Phytochemistry, 1971, Vol. 10, pp. 945 to 952. Pergamon Press. Printed in England.

USE OF ^{75}Se-SELENITE FOR THE STUDY OF SELENIUM METABOLISM IN *ASTRAGALUS*

J. L. MARTIN*

Department of Biochemistry, Colorado State University, Fort Collins, Colorado 80521, U.S.A.

A. SHRIFT

Biology Department, State University of New York, Binghamton, New York 13901, U.S.A.

and

MARLENE L. GERLACH

Department of Biochemistry, Colorado State University, Fort Collins, Colorado 80521, U.S.A.

(*Received* 19 *May* 1970)

Abstract—Selenium metabolism in Se-accumulating species of *Astragalus* and in related nonaccumulators was studied by supplying ^{75}Se-labeled selenite directly to leaves and racemes excised from plants growing in their natural habitat. Analysis of the ethanol extracts by ion-exchange chromatography gave results comparable to earlier work with species of these plants grown from seed in the laboratory. Scans of the column effluents from accumulators showed radioactive peaks at the position of Se-methylselenocysteine; for several of the species, peaks were noted at the position of selenocystathionine. Nonaccumulators showed either very little or no radioactivity associated with these compounds. Where nonradioactive selenocystathionine was present, its sulfur analog, cystathionine, could not be detected. Field use of ^{75}Se-selenite offers a convenient biochemical method for a widespread metabolic and taxonomic survey of this large genus of plants.

INTRODUCTION

SURVEYS of selenium metabolism in the genus *Astragalus* have been hampered by difficulties encountered in the collection of seeds from so many species. More than 500 species, about 25 of which accumulate Se, are widely scattered throughout North America.[1-3] The seeds ripen, uncooperatively, at different times. They are subject to insect infestation, and under laboratory conditions there is no guarantee that the various seed batches will germinate or that the seedlings will then grow. Other survey methods are needed, therefore, for a more intensive analysis of the metabolic differences between accumulator and nonaccumulator species.

The method described in this paper carries the isotope to the plant growing in its natural habitat. Leaves were excised in the field, immediately supplied with radioactive selenite, and then killed and stored in 70% ethanol. The ethanol extract was then analyzed by ion-

* Present address: Department of Chemistry, Metropolitan State College, Denver, Colo. 80204, U.S.A.

[1] R. C. BARNEBY, *Atlas of North American Astragalus, Memoirs of the New York Botanical Garden*, Vol. 13, Part I and Part II (1964).

[2] IRENE ROSENFELD and O. A. BEATH, *Selenium*, Academic Press, New York (1964).

[3] S. F. TRELEASE and O. A. BEATH, *Selenium*, Published by the authors, New York (1949).

exchange chromatographic techniques.[4] The results obtained thus far with four Se-accumulating species and with 11 nonaccumulator species verify earlier results with leaves from seedlings grown under laboratory conditions.[5,6] Accumulator species of *Astragalus* characteristically synthesized Se-methylselenocysteine from selenite among their soluble components; nonaccumulators made less or apparently none of this compound. Selenocystathionine was prominent in one species as well as in *Stanleya pinnata*, another Se accumulator; small amounts of this amino acid were detected in several nonaccumulators. These findings substantiate the validity of this isotopic field technique and show promise for comparative studies of sulfate metabolism.

RESULTS

Table 1 categorizes the species of *Astragalus*, *Stanleya* and *Oxytropis* by their varying ability to synthesize selenoamino acids.

TABLE 1. CATEGORIES OF SELENOAMINO ACID SYNTHESIZING PLANTS

Selenoamino acids detected	Accumulators	Nonaccumulators*
Se-methylselenocysteine only	*Astragalus bisulcatus* *Astragalus racemosus*	*Astragalus drummondii*
Selenocystathionine only		*Astragalus caryocarpus* *Oxytropis sericea* *Oxytropis splendens*
Se-methylselenocysteine and Selenocystathionine	*Stanleya pinnata*† *Astragalus pectinatus*‡	*Astragalus adsurgens*
None		*Astragalus canadensis* *Astragalus missouriensis* *Astragalus scopulorum* *Astragalus utahensis* *Oxytropis lambertii*

* Nonaccumulator species in which selenoamino acids were detected synthesized relatively small amounts as compared to the amounts synthesized by accumulator species.

† Synthesized large amount of ^{75}Se-cystathionine and small amount of ^{75}Se-methylselenocysteine.

‡ Synthesized large amounts of ^{75}Se-cystathionine and ^{75}Se-methylselenocysteine.

Comparison of Accumulators

The *Astragalus pectinatus* extract contained high concentrations of ^{75}Se-methylselenocysteine and ^{75}Se-selenocystathionine (Fig. 1). The chromatogram revealed an additional radioactive peak which appeared in the early effluent; this peak at 50 ml, presumably, is γ-glutamyl-Se-methylselenocysteine, a peptide that has been reported in *A. bisulcatus*[7,8] and whose sulfur analog has been identified in seeds of 10 North American accumulator species.[9] Notable and typical is the difference in concentration of the sulfur analogs of these selenoamino acids. S-methylcysteine is well represented while cystathionine, which should precede methionine on the chromatogram, is not detectable.

[4] J. L. MARTIN and MARLENE L. GERLACH, *Anal. Biochem.* **29,** 257 (1969).
[5] A. SHRIFT and T. K. VIRUPAKSHA, *Biochim. Biophys. Acta* **100,** 65 (1965).
[6] T. K. VIRUPAKSHA and A. SHRIFT, *Biochim. Biophys. Acta* **107,** 69 (1965).
[7] S. N. NIGAM, JAN-I. TU and W. B. MCCONNELL, *Phytochem.* **8,** 1161 (1969).
[8] S. N. NIGAM and W. B. MCCONNELL, *Biochim. Biophys. Acta* **192,** 185 (1969).
[9] P. M. DUNNILL and L. FOWDEN, *Phytochem.* **6,** 1659 (1967).

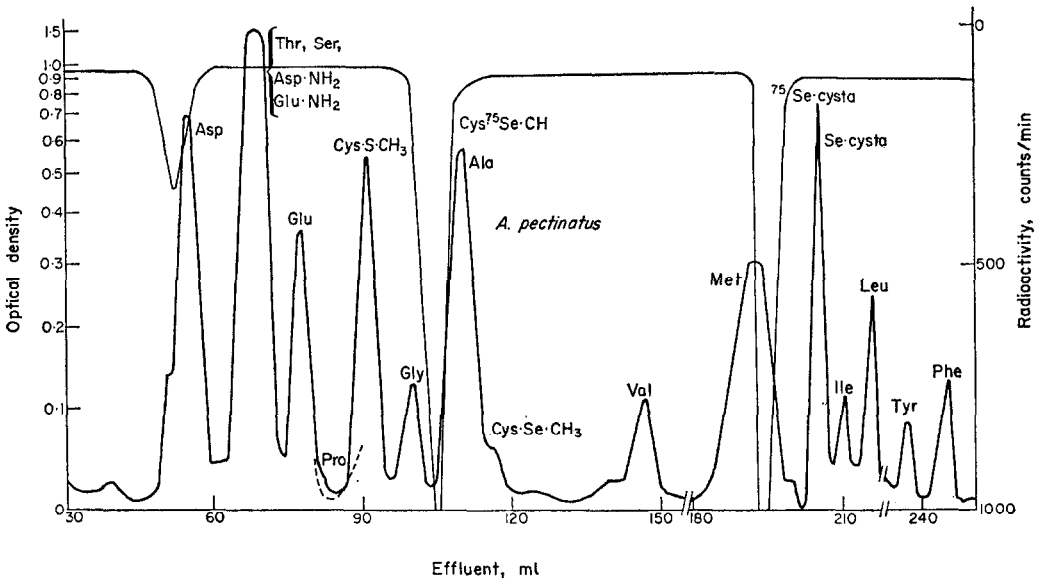

FIG. 1. PORTION OF A CHROMATOGRAM SHOWING ANALYSIS OF AN EXTRACT OF *Astragalus pectinatus*, A Se ACCUMULATOR.
The upper scan shows the radioactive peaks; the lower scan shows the ninhydrin peaks.

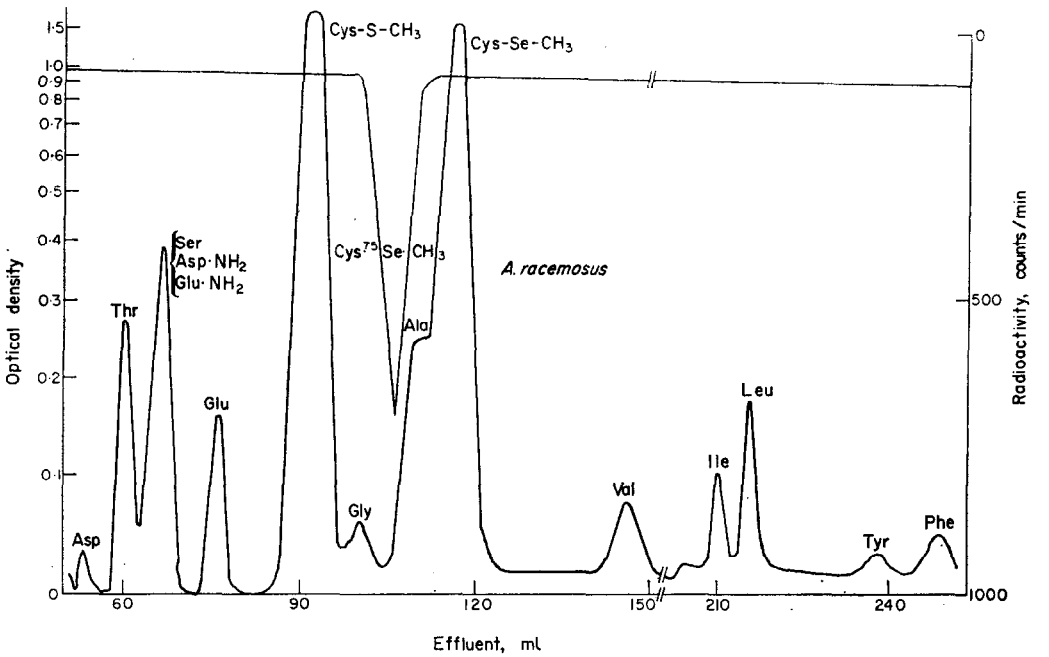

FIG. 2. PORTION OF A CHROMATOGRAM SHOWING ANALYSIS OF AN EXTRACT OF *Astragalus racemosus*, A Se ACCUMULATOR.
The upper scan shows the radioactive peaks; the lower scan shows the ninhydrin peaks.

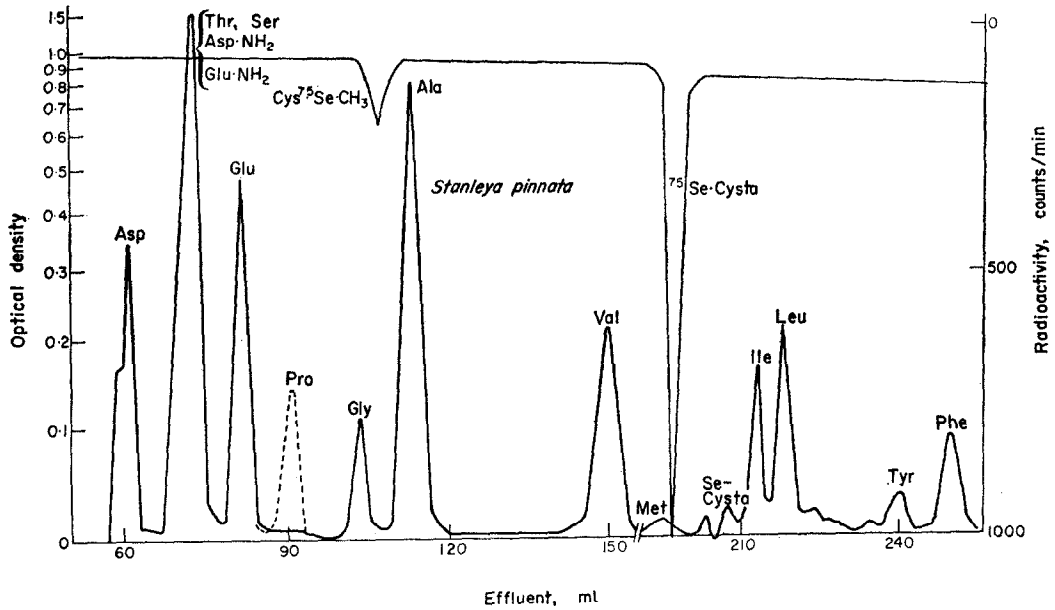

FIG. 3. PORTION OF A CHROMATOGRAM SHOWING ANALYSIS OF AN EXTRACT OF *Stanleya pinnata*, A Se ACCUMULATOR.

The upper scan shows the radioactive peaks; the lower scan shows the ninhydrin peaks.

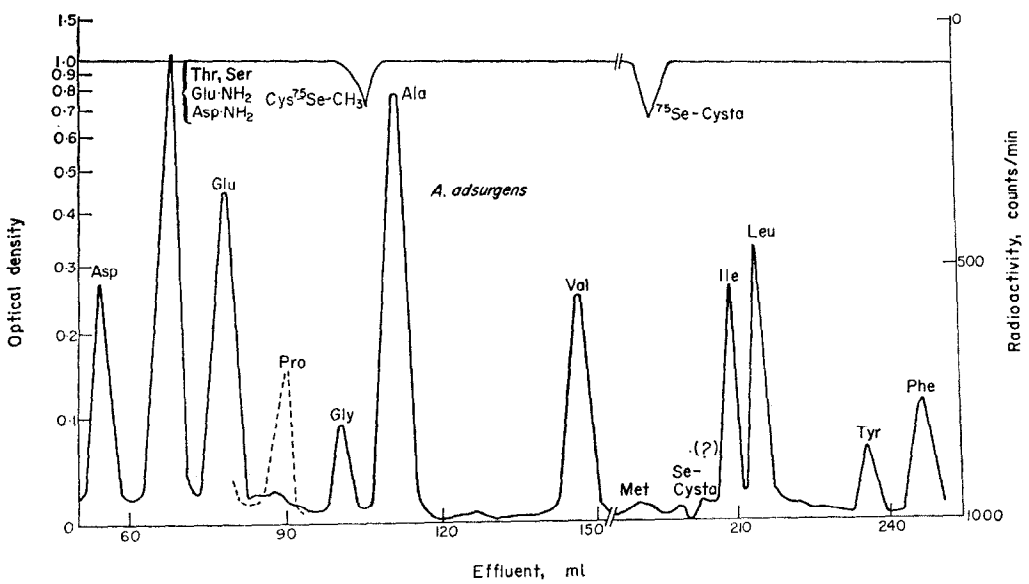

FIG. 4. PORTION OF CHROMATOGRAM SHOWING ANALYSIS OF AN EXTRACT OF *Astragalus adsurgens*, A NONACCUMULATOR.

The upper scan shows the radioactive peaks; the lower scan shows the ninhydrin peaks.

Selenocystathionine was not detectable in extracts of the accumulators *A. racemosus* and *A. bisculcatus* which, however, contained Se-methylselenocysteine (Fig. 2). *Stanleya pinnata*, another accumulator, synthesized large quantities of ^{75}Se-selenocystathionine (Fig. 3). A small corresponding ninhydrin peak probably represents selenocystathionine, but cystathionine was not evident. The elution pattern shows a small ^{75}Se-methylselenocysteine peak, but the corresponding ninhydrin peak of the nonradioactive compound which emerges just after alanine was absent. Nor could the sulfur analog, S-methylcysteine, which overlaps with proline, be seen.

Comparison of Nonaccumulators

Some of the plants investigated (Table 1) had no apparent selenoamino acid synthesizing ability. However, several of the other species classified as nonaccumulators showed a limited capacity to synthesize Se-methylselenocysteine, selenocystathionine or both (Table 1). Typical of all nonaccumulators in this category was the small size of the radioactive peak of the compound or compounds which the plant was able to synthesize; the corresponding ninhydrin peaks were not discernible with the possible exception of selenocystathionine in *A. adsurgens* (Fig. 4). The appearance of a radioactive peak in the absence of a ninhydrin peak demonstrates the ability of the plant to synthesize the specified selenoamino acid.

DISCUSSION

The results of this survey in several respects extend as well as corroborate findings reported earlier with *Astragalus* and other related plants.[4–6] Se-methylselenocysteine was the predominant nonprotein selenoamino acid found in selenium accumulating species. Selenocystathionine, already identified in many plants by a number of investigators,[4,10–15] was again found in *A. pectinatus* and in *Stanleya pinnata*. Smaller amounts of these two compounds occurred in several of the related nonaccumulators surveyed in this study.

The two sulfur and selenium analogs which almost always appeared together were S-methylcysteine and Se-methylselenocysteine. In some species S-methylcysteine was predominant, although larger amounts of Se-methylselenocysteine have been observed.[4] Whatever the reasons for these variations, the simultaneous occurrence of both compounds reflects the ability of S and Se to follow the same pathway as far as the synthesis of these two compounds is concerned.

There are instances, however, in which one or the other but not both of a pair of S and Se analogs are found.[5,6,16] Several of the present findings also showed this pattern. Methionine, for example, was detected in large amount in *A. pectinatus* (Fig. 1), and has been found in seeds of this species,[4] but there was no sign of selenomethionine which appears on a chromatogram between isoleucine and leucine. *Stanleya pinnata* demonstrated a limited ability to synthesize Se-methylselenocysteine but in an insufficient quantity to be detected as a ninhydrin peak. A ninhydrin peak for S-methylcysteine also was not detected. (Fig. 3).

[10] J. M. Horn and D. B. Jones, *J. Biol. Chem.* **139**, 649 (1941).
[11] T. K. Virupaksha and A. Shrift, *Biochim. Biophys. Acta* **74**, 791 (1963).
[12] L. Aronow and F. Kerdel-Vegas, *Separata de la Revista Dermatologia Venezolana* IV, 109 (1964).
[13] L. Aronow and F. Kerdel-Vegas, *Nature* **205**, 1185 (1965).
[14] F. Kerdel-Vegas, F. Wagner, P. B. Russell, N. H. Grant, H. E. Alburn, D. E. Clark and J. A. Miller, *Nature* **205**, 1186 (1965).
[15] P. J. Peterson, G. W. Butler, *Nature* **213**, 599 (1967).
[16] A. Shrift, *Ann. Rev. Plant Physiol.* **20**, 475 (1969).

Of particular interest is the apparent absence of cystathionine. This amino acid is an intermediate in the sulfur metabolism of bacteria, fungi and animals. It is also considered to be a precursor of methionine in plants,[17] but its occurrence in higher plants remains to be conclusively established.[16] Examination of the chromatogram tracings in the present study as well as those from earlier work in which seed extracts from three selenium accumulator species were analyzed[4] fails to reveal any clearcut ninhydrin peak at the position of cystathionine; the compound emerges from the column just before methionine.

The apparent absence of cystathionine from all scans which show selenocystathionine, especially when large amounts of selenocystathionine can be detected, signifies that differences in the metabolism of sulfur and selenium are even more widespread than have previously been suggested.[16] It is not known whether these differences reflect unequal rates of synthesis or utilization.*

Several of the present results are at variance with results reported earlier. Se-methylselenocysteine, for example, was absent from *A. canadensis* in the current study, but in an earlier study had been synthesized from both selenate and selenite.[6] Se-methylselenomethionine, which had been found in four nonaccumulator species,[6] was not detected in the extracts of any of the plants analyzed here, including *A. canadensis* which was common to both studies. These differences can perhaps be attributed to experimental conditions; in the earlier work, excised leaves had been exposed to selenite or selenate for 3 days, whereas here the excised leaves were exposed for only several hours. The storage conditions used in the current work may also have been unsuitable for this compound.

When the synthetic abilities of the various species are compared it becomes increasingly clear that selenium accumulator species differ biochemically from nonaccumulator species. There are, however, a number of nonaccumulators which appear to be intermediate in character. Some of the nonaccumulator species of *Astragalus* (as well as several species of *Oxytropis*, a related genus of legumes) contained Se-methylselenocysteine, selenocystathionine or both, but the levels were lower than the levels found in accumulators. The biochemical distinction between the two types of species is, therefore, not as sharp as the physiological distinction based on their accumulation of selenium under natural conditions.[2,3]

This paper describes a relatively simple application of isotopes in the field, for which as little as one leaf can be used. A virtually unlimited supply of plant material thus becomes available and makes feasible a thorough investigation of the many *Astragalus* species. Two of the selenium compounds which distinguish accumulator from nonaccumulator species are apparently stable enough to withstand the conditions of treatment and handling in the field. It should now be possible to survey many other selenium accumulators such as woody aster (*Xylorhiza*), goldenweed (*Oonopsis*) and a variety of secondary selenium absorbers also hazardous to livestock.[2,3]

EXPERIMENTAL

Plant Materials

The species investigated in this study are listed below. The locations where they were collected may be obtained from one of the authors (A.S.).

Accumulators. Astragalus racemosus Pursh, var. *racemosus*; *A. pectinatus* Douglas; *A. bisculcatus* (Hood) var. *Haydenianus* (Grey) Barneby; *Stanleya pinnata* (Pursh) Britt.

* In a preliminary experiment we supplied $^{35}SO_4^{-2}$ to excised leaves of *A. bisulcatus* and *Stanleya pinnata* and found small radioactive peaks at the elution position of cystathionine; further work is needed to establish the compound as cystathionine.

[17] J. F. THOMPSON, *Ann. Rev. Plant Physiol.* **18**, 59 (1967).

Nonaccumulators. A. canadensis; *A. caryocarpus*; *A. missouriensis* Nutt.; *A. scopulorum* (Porter); *A. utahensis* (Torr.) T. & G.; *A. Drummondii* Hook; *A. adsurgens* Pall. var. *robustior* Hook; *Oxytropis lambertii* (variety unknown); *Oxytropis lambertii* Pursh var. *lambertii*; *Oxytropis Sericea* Nutt., and *Oxytropis splendens* Doug.

Chemicals

^{75}Se-selenite, specific activity 83,000 mc/g, was purchased from General Electric Co., Pleasanton, California. The seleno and sulfur amino acids used as standards for the amino acid analyzer were obtained from the same sources described previously.[4]

Uptake of Isotopes

A single leaf or flowering stalk was excised at the base of the petiole and immediately placed in a vial that contained 1 μc of ^{75}Se-selenite in a volume of 0·5 ml of glass distilled water. No environmental controls were attempted during absorption of the radioactive solution. The rack of vials was taken in and out of the car as each new specimen was treated. When the radioactive solution was exhausted, glass distilled water was added to the vial. After several hours the surface of the petiole was rinsed with water and the entire leaf transferred to a vial that contained 5–7 ml of 70% alcohol. The vials were kept in the dark and on return to the laboratory were stored in a freezer until needed for analysis.

Preparation of Materials for Chromatographic Analysis

The preserved radioactive tissues were macerated in the storage vial with a glass rod and the extract was decanted into a beaker. The residue was then washed 3 × with 0·5 ml of 70% ethanol and the washings were pooled with the initial extract. The volume was reduced to 0·5–1·0 ml on a hot plate. After the pH was adjusted to 2 with 0·1 N HCl, 4 ml of sodium citrate buffer, pH 2·2, were added. An anion exchange column of Dowex 2- × 8 properly ionized with 0·1 N HCl and rinsed with distilled water to pH 5 was used to remove excess $^{75}SeO_3^{-2}$. All of the sample was used for the amino acid assays.

TABLE 2. METHODOLOGY USED FOR ANALYZING SAMPLE CONTAINING AMINO ACIDS IN A PROTEIN HYDROLYSATE TO WHICH ADDITIONAL SULFUR AND SELENIUM AMINO ACIDS WERE ADDED

Column size:	0·9 × 69 cm
Resin type:	Aminex A-4 (Bio-Rad)
Resin height:	56 cm
Column flow rate:	60 ml/hr
First buffer:	3·255 ± 0·005 (0·2N)
Second buffer:	4·25 ± 0·02 (0·2N)
Buffer change time:	145 min
Temperature:	56°

Chromatography

The radioactive plant extracts were chromatographed on a modified Beckman/Spinco Model 120B amino acid analyzer supplied with a Nuclear-Chicago Chroma-Cell Detector Assembly. Buffer flowed through a 1-ml cell (Packard Instrument Co., Inc.) packed with anthracene as the scintillator. Even though the counting efficiency of this assembly was no more than 1–2 per cent, sufficiently high quantities of radioselenium were made available to the plants so that radioactive peaks of selenoamino acids were easily discernible when their corresponding ninhydrin peaks, which are detectable at 0·02 μm were not.

Ion exchange procedures were used which had been developed to resolve selenoamino acids from their sulfur analogs. The elution protocol shown in Table 2 was modified slightly from the one described previously;[4] the effect of the increase in pH from 3·236 to 3·255 was to elute cystathionine before methionine. Fig. 5 illustrates the elution pattern of the amino acid mixture to which had been added additional sulfur and selenium amino acids. Radioactive peaks rather than ninhydrin peaks were used as criterion to determine the capacity of plants to synthesize selenoamino acids. Only the analyses of the acidic and neutral amino acids are shown. No attempt was made to analyze protein hydrolysates since the only selenoamino acids identified are of the nonprotein type.

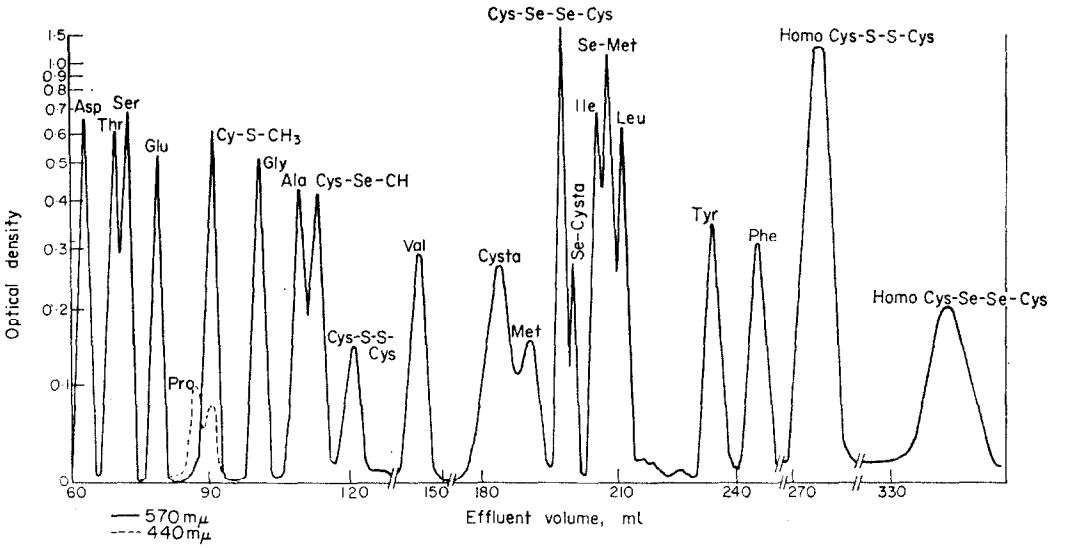

FIG. 5. CHROMATOGRAM SHOWING THE ELUTION OF A STANDARD AMINO ACID CALIBRATION MIXTURE (BIO RAD LABORATORIES, RICHMOND, CALIFORNIA, U.S.A.) PLUS ADDITIONAL SULFUR AND SELENIUM AMINO ACIDS. THE CONCENTRATION OF AMINO ACIDS IN THE STANDARD WAS 0·5 μM EXCEPT CYSTINE (0·25 μM). THE CONCENTRATION OF THE ADDITIONAL AMINO ACIDS WAS APPROXIMATELY 0·5 μM EXCEPT CYSTATHIONINE (1 μM) AND SELENOCYSTATHIONINE (0·25 μM).

Acknowledgements—The authors wish to thank Dr. Charles Taylor, S.D. State College, and Dr. Harold Eppson, University of Wyoming, for help in locating some of the plants, and Dr. R. C. Barneby, of the New York Botanical Garden for identifying many of the species. The able technical assistance of Mr. Paul Lieu in the preliminary work is gratefully acknowledged. This work was partly supported by Grant R01-00254-08 of the Division of Environmental Health Sciences, National Institutes of Health (to J. L. M.) and partly by Grant S-68-40-008 from the Research Foundation of State University of New York (to A.S.).