and Franke and Painter in 1935 (2) reported that these "toxic" proteins contained selenium which was in organic combination in the protein. There has been much speculation regarding the possibility of selenium replacing sulfur in the amino acids cystine and methionine. The toxicity of selenium in selenium analogs of these compounds is similar to that of selenium occurring naturally in proteins of seleniferous cereals, which indicates that the selenium may be present in the proteins as the analogs of these sulfur amino acids. Selenium-cystine has been prepared by Fredga (3), and Painter (4) has prepared both selenium-cystine and selenium-methionine.

Work in the South Dakota Experiment Station Chemistry Laboratory has shown that the selenium of seleniferous protein hydrolysates follows both cystine and methionine in fractionations for separation of the amino acids. Attempts to isolate the selenium-containing compounds from the hydrolysates of seleniferous cereal proteins have, however, been unsuccessful. Paper partition chromatography has been applied to the problem of separating these selenium compounds from proteins of seleniferous wheat, seleniferous corn, and Astragalus bisulcatus.

EXPERIMENTAL

Wheat gluten was prepared from a sample of highly seleniferous wheat (selenium content, 26.2 p.p.m.) and from control wheat, according to the method described by Morrow and Sandstrom (5), as follows:

Preparation of Wheat Gluten. Gradually add water to ground wheat until a stiff dough is formed. This requires about 60 ml. of water for each 100 g. of wheat. Knead the dough thoroughly and allow it to remain under cold water for at least one-half hour. Then wash out the starch by kneading it in a stream of cold water.

The above samples of protein were hydrolyzed by 8% hydrochloric acid according to the method of Painter and Franke (6). Selenium contents of these hydrolysates were found by analysis to be 15.5 p.p.m. and 1.1 p.p.m., respectively.

Hydrolysis of Protein. Digestions of proteins are carried out in a round bottom flask fitted to a water condenser with ground glass joint. The flask is heated on a sand bath. The humin is filtered and washed several times with hot water. Proteins are hydrolyzed by: (a) Boiling for 20 hours in 8% hydrochloric acid, with a volume of about 80 ml. of acid to 10 g. of protein, or (b) Boiling for 14 hours in three times their weight of concentrated sulfuric acid and six times their weight of water.

For later trials with Amberlite IR-4B acid ion exchange resin, the chloride present in the selenium gluten hydrolysate was precipitated with silver sulfate solution, with the result-

ing sulfate being precipitated by barium hydroxide solution; removal of dicarboxylic amino acids (aspartic and glutamic acids) is not possible in the presence of chloride ion. The dilute solutions were evaporated to the original volumes by vacuum distillation. Concentrations used on most of the paper chromatograms were one-fourth those of the original hydrolysates.

Corn protein was prepared from seleniferous corn by the method of Franke and Moxon (7), as follows:

Extraction of seed protein (Applied to corn and Astragalus bisulcatus). The ground seed is stirred into 0.2% aqueous sodium hydroxide solution in the ratio of 1 g. to 7 ml. of solution, standing, with stirring, 5 hours. The supernatant liquor is siphoned off and centrifuged to remove solid impurities. The protein is precipitated as the pH approaches its isoelectric point by the addition of hydrochloric acid.

This sample of protein was hydrolyzed in sulfuric acid according to the method of Painter and Franke (6) and neutralized by precipitation of sulfate ion with barium hydroxide solution for later acid ion adsorption trials. The dilute solution was evaporated to original volume by vacuum distillation. Selenium contents of the dry corn protein and its hydrolysate, hereby designated as Corn I, were 199.6 p.p.m. and 9.1 p.p.m., respectively.

A later sample of corn protein (selenium content, 149.5 p.p.m.) was prepared as above, and from it were prepared hydrolysates described and designated below:

Corn II. A sulfuric acid hydrolysate by the method of Painter and Franke (6), neutralized by precipitation of sulfate

ion by barium hydroxide solutions and used in that dilute concentration. The selenium content of the original hydrolysate was 1.4 p.p.m.

Corn III. A sulfuric acid hydrolysate by the method of Painter and Franke (6). The selenium content was 5.4 p.p.m. The solution was used on paper chromatograms in water-diluted (one-fourth) concentration and in about one-third concentration by near neutralization with sodium hydroxide solution. The acid titre was determined with one-fourteenth normal sodium hydroxide solution.

Corn IV. A hydrochloric acid hydrolysate by the method of Painter and Franke (6). The selenium content was 5.2 p.p.m. The solution was used on paper chromatograms in about one-fourth concentration by near-neutralization with sodium hydroxide solution. The acid titre was determined with one-fourteenth normal sodium hydroxide solution.

After water extraction of ground Astragalus bisulcatus seed (selenium content, 1130 p.p.m.), the seed protein was extracted and prepared by the method of Franke and Moxon (7). The isoelectric point of this protein was found by means of the Beckman pH meter to be at pH 3.8. This protein was hydrolyzed by 8% hydrochloric acid by the method of Painter and Franke (6). Selenium contents of the wet protein and its hydrolysate were 53.1 p.p.m. and 0.0 p.p.m., respectively. The hydrolysate was used on paper chromatograms in about one-fourth and about one-half original concentrations by near neutralization with sodium hydroxide solutions.

Water extracts and a two-normal hydrochloric acid extract of Astragalus bisulcatus plant tops were prepared.

In early work with paper chromatography, hydrolysis of 0.1% glutathione by warming in dilute hydrochloric acid was attempted for separation of its component amino acids, cysteine, glycine, and glutamic acid.

Known compounds (0.1% in water) for comparison with the protein hydrolysates on the paper chromatograms were aspartic and glutamic acids, alanine, glycine, methionine, selenium-methionine, cystine and selenium-cystine. Barely sufficient hydrochloric acid was added for solubility of the latter two compounds. Other compounds (0.1% in water) prepared for extra studies were L- and DL-α-alanine, β-alanine, L- and DL-methionine, L- and DL-serine, cystathionine[1], homolanthionine[1], cysteine, selenium di-cysteine[2], selenium tetra-cysteine[2], glutathione, oxidized glutathione, selenium tetra-glutathione[2], selenium hexa-glutathione[2], selenious acid, sodium selenite and sodium selenate. Barely sufficient hydrochloric acid was added for solubility when necessary.

The capillary ascent paper partition chromatography technique of Williams and Kirby (8), wherein the paper is formed into a cylinder by stapling, was used throughout this work, as was Whatman's No. 1 filter paper. Drops (0.01 ml.) of hydrolysates and known solutions (10 micrograms of amino acids) were placed

1. Samples of cystathionine and homolanthionine were kindly donated by Dr. J. A. Stekol, Lankenau Hospital Research Institute, Philadelphia, Pennsylvania.
2. South Dakota Experiment Station Chemistry Laboratory preparation believed to have this composition.

by pipette and labeled at about one-inch intervals, on the origin line, parallel to the base of the cylinder at a height of about one inch from the base of the cylinder. Drops were not placed closer than one inch to the vertical edges of the paper, as the solvent held back at the edge, causing distorted rising.

When dry, the cylinder, if the diameter was small, was set vertically in a beaker containing the developing solvent, enclosed in a tall, sealed jar, or set directly in the solvent contained in the bottom of the jar if the diameter was large, as shown in Figure I. The solvent was used at a depth of about one-fourth inch. When the solvent had risen to the top of the paper or had reached a stationary level, the paper was removed and dried in the hood before drying in the oven at 100° C. until excess solvent was removed (five minutes). Locations of the dispersed amino acid spots were revealed by the characteristic red colors produced by spraying with 0.1% aqueous ninhydrin solution, short drying at room temperature and heating in the 100° C. oven for five minutes.

Some of the later work was done by two-dimensional chromatography, in which the paper, with only one sample spot on an origin corner, is developed by one solvent, dried, and formed into another cylinder so that it can be developed by a different solvent in a direction perpendicular to the first. A greater separation is shown by the scattered pattern characteristic of the two solvents used.

All of the quantitative selenium analyses reported were carried out by a South Dakota Experiment Station Chemistry

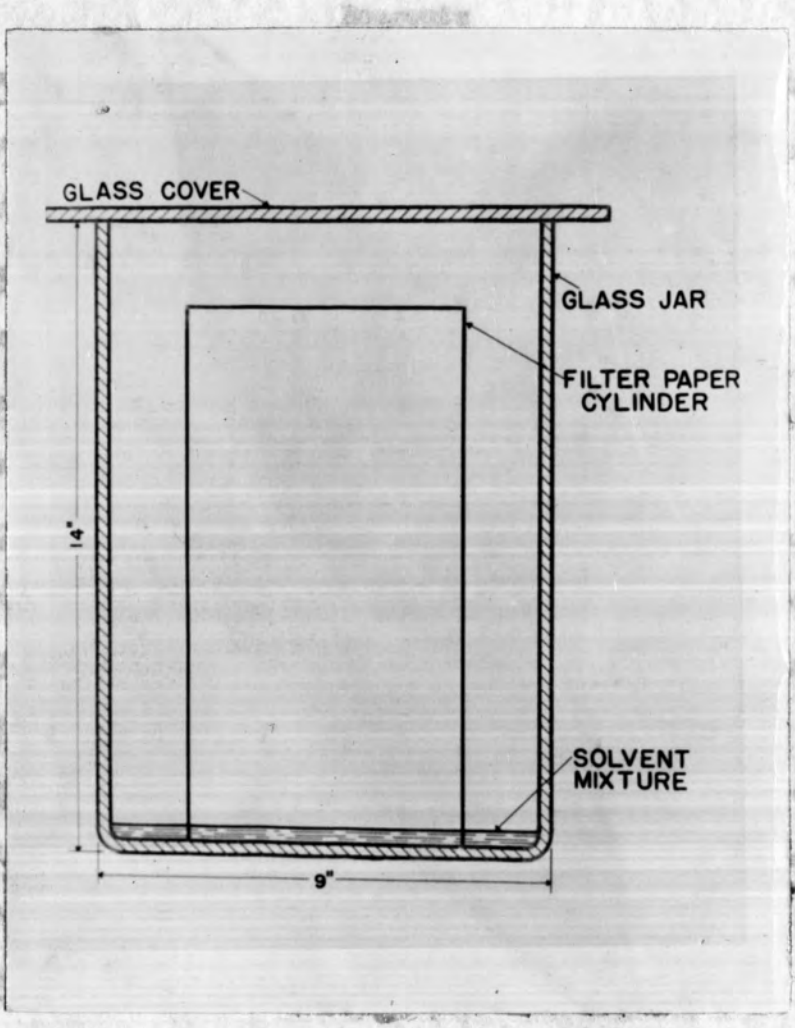

FIGURE I. A stapled filter paper cylinder standing in solvent solution in a sealed glass jar.

Laboratory modification of the Association of Official Agricultural Chemists method of Klein (9), as follows:

Modified Selenium Determination Method

Reagents

(a) Sulfuric-nitric acid. Add concentrated nitric acid to concentrated sulfuric acid in the ratio 2:1. Cool the mixture before using.

(b) Mercuric oxide. Dissolve the oxide in concentrated nitric acid in the ratio of 5 g. to 100 ml. of acid.

(c) Ammonium oxalate. One-fourth saturated, prepared from saturated water solution.

(d) Hydrobromic acid-bromine. Concentrated. Mix 10 ml. of bromine with 990 ml. of constant boiling hydrobromic acid.

(e) Sulfur dioxide. Commercial compressed cylinder gas.

(f) Hydroxylamine hydrochloride. 10% W/V in water.

(g) Hydrobromic acid-bromine. Dilute. To 5 ml. of hydrobromic acid add 10 ml. of saturated bromine-water and dilute to 100 ml. with water.

(h) Phenol. 5% W/V in water.

(i) Standard sodium thiosulfate. 0.002 normal, prepared from Acculute 0.1 normal reagent. One ml. of 0.002 normal thiosulfate is equivalent to 39.6 micrograms of selenium.

(j) Standard iodine. 0.002 normal, prepared from 0.1 normal reagent. Before final dilution add potassium iodide in the ratio of 20 g. per liter.

(k) Soluble starch indicator. 0.5% W/V in water.

Apparatus

An all-glass distillation apparatus consisting of a 250 ml. round-bottomed flask, still head, and condenser with dipping end.

Determination

Place the sample in a 600 ml. beaker and add 200 ml. of the sulfuric-nitric acid followed by 10 ml. of the mercuric oxide fixative. Carefully heat the beaker on an electric hot plate until the sample is completely digested, all fumes from nitric acid are driven off, and sulfur trioxide fumes begin to come off, when the sample should be crystal clear or have a pale yellow color. If the sample turns dark, remove it from the hot plate, cool, add 25 ml. of nitric acid and heat again until sulfur trioxide is driven off. Remove the beaker from the hot plate, cool, and add 50 ml. of one-fourth saturated ammonium oxalate solution.

Transfer the digest with two 25 ml. portions of water to the distilling flask. Rinse the beaker carefully with 25 ml. of the concentrated hydrobromic acid-bromine and add to the cooled digest and washing. After swirling the flask, distill until the residual liquid becomes colorless or pale yellow, and gives off sulfur trioxide fumes, the distillate being collected in a 125 ml. Erlenmeyer flask containing 5 ml. of water and surrounded by cold water.

Saturate the distillate with sulfur dioxide and after adding a few small crystals of hydroxylamine hydrochloride, cap the flasks with watch glasses and set on the steam bath

for thirty minutes. Place the flasks in cold water for thirty minutes, and then with suction collect the selenium on an asbestos pad contained in the filtration vessel. Rinse the precipitation flask and pad with five successive one ml. portions of water from a pipette.

Insert the filtration vessel into a titrating tube and dissolve the selenium with three one ml. portions of the dilute hydrobromic acid-bromine, first adding the reagent from a pipette to the flask and then transferring to the pad. Rinse the flask and pad with four successive one ml. portions of water, collecting the filtrate before each addition.

Agitate the filtrate with a pipette stirrer and dispel excess bromine with three drops of the phenol solution. Using the stirrer as a pipette, rinse the walls of the vessel several times with the solution to neutralize every trace of bromine. Immerse the titration tube up to two-thirds of its length in hot water for five minutes, stirring intermittently.

Using the original precipitate of selenium as a guide, add at least a 50% excess of the standard thiosulfate reagent and three drops of the starch indicator. After stirring, add the standard iodine until a permanent blue color appears. If less than one ml. of iodine is required, add sufficient thiosulfate so that at least one ml. of iodine is required. Then titrate to a colorless endpoint with thiosulfate, adding the reagent in increments of 0.01 ml. as the endpoint is approached.

Calculations and Standardization

To three ml. of dilute hydrobromic acid-bromine(5+95) add four ml. of water and three ml. of standard iodine. Titrate with the standard thiosulfate and toward the end add three drops of the starch indicator. Then titrate to a colorless endpoint with thiosulfate, adding the reagent in increments of 0.01 ml. as the endpoint is approached. Calculate the thiosulfate equivalent of the iodine.

Sample Calculation

From the total quantity of standard thiosulfate added to the sample, subtract the product of the quantity of standard iodine added to the sample multiplied by the thiosulfate equivalent of the iodine, to obtain the net thiosulfate sample titre. From that quantity subtract the quantity of standard thiosulfate (usually 0.30 ml.) needed to titrate a blank sample. Multiply that difference by the selenium equivalent (39.6 micrograms per ml.) to obtain the quantity of selenium in the sample.

Developing Solvents for Chromatography

The organic developing solvent solutions used were phenol (100 g. crystalline phenol: 18 ml. 10% aqueous sodium citrate: 0.5 g. ascorbic acid or no ascorbic acid) (10), n-butanol (80 ml. n-butanol: 20 ml. ethanol: 20 ml. water) (10), lutidine 1:1 with water, and s-collidine 1:1 with water. Excess water was removed by decantation or filtering. Sometimes the papers were set on microscope glass slides in the beakers to keep the bottom edges out of the excess water at the bottom of the beakers.

RESULTS

The best data concerning chromatography of the seleniferous protein hydrolysates were shown by quantitative selenium analyses of the horizontal strips cut at visible boundaries from a phenol chromatogram of a full horizontal band of the hydrolysate placed on the origin line.

Analyses of chromatograms of the selenium gluten hydrolysate (Figure II), having samples of selenium-cystine and selenium-methionine solutions for comparison, showed the highest concentrations of selenium occurring at levels corresponding to those of the known selenium-substituted amino acids, with some selenium, in an unknown form, appearing at the top of the paper. It was shown that in that particular hydrolysate, most of the selenium occurred as selenium-cystine. The plus signs were used to indicate relative quantities of selenium, since the actual minute quantities indicated by the selenium determination method were reliable only to that extent. The actual size of those papers, chromatographed in duplicate to accomodate more hydrolysate, was 28 cm. by 46 cm.; only a part of such a paper is shown as a tracing in Figure II. The concentration of the hydrolysate applied to the paper could not be increased without greatly blurring the whole paper due to higher concentrations of all the amino acids present.

When a similar phenol chromatogram (Figure III) was run with 0.30 ml. of control gluten hydrolysate, with 0.30 ml. of 0.1% selenium-cystine (141 micrograms of selenium) and 0.30 ml. of 0.1% selenium-methionine (120 micrograms of selenium) added,

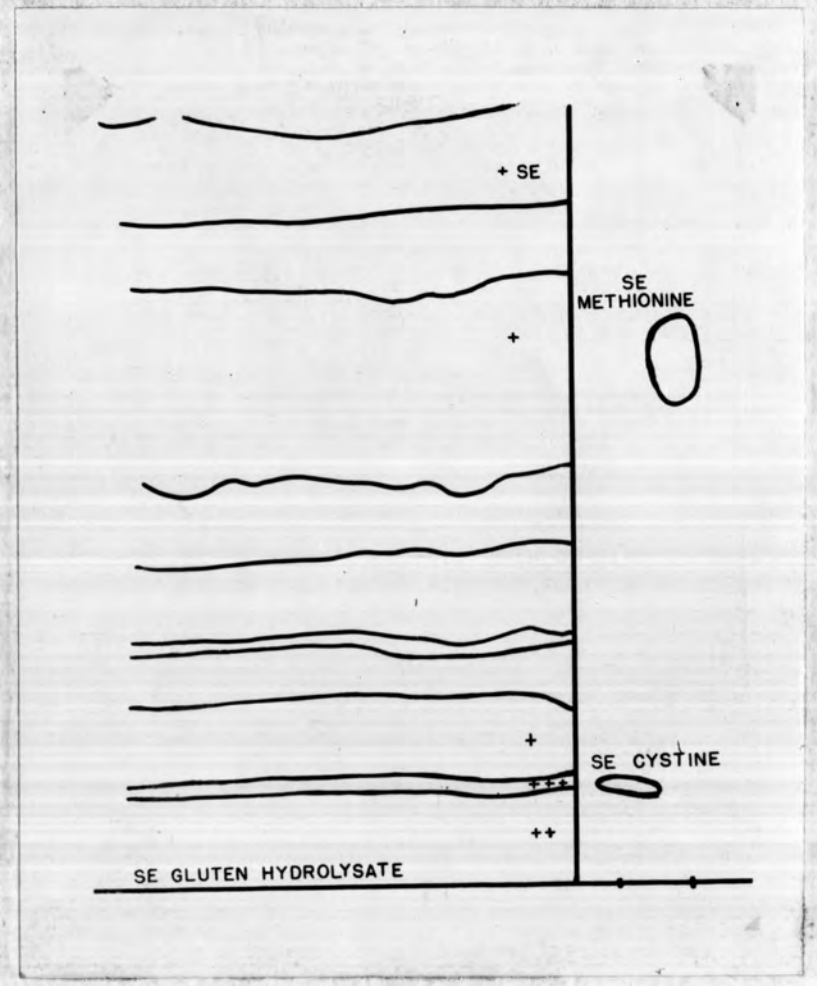

FIGURE II. A tracing of a portion of a phenol chromatogram of a horizontal band of seleniferous gluten hydrolysate on the origin, with comparison spots of selenium-cystine and selenium-methionine at the right. The plus signs indicate the relative quantities of selenium found in the strips cut at all the boundaries.

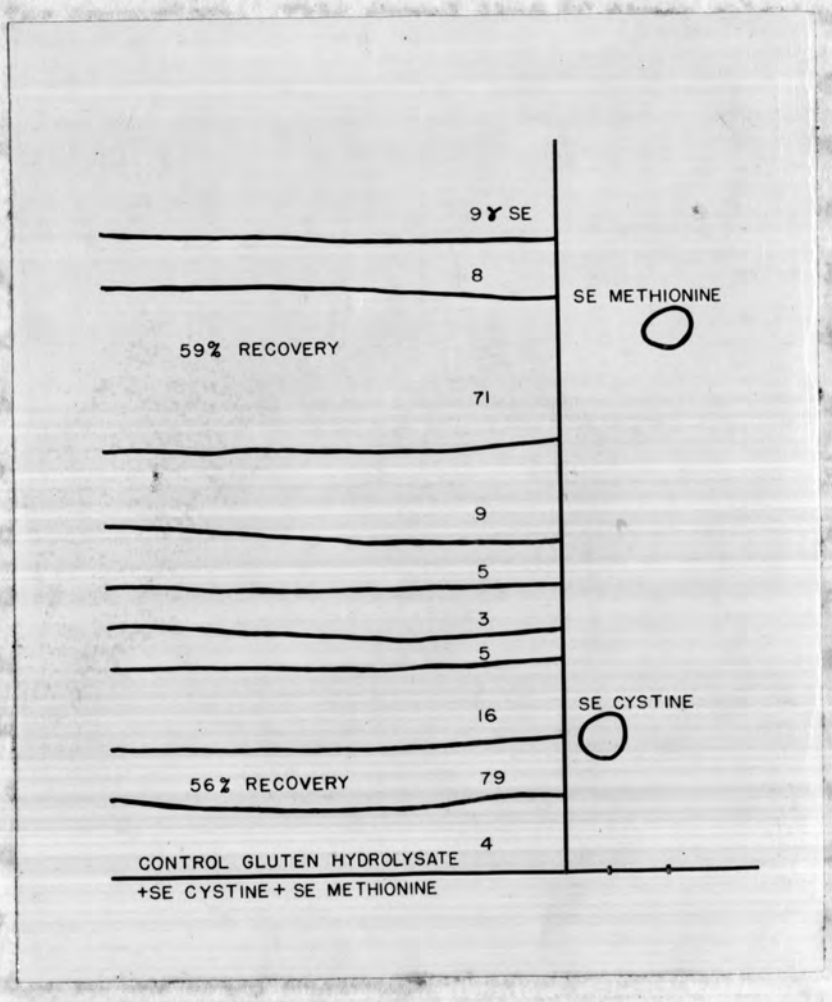

FIGURE III. A tracing of a portion of a phenol chromatogram of horizontal bands of control gluten hydrolysate, 0.1% selenium-cystine and 0.1% selenium-methionine (0.30 ml. of each, superimposed on the origin). The total selenium recovery was 81%. The quantities of selenium found are shown for all the strips.

analyses of the cut strips showed selenium recoveries of 56% and 59%, respectively, of the applied selenium compounds at levels corresponding directly to those of the spots of single compounds used for comparison. This showed that if those selenium compounds were present in a protein hydrolysate, they could be separated and concentrated at the heights of the spots of single compounds.

In the study of the hydrolysates of seleniferous corn proteins, it was found that only the sample called Corn I had a selenium content high enough to make paper chromatography useful. Analyses of pooled corresponding horizontal strips cut from four large phenol chromatograms of full horizontal bands of Corn I (Figure IV) again showed the highest concentrations of selenium at heights corresponding to those of selenium-cystine and selenium-methionine and also at the top of the paper; the nature of the latter is unknown. These results show that in that particular corn protein hydrolysate, most of the organic selenium occurrs as selenium-cystine and selenium-methionine. Black and white photography of such a chromatogram proved to be unsuccessful, due to the heavy coloring.

Large duplicate phenol, (without ascorbic acid), chromatograms of the water extract of the seed of Astragalus bisulcatus (Figure V) showed by analyses of the cut strips that the selenium, mostly organic (11), was concentrated in the mid-portion of the paper, with no strict correlation with the locations of the spots of selenium-cystine or selenium-methionine.

It had been found previously by the above procedure that sodium selenite was carried mostly up to the mid-portion of the

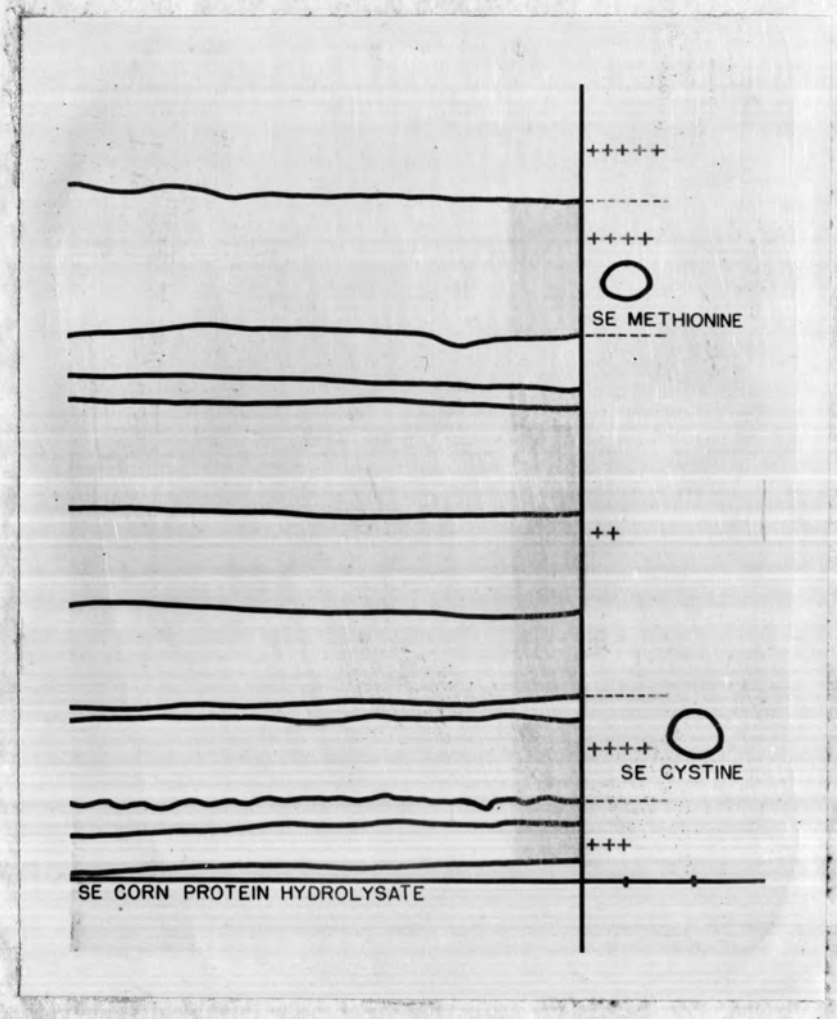

FIGURE IV. A tracing of a portion of a phenol chromatogram of a horizontal band of seleniferous corn protein hydrolysate on the origin. The plus signs indicate the relative quantities of selenium found in the strips cut at the dotted lines. The narrow shaded portions at the right show which horizontal strips are all colored.

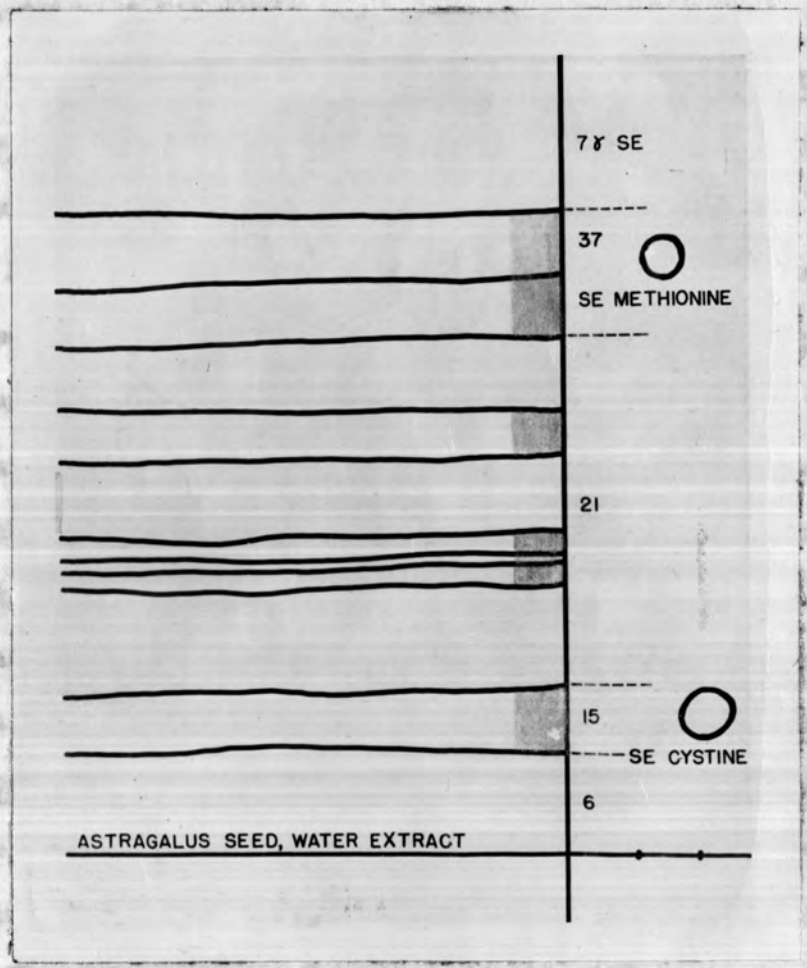

FIGURE V. A tracing of a portion of a phenol chromatogram of a horizontal band of a water extract of Astragalus bisulcatus seed on the origin. The quantities of selenium found in the strips cut at the dotted lines are shown. The narrow shaded portions at the right show which horizontal strips are all colored.

paper by phenol solution containing no ascorbic acid, while phenol containing ascorbic acid left the selenite almost entirely at the bottom of the paper. The phenol solution containing no ascorbic acid also left sodium selenate almost entirely at the bottom of the paper.

The hydrochloric acid hydrolysate of the Astragalus bisulcatus seed protein was not chromatographed for analyses of the cut strips because it contained no selenium.

A water extract of Astragalus bisulcatus plant tops, subjected to the above procedure, showed on the chromatogram (Figure VI) twelve horizontal bands. Analyses showed the selenium, chiefly organic (11), distributed over the entire height of the paper, there being no correlation with the selenium-cystine and selenium-methionine spot locations. A one-dimensional phenol chromatogram of spots of a water extract and a two-normal hydrochloric acid extract of Astragalus bisulcatus plant tops showed differing trails of ascending spots, indicating that the two extracts contain different ninhydrin-reactive compounds, although they were not identified.

In early reports of workers (8, 12, 13) in paper chromatography of amino acids, considerable attention was paid to R_f values (ratio of the travel distance of the compound to that of the solvent) of the various amino acids as a means of identification. Those values vary from 0.00 to about 0.90 and vary, for a certain compound, with different solvent solutions. Although the R_f values are quite consistent, it was observed, in agreement with Dent (14), that there are variations in R_f

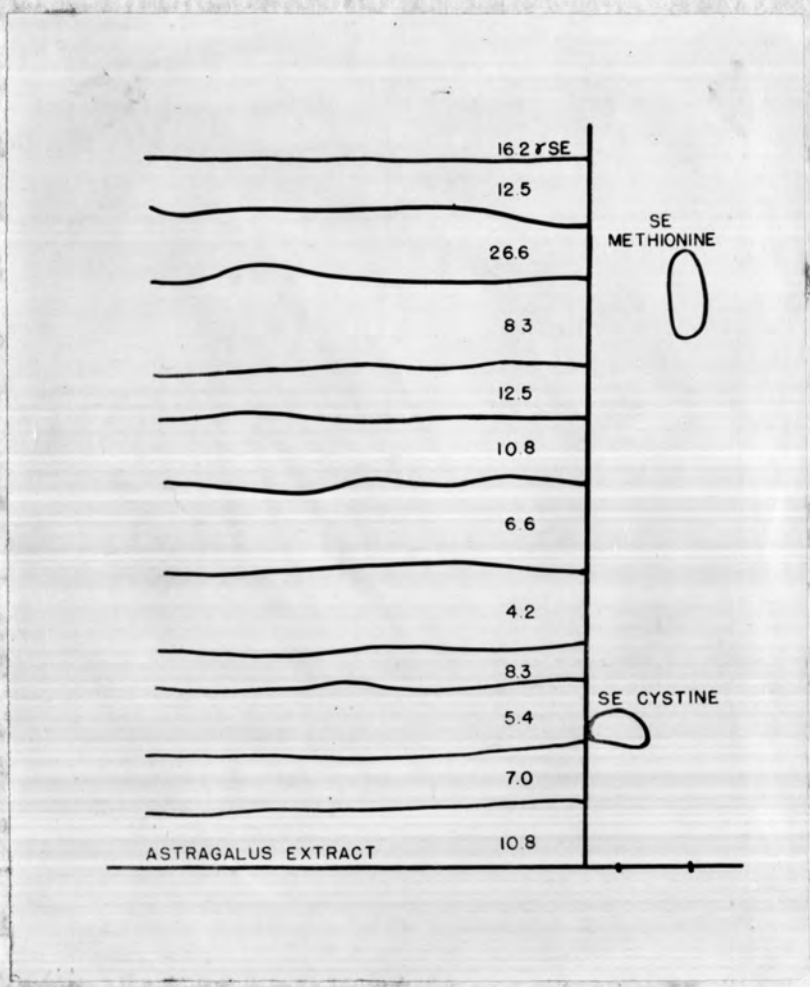

FIGURE VI. A tracing of a portion of a phenol chromatogram of a horizontal band of a water extract of Astragalus bisulcatus plant tops placed on the origin. The quantities of selenium found are shown for all the strips.

values, even on papers developed simultaneously in the same jar, and that the spot pattern is more accurate and more important for identification of the spots, especially in two-dimensional chromatography.

Because the present work was done chiefly with cystine, selenium-cystine, methionine and selenium-methionine, and since R_f values of the selenium compounds have not previously been published, all are shown, with respect to different solvents, in Table I.

TABLE I

Solvent	Cystine R_f	Se Cystine R_f	Ave. R_f Diff.	Methionine R_f	Se Methionine R_f	Ave. R_f Diff.
Phenol (ascorbic)	.14	.15	.02 6/7 prs.	.79	.76	.02 2/2 prs.
Phenol (no ascorbic)	.15	.18	.03 3/3 prs.	.77	.74	.01 4/5 prs.
n-Butanol	.00	.01		.49	.57	.07 3/3 prs.
Lutidine	.28	.34		.66	.72	
s-Collidine	.22	.23		.33	.38	

Although the analogs were not paired in a high number of trials, and although the separations are not wide, the average differences of R_f values (Table I) best show the degree of separation between analogs. With phenol containing ascorbic acid, the average R_f differences between the cystines and between the methionines place the selenium analogs at an R_f value 0.02 higher than the sulfur

compounds. In phenol without ascorbic acid, the average R_f differences place selenium-cystine at an R_f value 0.05 higher than that of cystine and selenium-methionine at an R_f value 0.01 higher than that of methionine. With the butanol solution, the average R_f differences show the R_f value of selenium-methionine to be 0.07 higher than that of methionine, with no separation of the cystines. Lutidine and collidine solutions both show greater R_f differences for both sets of analogs on one paper, but the actual vertical separation is small, since the compounds rise only a short distance.

Chromatograms in phenol solution and in butanol solutions show that methionine in 5% hydrochloric acid rises higher than methionine in 5% hydrochloric acid with ammonia blown on the spot on the origin, which in turn rises higher than methionine in water solution. In phenol the R_f values for those solutions of methionine, in order, are 0.79, 0.75 and 0.68.

In general, the R_f values of all the amino acids chromatographed agree quite well with those of Williams and Kirby (8), who used Whatman's No. 1 filter paper.

From the only two chromatograms using Schleicher and Schuell No. 604 filter paper in phenol solution without ascorbic acid, it was found that the R_f values of all the amino acids used were a little higher than those on Whatman's No. 1 filter paper. The R_f values for cystine, selenium-cystine,

methionine and selenium-methionine were 0.20, 0.23, 0.81 and 0.82, respectively. Still higher R_f values for all of the same amino acids resulted from the paper being left in the solution long after it had reached the top. The phenol solvent solution rose 28 cm. on the Schleicher and Schuell No. 604 paper in seven hours instead of the twenty-six hours required on the Whatman's No. 1 paper, a denser paper.

It was generally found that the selenium-substituted amino acids had R_f values slightly higher than those of their sulfur analogs.

Additional Applications of the Separation of Amino Acids and Selenium Complexes by Paper Partition Chromatography

A butanol chromatogram showed that it was impossible to separate L- and DL-α-alanine, L- and DL-methionine or L- and DL-serine by this means. The R_f value of β-alanine was slightly lower than that of α-alanine.

Phenol chromatograms of two more sulfur bearing amino acids, cystathionine and homolanthionine, showed R_f values of 0.17 and 0.22, respectively, for those compounds. Selenium analogs of those compounds have not been chromatographed.

Two-dimensional chromatograms developed in phenol and then in lutidine showed that a water extract of the roots of corn plant fed extra nitrogen as ammonium sulfate contained compounds believed to be asparagine and leucine in addition

to the phenylalanine, valine, alanine, glycine, aspartic and glutamic acids contained also by a water extract of the roots of corn plants starved of nitrogen. The same procedure showed that a water extract of the tops of corn plants also fed ammonium sulfate contained compounds believed to be asparagine and tryptophane in addition to the phenylalanine, leucine, valine, alanine, glycine, aspartic and glutamic acids contained also by a water extract of the tops of corn plants starved of nitrogen.

One-dimensional chromatograms in phenol solution and in lutidine solution showed that among the selenium complexes prepared at the South Dakota Experiment Station Chemistry Laboratory, those called selenium-di-cysteine and selenium-tetra-cysteine contain two entities each, which may indicate decomposition or impurity of the original material. Those substances called selenium-tetra-glutathione and selenium-hexa-glutathione appeared to consist of one entity, since each showed only one spot on the chromatograms.

DISCUSSION

Amino acids can be separated by paper partition chromatography because they have different coefficients of partition or distribution (ratio of concentration in water to that in another immiscible solvent) between the mobile organic solvent phase and the water-saturated cellulose, not because of differences of adsorption by the cellulose (12, 15). Several possibilities as to the organic solvent were mentioned by Consden, Gordon and Martin (12) in their initial paper, which is the primary reference in the study of paper chromatography; solvents immiscible or slightly miscible in water were recommended. However, the early literature was extremely vague as to the ratios of solvent to water. Nothing was accomplished in the present work prior to a private communication from Williams and Kirby (10), of the University of Texas, who recommended the phenol and n-butanol solutions which were used in this work.

As found by other workers (16), the phenol solvent mixture (Mallinkrodt's phenol was satisfactory without purification) gave the most satisfactory results, in that the samples of hydrolysates and known compounds were most widely separated vertically; all compounds were moved upward from the origin line. The n-butanol solution did not move aspartic or glutamic acids, cystine or selenium-cystine from the origin line. The lutidine and collidine solutions increased in efficiency of separation with increased water content up to a ratio of 1:1, which moved all compounds upward from the origin line. The lutidine solution was next best to the phenol solution in separation of the

amino acids. It was used after the phenol solution in two-dimensional chromatography for maximum separation, as it was by other workers (14, 17, 18) who published maps of such chromatograms.

In later work with the Williams and Kirby phenol solution (10), the ascorbic acid was omitted from the solution because, when heated after the ninhydrin solution spray, it produced a wide, dark pink horizontal band across the bottom of the paper, obscuring the spots of the low-rising amino acids. That property greatly discounted the advantage of the ascorbic acid in slightly preventing diffusion of the spots and its advantage in prevention of oxidation of the phenol, which the sodium citrate did also.

Since none of the solvent solutions separated cystine or methionine from their respective selenium analogs to an extent which would be dependable in distinguishing the compounds in a mixture, especially in the presence of the other amino acids in plant protein hydrolysates, it was apparent that some means other than difference of individual R_f values would be necessary for distinction of the analogs. It was found that although ascorbic acid solution would reduce a selenate or selenite, forming a brown spot of elemental selenium on the paper, such reduction could not be effected with the selenium amino acids. There was more promise in a test by which selenium amino acid spots were treated with hydrogen peroxide and later moistened with water and treated with sulfur dioxide, resulting in the appearance of a bordering red-brown ring of elemental selenium.

However, results were not constant enough to be dependable.

The McCarthy and Sullivan (19) test for methionine distinguished between solutions of methionine and selenium-methionine in test tubes, but it did not distinguish on paper between spots of those compounds. After the ninhydrin solution spray, selenium amino acids, as well as their sulfur analogs, gave positive results to the test described by Chargaff et al. (20), by decolorizing the iodine-sodium azide reagent. It was found that the colors on papers sprayed with the iodine-sodium azide reagent did not fade nearly as much as the colors on other papers, which soon became faint.

Cystine and homocystine, reduced by sodium cyanide solution, were distinguished from their respective selenium analogs by the sodium nitroprusside test (21), but it was found that the components of a mixture of such compounds could not be sufficiently separated on a chromatogram to warrant any conclusion from such a test. It was necessary, in view of the results of the above test, to resort to selenium analyses of horizontal strips cut from a chromatogram of a full band of hydrolysate.

Chromatograms of paired spots of the control and selenium gluten hydrolysates, with both butanol and phenol as solvents, were nearly identical in each of several trials, being nearly continous, with three dark spots. The phenol solution carried the trails of amino acids up about 85% of the height of the paper, while butanol carried them to a variable lesser height.

Because the high proportions of aspartic and glutamic acids (dicarboxylic) masked the spots of the cystines, near

the bottom of the paper, ammonia vapor was used in the jar with a few trials with phenol as the solvent. The ascent of the aspartic and glutamic acid spots was retarded, as Consden et al. (12) mentioned, but the paper was blackened, and separation of the cystines was not improved. The methionines rose to the upper limit of the trail, where phenylalanine and tryptophane interferred with the methionine positions.

Treatment of the selenium gluten hydrolysate with Amberlite acid ion adsorbent resin IR-4B (22), to remove the dicarboxylic acids, did not aid in separation of the cystines. A comparison of paired chromatograms of treated and untreated selenium gluten hydrolysate showed that the resin treatment, although not removing glutamic acid completely, did remove the traces of aspartic acid and the cystines also. Some amino acids corresponding in position to the methionines were also removed.

Chromatograms of seleniferous corn protein hydrolysate with phenol as the solvent showed a trailing distribution of amino acids much like that of the wheat gluten hydrolysates. The trail of spots was slightly less extended at both the top and the bottom and did not show spots for cystine or selenium-cystine.

The nearly neutralized corn protein acid hydrolysates showed much more definite separation of amino acids on phenol chromatograms than did the acid solutions.

Two dimensional chromatograms of seleniferous wheat gluten and seleniferous Corn I (Figure VII) hydrolysates developed in

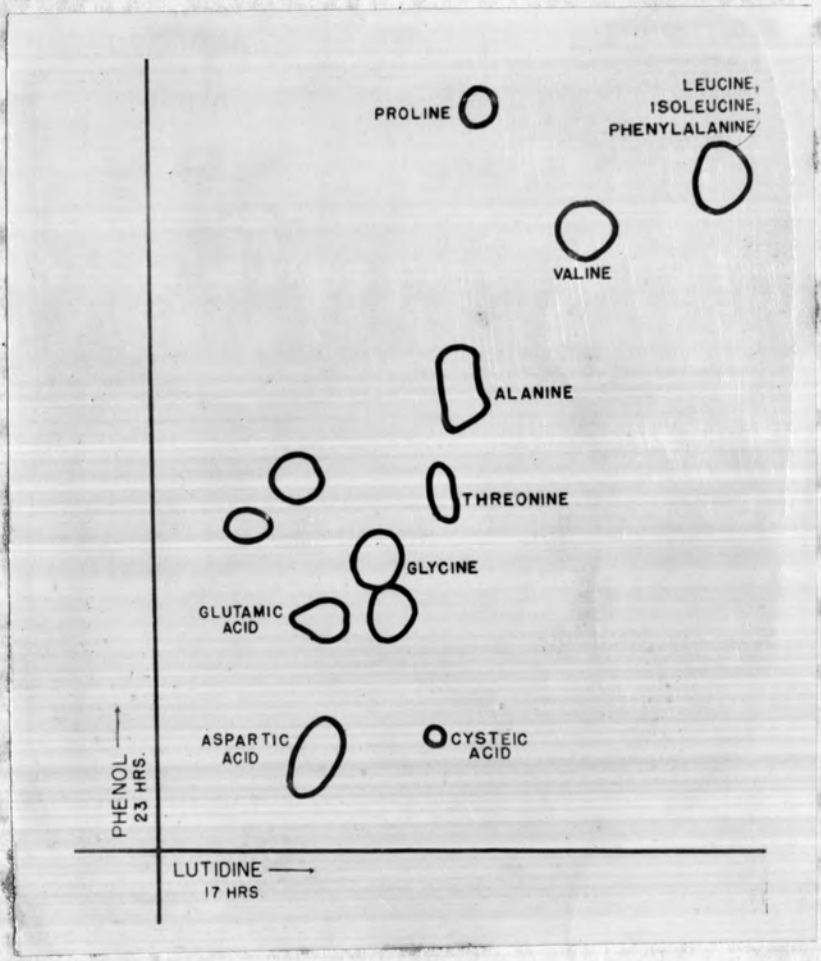

FIGURE VII. A tracing of a two-dimensional chromatogram of a seleniferous corn protein hydrolysate.

phenol solution and then in lutidine solution showed nothing conclusive regarding the selenium amino acids, since they are present in only very low concentrations and since the cystines are probably converted to the corresponding cysteic acids in the lutidine solution (14). However, the two-way separation was good, permitting identification of most of the amino acids of the Corn I hydrolysate from published "maps" (14, 17, 18) of such chromatograms.

Good guide spots for one- or two-dimensional chromatograms were formed by glutamic acid and by the glycine, alanine, valine and leucine, as mentioned by Dent (14). Figure VIII shows the pattern of such spots on a one-dimensional chromatogram.

The ninhydrin spray solution (0.40 g. ninhydrin: 10 g. phenol: 90 g. n-butanol) recommended by Bull, Hahn and Baptist (23) for its sensitivity to amino acids was not satisfactory. The aqueous solution alone produced much better colors.

A total of seventy-seven papers were chromatographed in this work. All of the papers and a record of their descriptions were kept for reference.

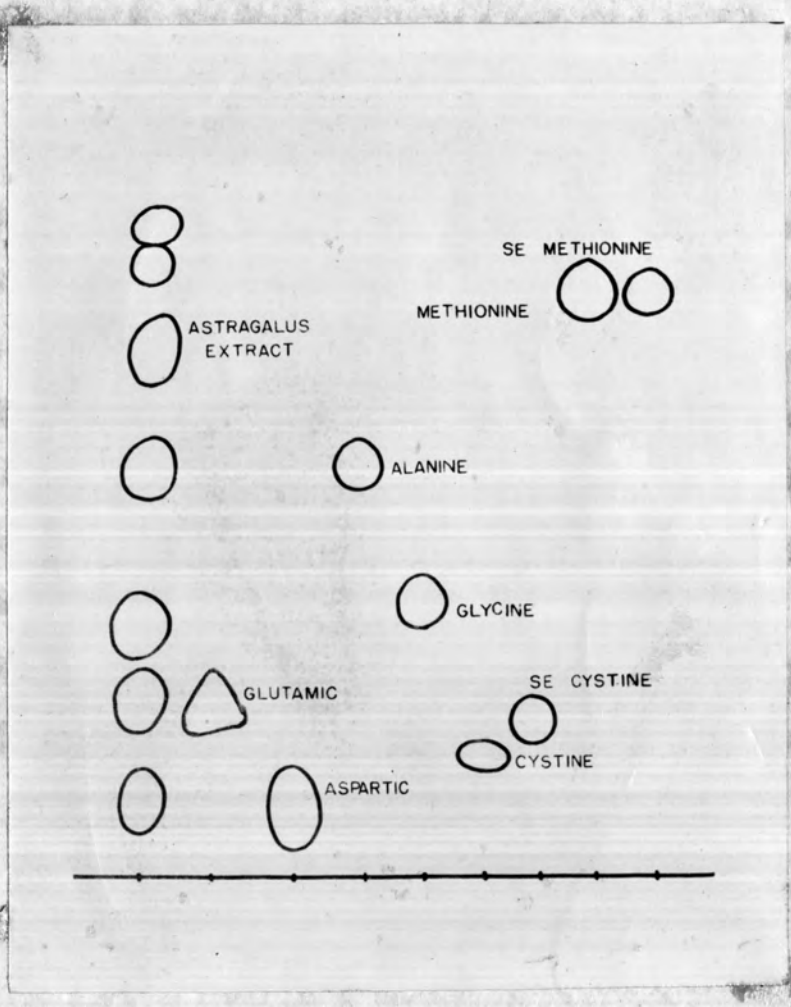

FIGURE VIII. A tracing of a phenol chromatogram of a water extract of the tops of Astragalus bisulcatus plants and amino acid solutions.

SUMMARY

1. The plant proteins of seleniferous wheat, seleniferous corn and Astragalus bisculcatus were prepared and hydrolyzed for use in attempts to isolate the selenium-substituted amino acids, selenium-cystine and selenium-methionine by paper partition chromatography.

2. Quantitative selenium analyses of large ascending phenol chromatograms of the hydrolysates of seleniferous wheat gluten and seleniferous corn protein showed selenium concentrated to a considerable extent at heights corresponding to those of selenium-cystine and selenium-methionine, indicating that those compounds are probably present in the hydrolysates.

3. The selenium analysis of the hydrolysate of Astragalus bisculcatus seed protein showed no selenium content.

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