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INVESTIGATIONS ON SELENIUM IN WHEAT

O. E. OLSON, E. J. NOVACEK, E. I. WHITEHEAD and I. S. PALMER

Biochemistry Department, South Dakota State University,
Brookings, South Dakota 57006, U.S.A.

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Abstract—Pronase hydrolysates of crude gluten preparations from naturally seleniferous wheat seeds were subjected to ion-exchange chromatography and the eluates were examined chemically for selenium. Almost half of the selenium in the hydrolysate was accounted for as selenomethionine, but selenocystine was not detected. More detailed studies were then made on wheat gluten, seeds and straw from plants grown on soil to which ^{75}Se-selenate had been added. On pronase hydrolysis of the seeds or the gluten, about half of the selenium could again be accounted for as selenomethionine. On examining protein-free, hot-water extracts of the seeds or straw of these plants, no free selenomethionine could be detected, suggesting the rapid incorporation of this amino acid into protein. Neither selenocystine, the seleno-half-cystine moiety nor Se-methylselenocysteine was detected in the hydrolysates or in the water extracts. The results suggest, however, the presence of selenocysteic acid in significant amounts in the gluten, the seeds and the straw. Over half of the selenium in the straw could be accounted for as selenate, but selenite was not found. Selenium compounds eluted shortly after selenite, selenate and cysteic acid, suggesting the presence of oxides of selenomethionine.

INTRODUCTION

SELENIUM in grains and forages is important in animal health as a toxicant and as a nutrient.[1] Interest in the identification of selenium compounds in these grains and forages stems from the possible significance of chemical form of the element to its functioning as a toxin or as a nutrient.

Since wheat is an important crop in the seleniferous areas of South Dakota, it is of special interest to this laboratory. In 1934, Franke[2] found selenium in wheat associated with the protein. Studies on acid hydrolysates of the protein suggested that selenium replaced sulfur in cystine and methionine.[3] Later, Smith[4] studied acid hydrolysates of wheat protein with paper chromatography and found most of the selenium localized in the area of selenocystine, lesser amounts in the area of selenomethionine and some at other locations on the chromatogram. Whitehead *et al.*,[5] however, reported that selenocystine and selenomethionine could not be identified by starch or by Dowex-50 resin chromatography in acid hydrolysates of leaf proteins and of seed proteins from wheat grown on ^{75}Se-selenate.

More recently, Peterson and Butler[6] subjected dialyzed sap of wheat seedlings grown in ^{75}Se-selenite to paper chromatography and electrophoresis. They found one compound which moved identically with selenomethionine and another that moved with selenomethionine selenoxide. When leaf protein was enzymatically hydrolyzed, a particularly sharp peak of radioactivity correlated with selenomethionine on the chromatogram.

[1] I. ROSENFELD and O. A. BEATH, *Selenium*, Academic Press, New York (1964).
[2] K. W. FRANKE, *J. Nutrition* **8**, 609 (1934).
[3] E. P. PAINTER and K. W. FRANKE, *Cereal Chem.* 13, 172 (1935).
[4] A. L. SMITH, M.S. Thesis, South Dakota State College, Brookings (1949).
[5] E. I. WHITEHEAD, C. M. HENDRICK and F. M. MOYER, *Proc. S. Dak. Acad. Sci.* **34**, 52 (1955).
[6] P. J. PETERSON and G. W. BUTLER, *Australian J. Biol. Sci.* **15**, 126 (1962).

In 1964, McConnell and Wabnitz[7] reported the separation of selenomethionine and selenocystine from their sulfur analogs on ion-exchange resins. Walter *et al.*,[8] Benson and Patterson[9] and Martin and Gerlach[10] have studied this method further. It offered another approach to the identification of selenium amino acids in wheat, as well as to their quantitation, and was used in the studies on wheat which are described here.

RESULTS AND DISCUSSION

Chromatography of Selenium Compounds

At the outset, the chromatographic column was calibrated using a standard amino acid solution and several different selenium compounds. In view of the report of Walter *et al.*[8] concerning the reaction of thiols with diselenides, buffer systems with and without thiodiglycol were used in eluting compounds from the column.

In general, the positions of the selenium compounds on the chromatogram were what would be expected in view of previous reports.[7–10] It is of interest that, in the absence of thiodiglycol, selenite eluted with the selenate and selenocysteic acid ($CySeO_3^-$) in a sharp peak at about 10 ml, while in the presence of thiodiglycol it eluted as a broad peak at about 96 ml. The thiodiglycol did not affect the elution of the selenate or selenocysteic acid. Selenite probably reacts with the thiol of the thiodiglycol, as suggested by Painter[11] and confirmed by Ganther,[12] to form the selenotrisulfide.

The reaction product of selenocystine and cysteine, presumed to be sulfoselenocystine (CySSeCy), gave a broad double peak under valine with the buffer containing thiodiglycol and a single peak, again under valine, when the thiol was absent.

Gluten from Naturally Seleniferous Wheat

Preliminary work in this study was undertaken with a crude gluten preparation from a seleniferous wheat produced in western South Dakota. A pronase hydrolysate of the gluten was chromatographed on Aminex A-4 cation exchange resin and 2·0-ml portions of the effluent between 74 and 180 ml were chemically analyzed for selenium. The results are summarized in Fig. 1. About half of the selenium placed on the column eluted under selenomethionine. The small amounts of selenium found under cystine, near valine and under selenocystine were of doubtful significance, since the values obtained were so close to the lower limit of reliability for the method (0·02 μg).

An additional chromatogram on a different hydrolysate gave a very similar pattern at the selenomethionine location (43·7 per cent of the selenium placed on the column eluted under selenomethionine), and an analysis of the first 20 ml of effluent indicated the presence of a small peak there. In view of the failure to find clear evidence for selenocystine, it was decided that further studies should be made, using wheat containing radioactive selenium (^{75}Se-wheat).

Studies on ^{75}Se-wheat

Wheat was grown on ^{75}Se-selenate and divided into seed, spikes exclusive of seed, and straw (stem and leaves). A crude gluten fraction was prepared from a portion of the seeds,

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[8] R. WALTER, D. H. SCHLESINGER and I. L. SCHWARTZ, *Anal. Biochem.* **27**, 231 (1969).
[9] J. V. BENSON, JR. and J. A. PATTERSON, *Anal. Biochem.* **29**, 130 (1969).
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[11] E. P. PAINTER, *Chem. Reviews* **28**, 179 (1941).
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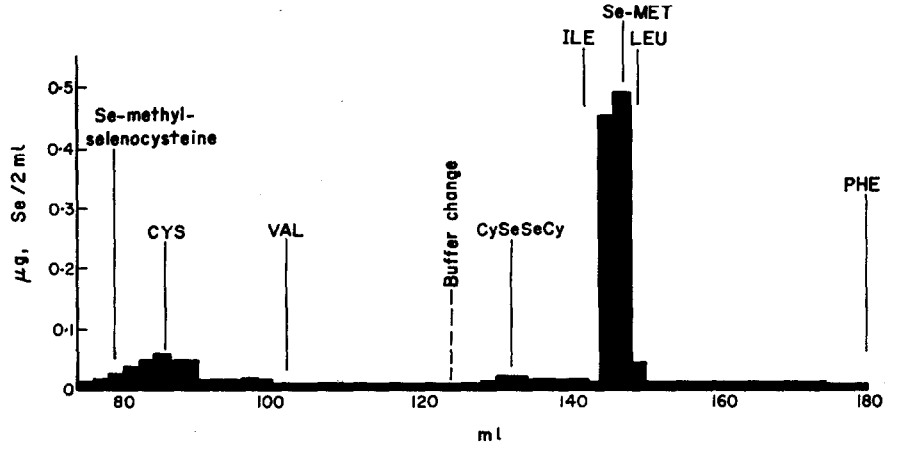

FIG. 1. SELENIUM CONTENT OF 2·0 ml FRACTIONS OBTAINED ON CHROMATOGRAPHING THE PRONASE HYDROLYSATE OF GLUTEN OBTAINED FROM NATURALLY SELENIFEROUS WHEAT.

The pH 3·30 and pH 4·25 buffer system with thiodiglycol was used in developing the chromatogram. Underlined amino acids were located as explained under the Experimental section. The selenium eluted at the location of selenomethionine represented 47·3% of that placed on the column and 41·4% of that in the gluten.

subjected to pronase hydrolysis and chromatographed. Finely ground seed, which had been continuously extracted with ether for 16 hr, was treated in a like manner. In addition to the pronase digests of the gluten and defatted seeds, protein-free, hot-water extracts of the defatted seeds and of the straw were also chromatographed. In all cases, 2·0-ml fractions were collected and the ^{75}Se was determined by counting. A summary of some of the data is given in Table 1. Chromatograms of the pronase hydrolysate of wheat gluten are shown in

TABLE 1. SUMMARY OF DATA FOR ^{75}Se-WHEAT DIGESTS AND EXTRACTS*

	Pronase hydrolysates		Protein-free, hot-water extracts	
	Gluten	Seed	Seed	Straw
% ^{75}Se in gluten, seed or straw recovered in hydrolysate or extract	92·4	80·3	19·5	83·5
^{75}Se placed on column (cpm)	315,867	197,253	38,142	109,924
^{75}Se in effluent collected (% of total on column)	94·1	101·9	49·2	94·8
^{75}Se in 0–50 ml of effluent (% of total on column)	15·1	24·6	40·9	88·2
^{75}Se under selenomethionine				
(% of total on column)	55·7	58·9	0·0	0·0
(% of total in gluten, seed or straw)	51·5	47·3	0·0	0·0

* For data obtained with pH 3·30 and pH 4·25 buffers containing thiodiglycol.

Fig. 2 for both the two-buffer system with thiodiglycol and the one-buffer system without thiodiglycol. A chromatogram of the protein-free, hot-water extract of wheat straw prepared with the two-buffer system with thiodiglycol is shown in Fig. 3. The positions where certain selenium compounds would be expected to appear on the chromatograms are indicated. In addition, the positions of certain amino acids are shown. These latter were verified by

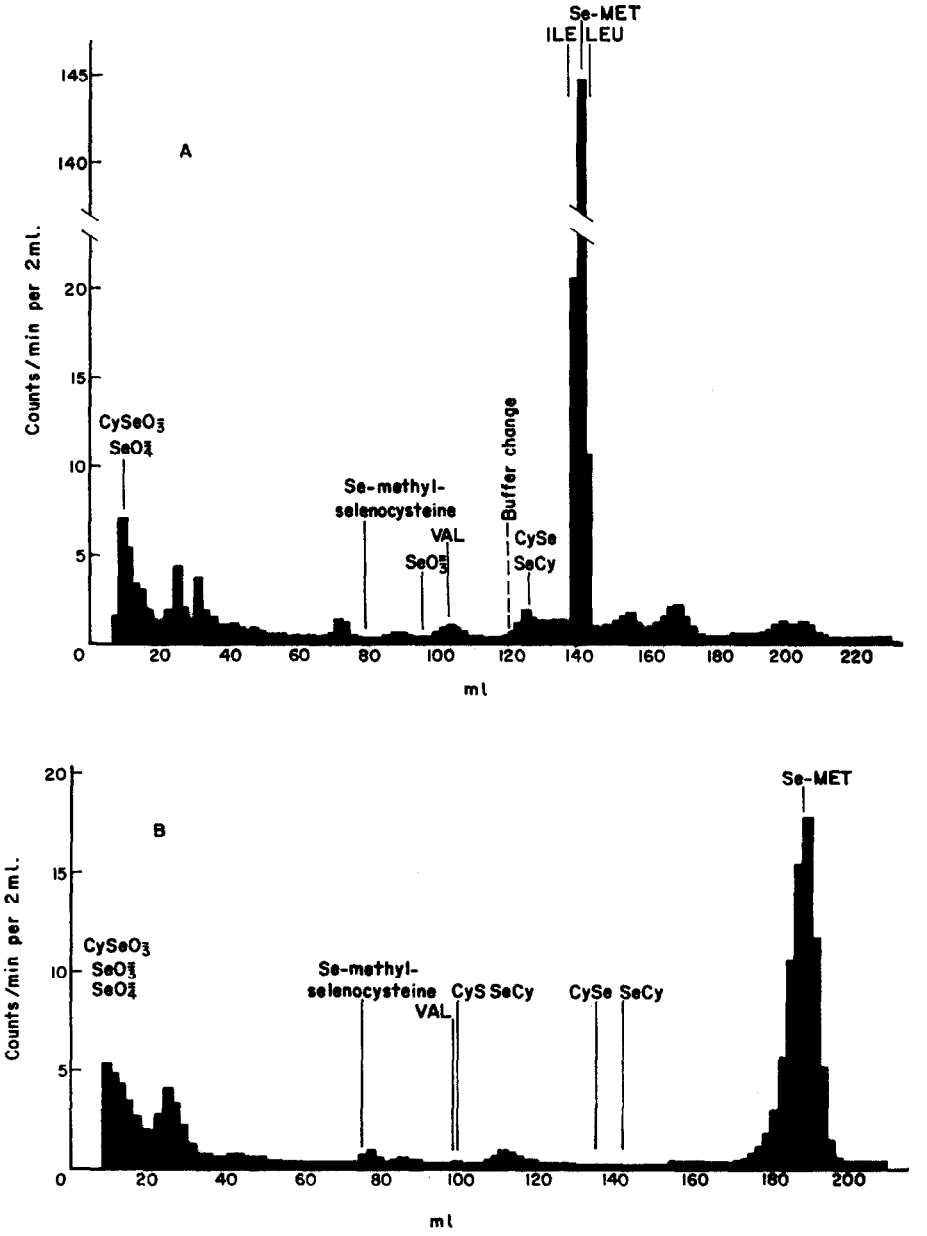

FIG. 2. CHROMATOGRAMS OF ^{75}Se IN PRONASE HYDROLYSATES OF WHEAT GLUTEN.
A. pH 3·30 and pH 4·25 buffers with thiodiglycol used for elution.
B. pH 3·30 buffer without thiodiglycol used for elution.

rechromatography, as explained in the Experimental. Chromatograms of the pronase hydrolysate and of the protein-free, hot-water extract of the wheat seeds were very similar to those shown in Figs. 2 and 3, respectively.

The high proportion of ^{75}Se of the wheat gluten and seeds appearing under the selenomethionine is immediately obvious. The results (Table 1) indicate that about half of the

selenium in gluten or in the seed is in the form of selenomethionine. The presence of selenomethionine in the gluten hydrolysate was confirmed by paper chromatography. The hydrolysate was streaked on filter paper and, with authentic selenomethionine as marker, was chromatographed in *n*-butanol–acetic acid–water (4:1:1, v/v/v). The selenomethionine area was located by treating the edges of the chromatogram with ninhydrin. This area of the chromatogram was then cut out and eluted, concentrated and co-chromatographed with authentic selenomethionine two-dimensionally in phenol–water (73:27, w/w) followed by *n*-butanol–acetic acid–water. The chromatogram was placed next to X-ray film for 1 month to locate the radioactivity and then sprayed with ninhydrin to locate the selenomethionine.

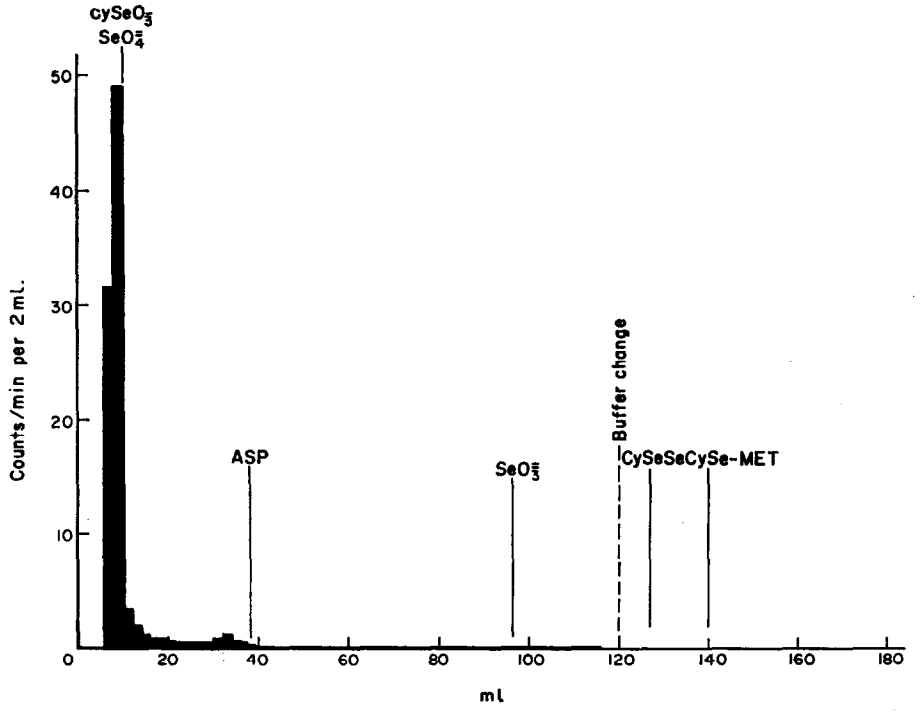

FIG. 3. CHROMATOGRAM OF ^{75}Se IN PROTEIN-FREE, HOT-WATER EXTRACT OF WHEAT STRAW, USING pH 3·30 AND pH 4·25 BUFFERS WITH THIODIGLYCOL.

Except for two very light concentrations occurring at the locations of the methionine sulfoxides, the radioactivity coincided with the selenomethionine.

The absence of selenomethionine in the protein-free, hot-water extracts of seed and straw suggests that, once formed, the amino acid is quickly incorporated into proteins and peptides. Analysis of these extracts showed the presence of the other free amino acids, but methionine was not detected (less than 0·001 μM). For example, alanine was found at levels of 0·030 and 0·077 μM and glutamic acid at levels of 0·044 and 0·090 μM in 4 ml of the seed and straw extracts, respectively. Apparently, methionine itself is present at very low levels in the soluble amino acid pool.

A significant peak at about 10 ml on all chromatograms suggested the presence of anionic or neutral selenium compounds. On chromatographing an aliquot of the protein-free, hot-water extract of straw on Dowex-1 anion exchange resin, essentially as described by Shrift

and Virupaksha,[13] 65 per cent of the selenium in the extract could be accounted for in the fraction where selenate would elute. This represented 54 per cent of the selenium in the straw. No selenate was found in the pronase hydrolysates of wheat gluten. About 3, 5 and 7 per cent of the selenium in the wheat straw extract, wheat gluten hydrolysate and wheat seed hydrolysate, respectively, was eluted from the Dowex-1 column in the fractions where both selenite and selenocysteic acid would be eluted. However, the data in Fig. 2A and 3 indicate that selenite is absent. It appears, therefore, that selenate and/or selenocysteic acid are present in the wheat.

Other peaks in the first 50 ml of eluate may represent oxides of selenomethionine. The possibility that neutral selenium compounds are present cannot be overlooked. The compounds in this portion of the chromatogram deserve further study. While some ^{75}Se appears under or near where Se-methylselenocysteine would be eluted in the chromatograms of the hydrolysates, this is not the case with the protein-free water extracts and the data do not justify concluding that this compound is present in wheat.

Where the two-buffer system was used, good recovery of ^{75}Se from the columns in the cases of the pronase digests and the protein-free, hot-water extract of straw suggests that a rather low concentration of peptides containing selenium was present in these preparations. In the case of the protein-free, hot-water extract of the seed, however, a fairly large proportion of the selenium was apparently associated with peptides. No convincing evidence was found for the presence of selenocystine in the hydrolysates or in the extracts. A small peak of ^{75}Se activity under selenocystine in Fig. 2A suggests its presence. However, on using the one-buffer system without thiodiglycol, the ^{75}Se under selenocystine (Fig. 2B) has disappeared.

It would be surprising to find selenocystine in biological material of relatively low selenium content and comparatively high sulfur content. In the presence of an excess of sulfur compounds, seleno-half-cystine should occur almost entirely as a sulfoseleno compound. The ^{75}Se peak under valine in the chromatogram of the pronase digest, using the two-buffer system with thiodiglycol (Fig. 2A), suggests the presence of sulfoselenocystine, but this peak was absent from the one-buffer system chromatogram (Fig. 2B). It is possible, of course, that seleno-half-cystine combines with other thiols. Treatment of the pronase hydrolysate of the seed with cysteine in excess by a method similar to that used in preparing sulfoselenocystine gave, however, a very small increase in the radioactivity under CySSeCy (less than 1 per cent of the selenium in the hydrolysate), and it cannot be concluded that such compounds are indeed present.

Huber and Criddle[14] have concluded from their studies on the stability of selenocystine that reports of the recovery of this compound from acid hydrolysates of biological material should be discounted. This study presents no firm evidence for its presence in wheat under conditions of isolation and analysis that should not cause its destruction. It appears that if selenocystine or the seleno-half-cystine moiety does exist in wheat, it is present in only very small amounts as compared to selenomethionine or other selenium compounds.

EXPERIMENTAL

Selenium Compounds

DL-Selenomethionine, DL-Se-methylselenocysteine and DL-selenocystine were purchased from Cyclo Chemical Corp., Los Angeles. ^{75}Se-selenious acid was obtained from ICN-Nuclear Science Division, Pittsburgh. ^{75}Se-selenic acid was prepared from the ^{75}Se-selenious acid by the method of Gilbertson and King.[15]

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[14] R. E. HUBER and R. S. CRIDDLE, *Arch. Biochem. Biophys.* **122**, 164 (1967).
[15] L. J. GILBERTSON and G. B. KING, *J. Am. Chem. Soc.* **58**, 180 (1936).

Potassium selenate used in growing the plants was prepared as described by Trelease and Beath.[16]

A solution containing a mixture of selenocystine, cystine and presumably sulfoselenocystine was prepared by mixing equimolar parts of L-cysteine and DL-selenocystine at pH 2·2. The mixture was allowed to stand at room temp. for 10 min and the pH was adjusted to 7·2 with NaOH. After 1 hr standing with occasional shaking, the pH was adjusted to 2·2. On chromatographing on the amino acid analyzer, this mixture gave peaks at the location of cystine, selenocystine and about midway between the two. The middle peak was probably the sulfoselenocystine (2,7-diamino-4-thia-5-selenaoctanedioic acid of Walter *et al.*).[8]

Selenocysteic acid was prepared by the method of Gortner and Hoffman[17] used to prepare cysteic acid from cystine. An excess of Br_2 water was added to solid selenocystine, the mixture was allowed to stand at room temp. for 5 min and then the excess Br_2 was removed *in vacuo*. Diluting buffer[18] (pH 2·2) containing no thiodiglycol was added to this preparation and an aliquot was subjected to amino acid analysis. Selenocystine was not found in the solution, but a peak did appear at the location where cysteic acid would appear on the chromatogram. The reaction product was presumed to be selenocysteic acid.

Chromatographic Methods

The various solutions were chromatographed on a Beckman Model 120 amino acid analyzer modified for accelerated analysis. A 50 × 0·9 cm column was packed with Aminex A-4 cation exchange resin (Bio-Rad Laboratories, Richmond, California). The buffer flow rate was set at 60 ml/hr, and the temperature of the jacket was maintained at 50° throughout each chromatogram. For elution, pH 3·30 and pH 4·25 buffers were used. These and the ninhydrin were made as described by Moore *et al.*[18] The buffer change was programmed at 90 min. Thiodiglycol was omitted from the pH 3·30 buffer for some chromatograms and in these cases the pH 4·25 buffer was not used.

The various selenoamino acids were located on the chromatogram by first chromatographing a Beckman standard amino acid solution and then chromatographing each selenoamino acid alone, using alanine, isoleucine or leucine as reference compounds. Selenite and selenate were located by using the radioactive form and counting 2·0-ml portions of the effluent collected in the fraction collector.

The procedure used in examining the various hydrolysates and extracts was as follows. The effluent from the column was fed directly to a fraction collector programmed at 2·0 min. An appropriate volume of the solution to be examined was placed on the column and elution and fraction collection were begun simultaneously. The recorder was used to record time and for making notations during the determination. The original solution and the fractions collected were then assayed for selenium by counting or by chemical analysis. The buffer change was located by pH determinations on appropriate tubes.

The time lag in colorimetric detection and recording of amino acids chromatographed in the usual manner and the appearance of these amino acids in the fractions collected directly from the chromatographic column was found to be 7 min. However, in order to ensure correlation of each chromatogram obtained with the standard chromatogram, one of the following methods was used. For the fractions from the naturally seleniferous wheat, where chemical analysis was used to locate the selenium, it was necessary to analyze essentially all of the solution in each tube. Therefore, about 10 μl from each tube was spotted on filter paper, allowed to dry and then sprayed with ninhydrin to locate tubes containing amino acids. In addition, a chromatogram of the hydrolysate was made using ninhydrin and the recorder. It was possible with these two pieces of information to locate the position of a number of the amino acid peaks in the tubes within about 2 ml. A different system was used for chromatograms of the radioactive wheat. The positions of amino acid peaks could be verified within about 1 ml by rechromatography of two or three tubes at the presumed location of a selected amino acid. For this, the fractions were adjusted to pH 2·2 before being placed on the analyzer column. It was found that the variation in the location of the amino acids from chromatogram to chromatogram was very small.

Selenium Analysis

Selenium was determined chemically by the method of Watkinson[19] with some modifications.[20] ^{75}Se counting was done with a Packard Auto-Gamma Spectrometer.

Plant Materials

Naturally seleniferous wheat (*Triticum aestivum* L.), seed of unknown variety, was obtained from seleniferous land in Lyman County, South Dakota. It was ground in a Wiley mill through a 40-mesh screen. On analysis, it contained 31 ppm Se.

Wheat containing ^{75}Se was raised as follows. Glazed 3 gal jars were filled with 15 cm of a 1:2 mixture of sand and sandy loam soil on top of 5 cm of sand and sterilized by autoclaving. Ten seeds of Red River 68

[16] S. F. Trelease and O. A. Beath, *Selenium*, p. 266, Champlin Printers, Burlington, Vt. (1949).
[17] R. A. Gortner and W. F. Hoffman, *J. Biol. Chem.* **72**, 435 (1927).
[18] S. Moore, C. H. Spackman and W. H. Stein, *Anal. Chem.* **30**, 1185 (1958).
[19] J. H. Watkinson, *Anal. Chem.* **38**, 92 (1966).
[20] O. E. Olson, *J. Assoc. Offic. Anal. Chemists* **52**, 627 (1969).

wheat seed were distributed on the surface in each jar. The seeds were covered with 2·5 cm of sand. The jars were placed in a plant-growing chamber at 20° and illuminated 16 hr per day at 4·6 mw/cm². They were watered daily with deionized water. N, P and K additions were made at about 4-week intervals. At 34 days, when the plants were in the boot stage, 100 ml of a solution containing 0·1 mg Se as K_2SeO_4 and 0·25 mc ^{75}Se as selenic acid was added to each jar. This was repeated at 37, 41 and 44 days. At 90 days, when the plants were well matured, the heads were removed and allowed to air-dry overnight. The seeds were separated and the spikes, exclusive of the seeds, were weighed and then ground through a 40-mesh screen in the Wiley mill. The seeds were weighed and a portion was ground in the Wiley mill. The stems and leaves were harvested together, allowed to air-dry overnight, weighed and ground. The roots and crowns were discarded. Yield and selenium data were as follows:

Fraction	Weight (g)	Kjeldahl N (%)	Selenium content (cpm/mg)	Selenium content (ppm)	Total selenium in fraction (cpm × 10^{-7})	Total selenium in fraction (mg)
Stems and leaves	262·5	0·50	4416	3·3	115·9	0·87
Spikes minus seeds	177·3	0·60	2934	2·7	52·0	0·48
Seeds	412·5	2·23	5414	3·8	223·3	1·57

Preparation of Wheat Gluten

A crude gluten fraction was prepared from the seeds essentially as described by Franke and Moxon.[21] This consisted of kneading the wheat after a short pre-soaking period in copious amounts of tap water. The process removed most of the bran and starch and should have removed essentially all water-soluble selenium compounds. The gluten was dried by lyophilizing and then finely ground with a mortar and pestle. The gluten from the naturally seleniferous wheat contained 131 ppm Se. That prepared from the ^{75}Se-wheat contained 34,127 cpm/mg.

Enzyme Hydrolysis

A 0·5 g sample of finely ground wheat gluten was added to 20 ml of 0·1 M phosphate buffer, pH 7·2, at 37°. After adding 10 mg pronase (Calbiochem, Los Angeles) and mixing well, the mixture was covered with a layer of toluene and incubated with stirring in a 37° water bath. At 24 hr, another 10 mg of pronase was added. A layer of toluene was maintained on the surface, and the pH was monitored with Hydrion paper (it did not fall below 7·0). At 72 hr, the pH was adjusted to 2·2 with HCl, the volume was adjusted to 25 ml with pH 2·2 diluting buffer[18] containing no thiodiglycol and an aliquot was centrifuged at 4500 *g* for 30 min. The clear supernatant liquid was removed and stored at −10°.

Finely ground wheat grain was also subjected to pronase hydrolysis, following 16 hr extraction with diethyl ether.

Protein-Free, Hot-Water Extracts

Protein-free, hot-water extracts of the seed and straw were prepared as follows. A 1·0 g sample was continuously extracted with diethyl ether for 16 hr. The residue was air-dried and then added to 30 ml of water at 80° and maintained at that temperature with stirring for 30 min. After cooling, the volume was brought to 45 ml with 20% sulfosalicyclic acid,[22] and the solution was centrifuged at 6000 *g* for 30 min. A 20 ml aliquot of the clear liquid was adjusted to pH 2·2 with NaOH and made to 22·5 ml with water. The extraction of ^{75}Se is summarized as follows:

	Seed	Straw
% of ^{75}Se in ether extract	0·051	0·033
% of ^{75}Se in sulfosalicylic acid clarified extract	19·5	83·5

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[22] W. D. BLOCK, M. E. MARKOUS and B. F. STEELE, *Proc. Soc. Exp. Biol. Med.* **122**, 1089 (1966).