1952

Experiments with Selenium 79 and Selenium 75 Preparatory to Studies of the Metabolic Pathways of Selenium in Plants Using Radioisotopes as Tracers

Ronald R. Johnson

EXPERIMENTS WITH SELENIUM 79 AND SELENIUM 75 PREPARATORY TO STUDIES OF THE METABOLIC PATHWAYS OF SELENIUM IN PLANTS USING RADIOISOTOPES AS TRACERS.

by

Ronald R. Johnson

Submitted to the Graduate Faculty

of

South Dakota State College of Agricultural and Mechanic Arts

in Partial Fulfillment of the Requirement for

the Degree of Master of Science

June, 1952

EXPERIMENTS WITH SELENIUM 79 AND SELENIUM 75 PREPARATORY TO STUDIES OF THE METABOLIC PATHWAYS OF SELENIUM IN PLANTS, USING RADIOISOTOPES

By

Ronald R. Johnson

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.

ACKNOWLEDGMENT

I wish to thank Dr. Alvin L. Moxon, Mr. Eugene I. Whitehead, Miss Frances Meyer, and Mr. Robert Pengra for the valuable suggestions and assistance they have given me during the course of this work.

The Author

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

Since 1935, selenium has been recognized as the toxicant naturally present in certain foodstuffs which cause the malady of livestock known as "alkali disease" (2,3,8). Although selenium is present in very small amounts in plants and in even smaller amounts in soils, feeding of seleniferous forage caused serious losses in livestock in many areas of the great plains.

Since its discovery as a toxicant, much work has been done in attempts to determine the forms in which selenium occurs in soils, plants, and animals. Early work by Franke (4) showed that the toxicant was almost wholly present in the protein fraction of wheat and corn. This was later confirmed by further experiments (5,6,23,24). Due to the similarity of selenium and sulfur, both in physical and chemical properties, investigators (23) suggested that it might replace sulfur in sulfur bearing compounds, particularly, the amino acids. Hurd-Karrer et al. (11,14), and Martin (17) have shown that if sufficient sulfur was present in either a culture solution or a soil, selenium (as selenate) injury to wheat plants was greatly inhibited. Franke and Painter (7) could not confirm part of these conclusions but later (25) agreed that selenium followed sulfur deposition more closely than nitrogen deposition.

Although plants may contain both inorganic and organic forms of selenium, most cereals and also plants of the Astragalus genus contain almost all organically bound selenium (31). Horn and Jones (10) attempted to isolate the compound or compounds which bear the selenium in toxic wheat gluten. They succeeded in showing that most of the selenium was

in the leucine fraction which would contain in addition valine and phenylalanine. However, due to the minute quantities of selenium present in wheat, they were unable to continue this isolation. They conducted a similar experiment on water extracts of Astragalus pectinatus which contained 1500 to 2000 parts per million of selenium. In this experiment they succeeded in isolating a complex which they believed to be a combination of two isomorphic amino acids, $HOOC-CH(NH_2)-CH_2-CH_2-Se-CH_2-CH(NH_2)-COOH$ and $HOOC-CH(NH_2)-CH_2-CH_2-S-CH_2-CH(NH_2)-COOH$ in a ratio of 2:1. Their isolation methods closely resembled those used for isolation of lanthionine, $HOOC-CH(NH_2)-CH_2-S-CH_2-CH(NH_2)-COOH$, the similarity in structure thus further confirming their belief. Also, the S-Se complex had properties resembling those of the leucine fraction of wheat previously mentioned, thus indicating a further relationship. Other investigators (31) have found that selenium tended to be associated with cystine although selenium was absent from the cystine fraction of wheat (10). From the information presented, it seemed that more work should be done with the isolation of the selenium bearing compounds in wheat, since this is the cereal grown predominately in seleniferous areas and also since this cereal accumulates selenium at a higher concentration than the other cereals.

Data on the concentration of selenium in wheat plants as related to the form of selenium and its concentration in the substrate can be readily found in the literature (11,17,30); however, data for selenium uptake as related to growth stage of the wheat plant, from seedling to maturity were not reported. Preliminary to the use of radioselenium as a tracer

in studies of selenium metabolism by wheat plants it seemed desirable to obtain this data for greenhouse grown wheat plants, and also to determine how many replications of a given soil selenium concentration are desirable in order to obtain significant data. Likewise, the soil selenium concentration yielding plants of high selenium content with minimum inhibition of growth should be established.

With this accomplished, a series of plants could be grown using radioactive selenium as a tracer which would enable one to detect small amounts of selenium in the individual amino acid fractions, after they had been fractionated. Results of such an experiment would be expected to supply a clue as to the compound or compounds in which selenium occurs in the protein and water-soluble fractions.

EXPERIMENT I

In the first experiment, five 2-gallon glazed crocks (with drainage provided) were used for each of the five soil mixtures (a coarse sandy loam) having the following selenium (as selenite) concentrations: 2 p.p.m., 4 p.p.m., 8 p.p.m., 16 p.p.m., and 32 p.p.m. Approximately 100 seeds of Rushmore wheat (treated with Semesan for root rot) were planted in each crock on October 31, 1950, and were given an initial watering of 500 ml. of distilled water. Subsequent minimal additions of distilled water were made throughout the growth and ripening period to replace transpiration and soil evaporation losses. This amounted to approximately 175 ml. of water daily. Because the plants were being grown under very crowded conditions, nutrient deficiencies occasionally appeared, at which time 200 ml.

of a modified Trelease nutrient culture solution (30) were added to each crock. In growing the plants, auxiliary lighting was employed with increasing length of photoperiod as growth progressed. The positions of the crocks were changed daily to assure equal light and temperature conditions for all crocks. Harvests of 20 to 25 plants (tops only) from each crock were taken on four dates (21 days, 44 days, 67 days, and 116 days after planting), the final sampling being made with the plants in the seed ripe stage. When harvested, the plants were immediately air-dried at 65°C. in a forced draft air-oven for 2 to 4 hours. By this method, loss of selenium by volatilization was held to a minimum (31). The air-dried tops were weighed and, with the exception of the fourth harvest, then ground in a semi-micro Wiley mill. The heads from the fourth harvest were removed from the tops and separated into kernels and hulls before grinding. Selenium content of these tissues was determined by the method outlined by Klein (15).

METHOD: Duplicate one gram samples of the air dried tops were placed in a 600 ml. beaker with 10 ml. of a mercuric nitrate fixative solution (5 g. of red HgO in 100 ml. of nitric acid) which prevents volatilization of selenium at high temperatures. To this, 150 ml. of digestion mixture (2:1 HNO_3:H_2SO_4) were added and the samples were digested on a hot plate. If charring occurred when the HNO_3 had been driven off, indicating incomplete digestion, 10 ml. additions of HNO_3 were made until the samples were completely digested. A small amount of $(NH_4)_2C_2O_4$ was added to decompose the excess HNO_3. After the digests had cooled, they were washed into a distillation flask with 50 ml. of H_2O and 50 ml. of concentrated HBr-Br_2 (10 ml. Br_2 in 990 ml. HBr). The contents were distilled into 200 ml. Erlenmeyer flasks until 75 ml. or more had distilled over, the selenium coming over as $SeBr_4$. The selenium was reduced to elemental form by saturation of the solution with SO_2 followed by an addition of hydroxylamine hydrochloride (the SO_2 reduces selenite to elemental selenium while the hydroxylamine reduces selenate). To complete the precipitation, the flasks were placed on the steam bath for 30 minutes. The precipitate was then collected on asbestos pads and washed several times with water by rinsing down the sides of the flask and pouring the rinse on the pad. Any SO_2

remaining in the flasks was removed by playing a stream of air into the flasks. The precipitate was then dissolved and washed through the pad into titration tubes by the addition of four 1 ml. washes of dilute HBr-Br_2 (10 ml. saturated Br_2 water and 5 ml. HBr made up to 100 ml.). The washes were first used to rinse the flasks in which the selenium was precipitated, since some selenium may cling to the glass surfaces. Three 2 ml. rinses of H_2O were used to insure a complete transfer. Six drops of 5 per cent phenol were added and the tubes placed in a boiling water bath for 5 minutes to react with any excess Br_2. After cooling, the selenium, which was then present as H_2SeO_3, was titrated with 0.001 N $Na_2S_2O_3$ (using microburettes) and back titrated with 0.001 N I_2 using 0.5 per cent starch as an indicator. Since the titration tubes were of a Nessler like construction, it was possible to look down the long axis of the body of the solution and detect small color changes, which provided an accurate method for the micro quantities used. One ml. of 0.001 N $Na_2S_2O_3$ was equivalent to 19.8 micrograms of selenium. Selenium was reported as parts of selenium per million parts of tissue or micrograms of selenium per gram of air-dried tissue.

DISCUSSION: Figures 1, 2, 3 and 4 are photographs, taken on each of the harvest dates, showing representative crocks of the five soil selenium concentrations. These photographs show very plainly the decreased growth of the wheat plants at the more toxic levels of selenium. Little difference can be noticed between the 2 p.p.m and 4 p.p.m. levels, a point which is briefly discussed later in this paper. However, the plants grown on selenium levels greater than 4 p.p.m. show a sharply decreasing growth rate with increasing selenium concentrations. Also, the plants grown at these more toxic levels began to show a snow-white chlorosis of the leaves which is a typical symptom of selenium injury (31). Plants of the 32 p.p.m. level showed very little growth between the 3rd and 4th harvests and no kernels were formed in the minute heads present.

Table 1 gives the mean dry weights per plant for each level of selenium in the soil. It can be seen that the weights for the plants grown on the 4 p.p.m. level of selenium soil do not differ greatly from those grown on the 2 p.p.m. level, thus confirming the statement made concerning

Figure 1. Wheat plants grown for 21 days on soils containing selenium (as selenite) at levels of 2 p.p.m., 4 p.p.m., 8 p.p.m., 16 p.p.m., and 32 p.p.m.

Figure 2. Wheat plants grown for 44 days on soils containing selenium (as selenite) at levels of 2 p.p.m., 4 p.p.m., 8 p.p.m., 16 p.p.m., and 32 p.p.m.

Figure 3. Wheat plants grown for 67 days on soils containing selenium (as selenite) at levels of 2 p.p.m., 4 p.p.m., 8 p.p.m., 16 p.p.m., and 32 p.p.m.

Figure 4. Wheat plants grown for 116 days on soils containing selenium (as selenite) at levels of 2 p.p.m., 4p.p.m., 8 p.p.m., 16 p.p.m., and 32 p.p.m.

Table I
Growth Data for Wheat Plants Grown in Soils with Varying Amounts of Selenium (as Selenite)

(Data expressed as milligrams dry-weight per plant)

Se Content of Soil		21 days	44 days	67 days	116 days
2 p.p.m.	Mean ± Dev	24.5 ± 1.3	65.6 ± 4.4	171.4 ± 13.6	277.7 ± 16.4
	(Range)	(26.3 - 23.3)	(71.8 - 61.5)	(189.0 - 152.5	(306.1 - 265.0)
4 p.p.m.	Mean ± Dev	23.2 ± 1.5	62.3 ± 3.6	149.3 ± 12.2	272.0 ± 45.9
	(Range)	(25.5 - 21.7)	(66.6 - 57.9)	(167.2 - 133.1)	(337.6 - 241.5)
8 p.p.m.	Mean ± Dev	23.3 ± 0.9	52.0 ± 5.4	135.3 ± 7.0	218.2 ± 15.9
	(Range)	(24.3 - 22.3)	(58.8 - 44.1)	(141.2 - 123.5)	(230.9 - 191.6)
16 p.p.m.	Mean ± Dev	18.6 ± 0.6	40.8 ± 2.6	87.6 ± 9.7	142.0 ± 26.1
	(Range)	(19.5 - 18.1)	(45.0 - 39.0)	(102.5 - 76.8)	(179.3 - 106.1)
32 p.p.m.	Mean ± Dev	8.7 ± 1.1	20.5 ± 3.7	49.7 ± 6.3	66.4 ± 5.2
	(Range)	(10.2 - 7.6)	(23.8 - 14.5)	(56.2 - 40.8)	(73.8 - 61.0)

these levels in the previous paragraph. Apparently plant injury by selenite selenium, as measured by plant dry-weights, is not significant until concentrations greater than 4 p.p.m. of selenium are present. Also, this evidence suggests that, had a control series been maintained, plants grown in selenium-free soil would not differ significantly in weight from the 2 p.p.m. and 4 p.p.m. plants; it is probable that the selenium-free plants might weigh slightly less, since Stanford and Olson (27) observed a slight growth stimulation by low concentrations of selenium. The suggestion that plant growth would decrease on levels of selenite selenium greater than 4 p.p.m. is apparent, when one observes that a marked decrease in plant weight is

shown for the 8 p.p.m. level. This trend is further accentuated throughout the 16 p.p.m. and 32 p.p.m. levels. In the 2 p.p.m. series, the plant weights nearly tripled between the 1st and 2nd harvests and between the 2nd and 3rd harvests, while in the other series, they approximately doubled in the same periods. However, since the plants were nearly full grown at the 3rd harvest (preflowering stage) and the kernels had but to fill and ripen, there was less percentage gain between the 3rd and 4th harvests. Upon analysis of the data in Table 1, it was found that 70 per cent of the weight values fell within the mean ± the standard deviations, which indicated a normal distribution. Since the standard deviation frequently exceeded 10 per cent of the mean value, it would seem that more than 5 replications of a given soil selenium concentration would be desirable for highly significant plant weight data.

The plant weights at the lower selenium levels were small even at maturity, when compared with similar data for greenhouse-grown wheat plants found in the literature. This perhaps can partially be accounted for by the fact that many plants were grown in each crock and because the winter season lighting is generally unfavorable for vigorous plant growth.

If one plots the dry plant weights against the days of growth, as is done in Figure 5, an even better picture of the growth can be obtained in the form of typical S-shaped growth curves for each level of selenium in the soil. This figure illustrates the proximity of the growth rates for the 2 p.p.m. and 4 p.p.m. selenium levels and also clearly shows how the rates drop with increasing concentrations of selenite selenium in the soil.

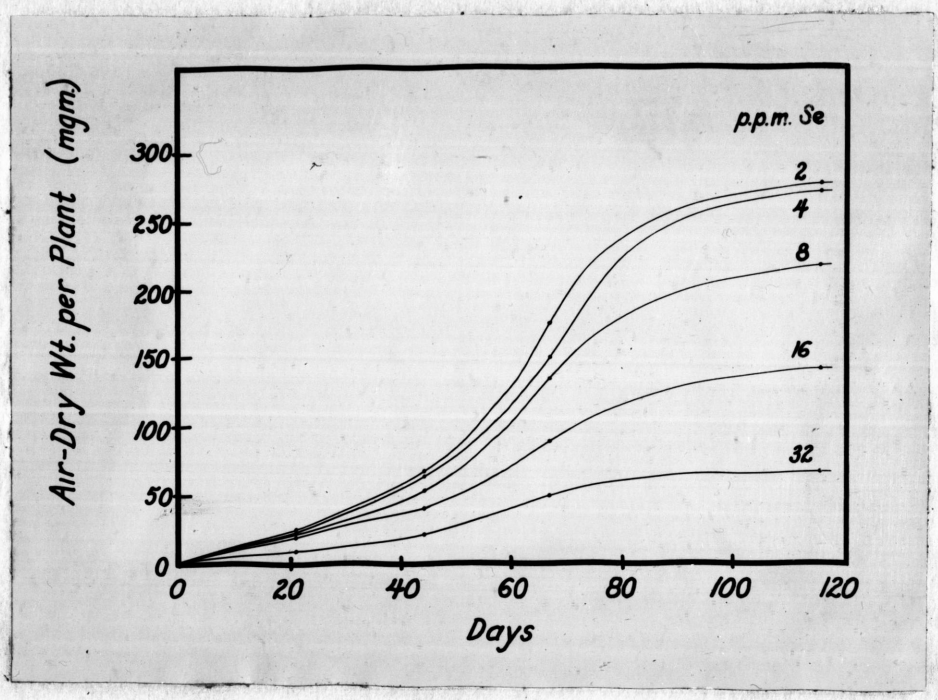

Figure 5. Growth curves for wheat grown on soils containing selenium (as selenite) at levels of 2 p.p.m., 4 p.p.m., 8 p.p.m., 16 p.p.m., and 32 p.p.m.

Table 2 presents the selenium contents (as parts per million of selenium per plant) for the tops of wheat plants at four successive harvests. It was noted that plant tissue obtained from the 2 p.p.m. selenium series showed a gradual decrease in selenium content during the growth period. This might indicate that, considering the number of plants raised in each crock of soil, 2 p.p.m. of selenium in the soil was not sufficient to allow selenium uptake by the plants to keep apace with plant growth rate. Also in this regard, one should consider the fact that selenite selenium is fixed to a marked degree by soil colloids (21) and certain iron compounds (22).

At the remaining four levels, it was evident that the amount of selenium (as p.p.m.) in the plant tissues remained relatively constant during the growth period and increased sharply at a time coincident with development

TABLE II
Selenium Content of Wheat Plants Grown in Soils with Varying Amounts of Selenium (as Selenite)

(Data for plants harvested at successive stages of maturity are given as p.p.m. of Se and as micrograms of Se per plant)

Se Content of Soil		21 days	44 days	67 days	116 days
2 p.p.m.	p.p.m.	13.5	10.1	9.4	8.03
	ug Se/plant	0.33	0.66	1.61	2.23
4 p.p.m	p.p.m.	14.1	13.8	14.8	15.5
	ug Se/plant	0.33	0.86	2.20	4.21
8 p.p.m.	p.p.m.	30.6	25.2	29.0	35.8
	ug Se/plant	0.71	1.31	3.91	7.82
16 p.p.m.	p.p.m.	55.1	56.7	51.5	82.2
	ug Se/plant	1.02	2.31	4.51	11.66
32 p.p.m.	p.p.m.	82.8	98.1	99.3	113.7
	ug Se/plant	0.71	2.02	4.94	7.55

of the head and kernels. The percentage of increase paralleled the increase in soil concentration of selenium except in the 32 p.p.m. series, where poor head and kernel formation could account for the smaller percentage increase. For all harvests, the total selenium content of the plants increased with increasing selenium content of the soil with the exception of the 32 p.p.m. series. Though plants grown in soil containing 32 p.p.m. of selenite selenium showed a higher tissue concentration of selenium as reflected in the p.p.m. data, the amount of selenium per plant fell below that recorded for the 16 p.p.m. series due to highly retarded plant growth in the former. This was especially notable in the last harvest.

The first and second plant harvest did not provide sufficient amounts of sample to permit separate selenium determinations of the plant tissues obtained from each crock; therefore, the samples gathered from each of the five crocks in a given series were combined for analyses. However, the third and fourth harvests did provide sufficient material to allow for detailed analysis. This data along with statistical considerations is presented in Table 3. The standard deviations for these data approximates five per cent of the mean values, exceeding ten per cent only in the selenium

TABLE III
Selenium Content of Wheat Plants Harvested after 67 Days and 116 Days of Growth in Soils with Varying Amounts of Selenium (as Selenite)

(Data extends that recorded in TABLE II for the third and fourth harvests)

Se Content of Soil	3rd Harvest (67 days) Se content in p.p.m.		4th Harvest (116 days) Stems		Kernels		Hulls	
			Se in p.p.m.	ug Se/stem	Se in p.p.m.	ug Se/head	Se in p.p.m.	ug Se/head
2 p.p.m.	Mean ± Dev	9.39 ± 0.32	8.31 ± 1.82	1.51	9.4	0.55	4.3	0.17
	(Range)	(9.78 - 8.92)	(11.39 - 6.73)					
4 p.p.m.	Mean ± Dev	14.85 ± 0.68	16.71 ± 1.28	3.05	15.9	0.90	7.5	0.26
	(Range)	(15.88 - 14.14)	(17.70 - 15.20)					
8 p.p.m.	Mean ± Dev	29.04 ± 0.69	40.6 ± 2.62	5.87	32.9	1.40	17.7	0.55
	(Range)	(29.50 - 27.95)	(43.0 - 36.7)					
16 p.p.m.	Mean ± Dev	51.51 ± 2.12	93.2 ± 3.57	9.44	68.5	1.40	41.4	0.82
	(Range)	(54.18 - 47.45)	(98.2 - 88.6)					
32 p.p.m.	Mean ± Dev	99.27 ± 5.38	118.6 ± 17.3	6.49	*	*	90.2	1.06
	(Range)	(103.64 - 91.68)	(137.0 - 100.8)					

* At the 32 p.p.m. Se level no filled kernels were set by the plants; only a few withered seeds were found amongst the chaffy hulls.

determinations on stems from the fourth harvest, 2 p.p.m. selenium series. In the data presented earlier on growth as measured by dry weights of plants, the deviations frequently exceeded ten per cent of the mean values. This would indicate that it is easier to obtain wheat plants of approximately uniform selenium content at each soil selenium level than it is to obtain plants of uniform weight. Therefore, fewer replications of a given soil selenium level would be necessary to obtain significant data with regard to selenium content than would be necessary for plant weight data. It is apparent that most of the sharp increase in selenium content in the fourth harvest can be accounted for by the stems, since they not only had a much higher selenium content but contained most of the selenium per plant also. It is also evident that the hulls contained considerably less selenium than the kernels. Since the kernels contain more protein than the hulls, this would be expected if the majority of the selenium was in the protein fraction, as was stated earlier.

EXPERIMENT II

In the second experiment, radioactive selenium 75 was employed. Certain volatile compounds are given off by plants which may or may not contain selenium (11,16,20). Though not harmful in their ordinary inactive form due to the minute quantities, they could be considered a health hazard when radioactive selenium is used in tracer experiments. To avoid this hazard, a glass chamber was constructed with a means provided for exhausting the air from the chamber to the outside. The chamber was five feet long, four feet wide, and four feet high. It was built with a wooden frame, double strength glass sides and ends, and a triple strength glass top. To one

upper corner of the chamber, a small exhaust blower was fastened and provided with an outlet pipe to the outside. The corners of the chamber rested on small slats so as to allow about a 1/4" slit all around the bottom edge where air could enter. When the chamber was filled with smoke, it was removed by the exhaust fan at a satisfactory rate. The floor of the chamber was painted with aluminum paint to reflect sunlight and prevent abnormally high temperature within the chamber. A photograph of the chamber is shown in Figure 6.

Figure 6. Glass chamber used for growing wheat plants in soils containing radioactive selenium.

Fifteen crocks were prepared as before using selenium-free soil. Approximately 100 seeds of Rushmore wheat (treated with Semesan to prevent root rot) were planted in each crock on April 6, 1951, and the crocks were placed in the chamber. Distilled water was applied daily to replace that moisture lost by transpiration from the plants and evaporation from the soil. Auxiliary lighting was used when needed. Again, when nutrient deficiencies were noted, a sulfate-free, modified Trelease nutrient culture solution (200 ml.) was added to each crock.

It was shown in the discussion of experiment I that at the 4 p.p.m. level of selenite selenium, there was not a large uptake of selenium by or injury to the plant. However, the level of 4 p.p.m. was chosen for use in this experiment for selenate selenium was to be used and it has been shown to be many times more toxic to wheat plants than selenite selenium (13). Hurd-Karrer (11,13) stated that an excess of sulfate in the soil somewhat inhibits the uptake and resulting toxicity of selenium. Although this has not been confirmed for South Dakota soils, it seemed advisable that the sulfur content of the soil be kept at a minimum for plant growth, first, by excluding it from the nutrient culture solution, and secondly, by adding a known amount of sulfate to provide approximately 20 p.p.m. of sulfur in the soil. Considering the analytical values[1] for the selenate compounds being used and having determined that there were about 7500 g. of air-dry soil in each crock, the amounts of selenate (labeled and non-labeled) and of sulfate required were calculated:

---

[1]Analysis of the technical grade Na_2SeO_4 on hand showed it to be 36.22 per cent selenium while analysis of the C.P. tracer $K_2Se^*O_4$ showed it to be 30.06 per cent selenium.

Na_2SeO_4 required per crock for 4 p.p.m. Se = 82.8 mg.

$K_2Se^*O_4$ required per crock for 4 p.p.m. Se = 100.0 mg.

K_2SO_4 required per crock for 20 p.p.m. S = 817.0 mg.

The design for the experiment was then set up as follows:

Compounds Added	Control (A)	Se as 100 °/o Se* (B)	50 °/o Se 50 °/o Se* (C)	75 °/o Se 25 °/o Se* (D)	100 °/o Se (E)
$K_2Se^*O_4$	none	100 mg.	50 mg.	25 mg.	none
Na_2SeO_4	none	none	41.4 mg.	62.1 mg.	82.8 mg.
K_2SO_4	817 mg.	817 mg.	817 mg.	817 mg.	817 mg.

(Three crocks were used in each series, that is A_1, A_2, A_3, B_1, etc.)

Three solutions were prepared:

Solution I: 1 gram of $K_2Se^*O_4$ in 100 ml. H_2O

Solution II: 0.828 gram of Na_2SeO_4 in 100 ml. H_2O

Solution III: 16.34 grams of K_2SO_4 in 4 liters of H_2O

The amounts of these solutions to be added to each crock in a series were calculated as follows:

	A	B	C	D	E
Solution I	----	10 ml.	5 ml.	2.5 ml.	----
Solution II	----	----	5 ml.	7.5 ml.	10 ml.
Solution III	200 ml.	200 ml.	200 ml.	200 ml.	200 ml.

All solutions for a single crock were mixed together and made up to 500 ml. before adding to the soil. This allowed for reasonably equal distribution throughout the soil. The solutions were added to the crocks on May 11, 1951,

and the first harvest was taken May 16, 1951, 40 days after planting. Succeeding harvests were taken on June 7, 1951 (63 days), and July 10, 1951 (95 days). Although nutrient culture solutions were added frequently a sulfur deficiency appeared about the time of the 2nd harvest. It was characterized by spotted yellow chlorosis of the leaves and stems. At that time, 200 ml. of the sulfate solution were added again to each crock, but since the plants were so near maturity, little, if any, alleviation of the deficiency was noted before the plants ripened. As in the previous experiment, 20 to 25 plants were cut on each harvest date and immediately air-dried at 65°C. in a forced draft air oven for 2 to 4 hours. The air dried tops were weighed and then ground in the semi-micro Wiley mill. The heads from the 2nd and 3rd harvests were removed from the stems and weighed--the kernels and hulls, being separated also, in the 3rd harvest. The selenium content of these samples was determined by the method outlined by Klein (15). Selenium 75 activity was measured by Geiger-counting of the solutions from the Klein determination. Aliquots of these solutions were placed in metal planchets and dried before counts were taken. In calculating the selenium content by this method, comparisons were made with the counting rate of selenium 75 standards prepared from the original solution applied to the soil.

DISCUSSION: In experiment I, it was shown that five or more replications of a given selenium level would be necessary if one desired to measure selenium toxicity by plant weight data, but three similar replications would be sufficient if selenium content of the plants was to be used. In experiment II, little emphasis was placed on plant weights, the primary objective being a measure of the selenium contents. Therefore, only three

replications were provided for a given series. No attempt was made to control growth conditions by providing equal temperature and lighting as was done in the first experiment. Plant weights were taken, however, and merit some discussion. This data is given in Table 4. There was a

TABLE 4

DRY PLANT WEIGHTS, EXPERIMENT II
DATA GIVEN AS MILLIGRAMS PER PLANT

Sample	1st Harvest 40 Days	2nd Harvest 63 Days		3rd Harvest 95 Days			
	Whole Plant	Whole Plant	Head	Whole Plant	Head	Kernel	Hull
A	128.0	208.7	67.48	212.8	77.12	47.01	30.11
B	134.0	202.3	55.22	187.8	60.57	32.02	28.55
C	121.8	190.3	50.89	196.5	63.94	35.23	28.71
D	136.7	202.4	53.09	192.5	59.34	31.48	27.86
E	127.8	204.4	60.25	196.3	69.56	38.98	30.58

significant increase in plant weights between the first and second harvests, since the plants were not headed at the time of the first harvest. There was no significant difference between the second and third harvests, however. Series A appears to have slightly higher plant weights than series B,C,D, and E, probably due to a slight retardation of growth in the latter four due to their selenium concentration. The selenium applied did not alter growth appreciably because the plants were near maximum stem growth at the time of its application; also, the roots were well

developed at this time and capable of supporting further plant growth. In general, there were no differences in plant weights so large that they could not be accounted for by variations in growing conditions within the glass cabinet and slight growth retardation due to selenium.

The data for selenium content as determined by the Klein method and by counting of the selenium 75 activity are presented in Table 5. Composite values for plants from the three crocks in a given series are given for all groups except the leaves and stems of the last harvest. For this group the data for plants from each crock of a given treatment are given and the averages are shown.

Although no selenium was added to the soil in series A, the first two harvests of this series contain minute amounts of selenium. A probable explanation of this is that the soil may have contained some selenium naturally, which would be sufficient to allow accumulation of these small amounts in the plants. Since no selenium was found in the third harvest of series A, that which had been present was apparently lost, possibly through volatilization during the ripening process.

The last four series of crocks had the same soil selenium concentration (4 p.p.m.) but contained varying amounts of labeled selenium. Previous work in Experiment I showed that selenium uptake was accelerated by head formation, and this fact is reflected again in the data in Table 5 for selenium content (Klein values) of the first and second harvests. The selenium contents of the leaves and stems increased between the second and third harvests, but the selenium contents of the heads did not change appreciably.

Klein Method of Selenium Analysis and by Counting Selenium 75 Activity

Harvested at:	40 days		63 days				95 days					
Plant Fraction:	Entire Plant		Leaves + Stems		Entire Heads		Leaves + Stems		Kernels		Heads (- kernels)	
Treatment[2]	Klein	Isotope	Klein	Isotope	Klein	Isotope	Klein	Isotope	Klein	Isotope	Klein	Isotope
A - 1							0	-				
2	3	0	2	-	2	-	0	-	0	-	0	-
3							0	-				
							Ave. 0					
B - 1							96	129				
2	52	57	70	72	110	111	114	132	111	131	116	120
3							120	129				
							Ave 110	130				
C - 1							98	58				
2	54	33	73	38	111	60	132	66	117	66	109	63
3							144	71				
		(x2 = 66)		(x2 = 76)		(x2 = 120)	Ave. 125	65		(x2 = 132)		(x2 = 126)
								(x2 = 130)				
D - 1							101	21				
2	51	16	70	-	113	29	104	45	115	31	113	32
3								33				
		(x4 = 64)				(x4 = 116)	Ave. 102			(x4 = 124)		(x4 = 128)
								(x4 = 132)				
E - 1							104	-				
2	60	-	82	-	108	-	104	-	86	-	110	-
3							82	-				
							Ave. 97					
Average for all selenium treated plants	54	62	74	74	110	116	108	131	107	129	112	125

[1]Separate values are recorded for leaf and stem tissue of plants from each crock of the series and harvested after 95 days of growth.

[2]Treatments: see description in text.

The selenium contents (Klein values) of the plants in the B,C, and D series compared well throughout a harvest. The range in the first harvest for these series was from 51 to 54 p.p.m., while in the second harvest, it was from 70 to 73 p.p.m. for the leaves and stems and 110 to 113 p.p.m. in the heads. The values for these series in the last harvest range from 102 to 125 p.p.m. in leaves and stems, 111 to 117 p.p.m. in kernels, and 109 to 116 p.p.m. in the hulls. The selenium contents of the plants in the E series vary somewhat from the values obtained for the other treated series, but the variations were probably within the limits of experimental error.

The accuracy of the determination of selenium by counting the selenium 75 activity was decreased by low count rates. Considering this fact, the values obtained by the counting method compare favorably with the Klein values. The data for the leaves and stems of the last harvest indicate that most of the deviations for either method of analysis were within the limits of experimental error. It appears, therefore, that either method would be useful for determination of selenium, especially if significantly high count rates were obtained.

EXPERIMENT III

In order to obtain a sample sufficient for amino acid isolation studies, a 9' x 9' plot on Reed's Ranch in Lyman County, South Dakota, was seeded with Rushmore wheat on April 17, 1951. The plot was divided into two sections: each section consisted of 6 rows of wheat plants, the rows being spaced 6" apart. This arrangement left a 2' aisle between the planted sections. The plot was covered with a screen enclosure to protect it against grasshoppers. Two photographs of the plot appear in Figure 7. On June 27, 1951, one half of the plot was treated with radioactive selenium using the following preparation: 1 g. $K_2Se^*O_4$, which contained 18.33 millicuries of activity per gram, and 3 g. $K_2Se^*O_4$, which contained 1.54 millicuries of activity per gram, were dissolved in water and diluted to 100 ml.[1] The other half of the plot was not treated and served as a control. A harvest was taken from each half of the plot on July 23, 1951, when the kernels were in the milk stage. The samples were air-dried at 65°C. in a forced draft air-oven for 2 to 4 hours. The heads were separated from the stems, and then threshed so enough of the shriveled green kernels were obtained for separate analysis. A second harvest was taken August 11, 1951, with the plants in the seed ripe stage. All the remaining plants were taken at this time and treated as before. The heads were threshed in a small mechanical thresher, and as a consequence, the hulls were lost from this harvest. The kernels of both harvests and

---

[1] A furrow about 2" in depth was made between the rows and just outside the first and sixth rows. Fifteen ml. of the solution were diluted to a gallon and applied to each of the furrows between the rows, and 7.5 ml. were diluted to a half gallon and applied to each of the two outside furrows. This was calculated to provide approximately 4 p.p.m. of selenium in the first few inches of soil.

Figure 7. Wheat plants grown on 9' x 9' plot on Reed's Ranch, Lyman County, South Dakota. Top photograph shows entire plot as viewed from a distance. Lower photograph shows a closeup view with the screen drawn back to show the plants.

the hulls of the first harvest were ground in a semi-micro Wiley mill, while the stems were ground in a large Wiley mill. Selenium content of the tissues was determined by the method of Klein and the selenium 75 activity determined in a manner similar to that described in experiment II.

DISCUSSION: No difference could be noted between the control and radioactive wheat plots at any time during growth. This was expected since the plants were in the milk stage when the selenium was applied. The ripened kernels from the radioactive samples were slightly more shriveled than the control kernels.

The total selenium content of the samples as determined by the Klein method and by counting selenium 75 activity (value for latter given in parentheses) is presented in Table 6.

Table 6. Selenium values by Klein method and Selenium 75 activity method (in parentheses) for treated and control Reed Ranch wheat.

Date	p.p.m. Selenium		
	Plants	Kernels	Hulls
July 23			
Control plot	11	11	9
Treated plot	93 (100)	105 (100)	92 (88)
August 11			
Control plot	7	15	-
Treated plot	97 (150)	105 (124)	-

It is apparent that the control plants contain significant amounts of selenium, up to 15 p.p.m. in the final kernels. This was expected since the Reed Ranch soil is naturally seleniferous. The selenium contents of the radioactive samples were comparable to those obtained in greenhouse wheat plants grown in soil containing approximately the same concentration of selenium. Although radioselenium was not applied until the heads were

in the milk stage, a considerable absorption of nutrients from the soil into the plants occurred after this stage, as was evidenced by selenium uptake.

EXPERIMENT IV

In a study of selenium and sulfur metabolism in plants, it was desirable to separate and measure the various amino acids present, with special emphasis being placed on the sulfur containing amino acids. Due to the minute amounts of these constituents present in both the leaf proteins and in water extracts of plant tissue, the separation of the amino acids was dependent upon chromatographic techniques. In these experiments, an adaptation of starch column partition chromatography, as developed by Stein and Moore (18,19,28,29), was used.

METHOD: A water extract of the finely ground, air-dried plant tissue was prepared as suggested by Pucher and Vickery (26). Two or four ml. (depending on the concentration of amino acids) of this extract were placed in a small beaker and evaporated on a steam bath. After cooling, 0.2 ml. of H_2O were added to the dried extract, mixing thoroughly with a glass rod to effect rapid solution of the residue. 1.8 ml. of 3:1 (n-propanol: 0.5 N HCl) solution were added to the dissolved residue and a fine suspension of the resulting mixture was produced by drawing it into a 3 ml. hypodermic syringe (equipped with a fine needle) and forcing it back into the beaker in a fine jet, repeating the process five or six times. This process results in some volatilization of the solvent, amounting to 12 ± 2 per cent, and data corrections were made for this loss. 0.5 ml. of this prepared suspension was placed on starch columns (0.9 cm. in diameter and 30 cm. in length) and the chromatograms were developed first with 1:2:1 (n-propanol:0.5 N HCl) solvent. The colors formed by the reaction between the amino acids and ninhydrin were read on a Coleman spectrophotometer, the blue colors being read at 570 mu, and the yellow to orange colors (proline) being read at 440 mu.

All amino acids except proline were calculated in terms of leucine equivalents. Proline was calculated as such. The count data in Figure 10 was obtained by duplicate determinations, using one set of effluents for

amino acid color development, and the other for counting after drying the second set of effluents in planchets. This procedure was abandoned, since it was apparent that the placement of the amino acid peaks varied slightly between chromatograms and superimposing selenium 75 counts upon the previously determined amino acid data seemed questionable. With the exception of leaves and stems (A) in Figure 11, the count data was obtained by diluting the effluent fractions (after ninhydrin color development) to 20 ml., counting the activity in the test tube with a Geiger dip-counter. The activity in the leaves and stems (A) sample was counted after placing twice the usual concentration of sample on the column, taking half of the effluent in each tube for ninhydrin color development and reserving the other half for counting by the planchet method. All dip-counting values were converted to planchet counting values (dip-counting yielded 3.5 counts per ug. of Se in 20 ml. of solution, while planchet counting gave 52 counts per ug. Se.

DISCUSSION: Following the chromatographic separation of the amino acids in a given sample and subsequent colorimetric measurement of the color intensity formed by the reaction of the amino acid with ninhydrin, the log of the optical density was plotted against the milliliters of effluent, resulting in a curve resembling those shown in Figure 8. The upper curve was obtained for the straw of the 2 p.p.m. series, second harvest wheat plants of experiment I, and the lower curve represents the straw of the 8 p.p.m. series of the same experiment and harvest. Confirmatory tests on the amino acid content of the effluent peaks have not been made, but the plotted peaks represent positions where specific amino acids or com-

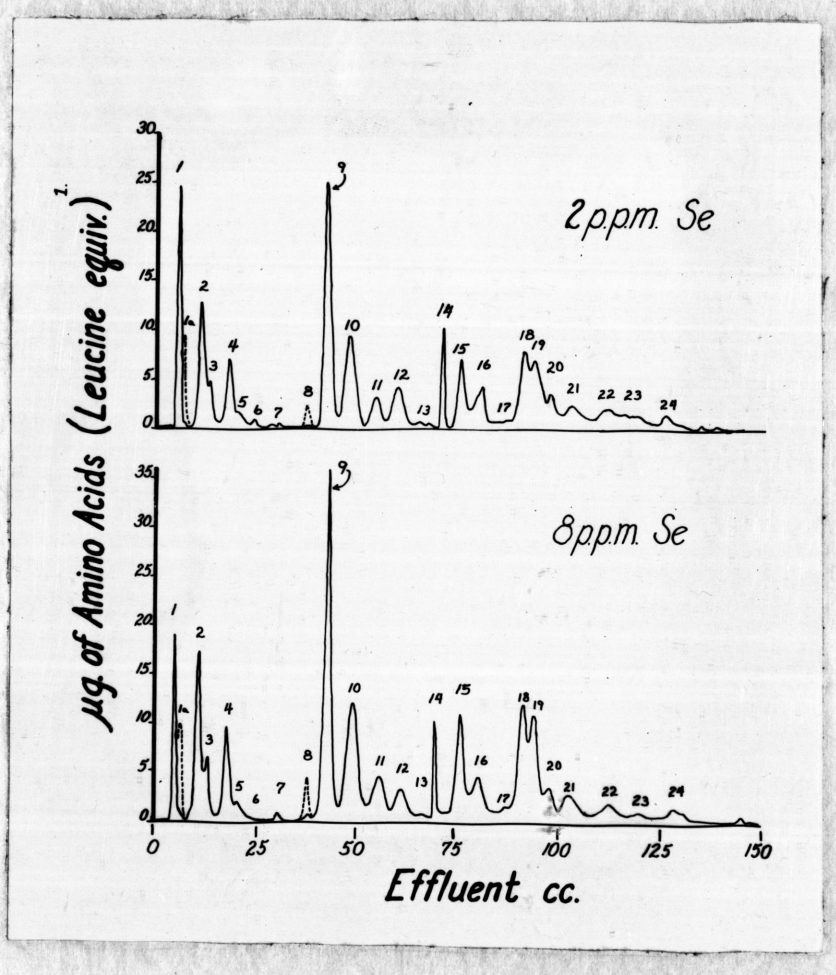

Figure 8. Graphs of amino acids in water extracts of second harvest wheat straw (Expt. I) grown on soils containing selenium (as selenite) at 2 p.p.m. and 8 p.p.m. levels. Amino acid data are given on basis of leucine equivalents per gram of air-dry sample.

The amino acid peaks are designated by the figures above each peak:

1. Unidentified
2. Leucine group
3. Phenylalanine and tryptophane
4. Valine
5. Methionine and tyrosine
6. Unidentified
7. Reduced glutathione ?
8. Proline
9. Glutamic acid and alanine
10. Unidentified
11. Threonine
12. Aspartic acid
13. β-alanine ?
14. Artifact peak due to solvent change
15. Serine and glutamine
16. Glycine
17. Unidentified
18. Ammonium
19. Asparagine
20. Glucosamine ?
21. Arginine ?
22. Lysine ?
23. Histidine ?
24. Cystine ?

bination of amino acids have been shown to emerge. The area under each peak is proportional to the amount of amino acid present. The first peaks emerging are thought to be caused by peptides, although they have not been definitely identified as yet. The first appeared as a sharp, blue peak, while the next peak (1a) was a less distinct peak giving yellow to orange colors. It is of interest to note that this peak, although present in the greenhouse grown wheat plants, did not appear in the field grown samples. In some plants, such as corn, another, but less distinct peak of yellow to orange colors appears; at no time did it appear in the wheat plants. Following these undetermined compounds, several amino acids appeared in rapid succession. The second peak combines leucine and isoleucine, while phenylalanine and tryptophane emerged together as the third peak to the side of the leucine peak. Valine appeared as a separate, distinct fourth peak with a smaller peak (No. 5 representing tyrosine and methionine) trailing from the side of the valine peak. Following the fifth peak and preceding the proline peak, an area of low amino acid content appeared, interrupted twice by small rises. The first (No. 6) is unidentified. The second rise (No. 7) was not large in either greenhouse plants or in field grown tissues but was of sufficient magnitude to be significant, since it might represent reduced glutathione. It has been determined in this laboratory (unpublished data) that reduced glutathione emerges in this position. Proline, which is shown by peak 8, formed yellow to orange colors with ninhydrin. Glutamic

acid and alanine appeared in a single, markedly high peak (No. 9) followed by a peak representing an unidentified compound (No. 10). Although the identity of the compound causing this tenth peak is not known, it is presumed to be an amino acid, possibly another form of glutamic acid, since glutamic acid is known to occur in more than one form in plants (1). The eleventh and twelfth peaks to emerge represent threonine and aspartic acid, respectively. Another unidentified compound emerged immediately after aspartic acid. Due to the similarity in the structures and properties of glutamic acid and aspartic acid, the latter might exist in more than one form and be responsible for this peak (No. 13). β-alanine also is a possibility. The unknown compound in peak 13 was present in larger quantities in field grown plants. A narrow artifact peak (No. 14) which resulted from the change of solvents, was found between peak 13 and the serine and glutamine peak (No. 15). Effluent peak 16, containing glycine, closely followed the serine-glutamine peak. In the short valley which followed glycine a small peak (No. 17) of unknown composition was found. Ammonium and asparagine appeared as peaks number 18 and 19, respectively. These compounds, due to their emergence proximity, did not always appear as two distinct peaks. Trailing from the asparagine peak, peak number 20 (possibly glucosamine) appeared. The last four peaks, numbers 21,22,23 and 24, are considered to represent arginine, lysine, histidine, and cystine, respectively. The latter part of chromatograms of water extracts have often been found to be ragged and erratic and difficult to characterize. Added to this source of error are the facts that many of these late emerging amino acids are not present in nicely measurable amounts in water

extracts of wheat plants and that several peptides are known to emerge throughout this region of the chromatogram. Therefore, the identity of the last five peaks will remain questionable until further proof of their identity has been obtained.

Similar curves are shown in Figure 9 which represents the amino acid data for the first and second harvests of the control (Reed's Ranch) field stems.

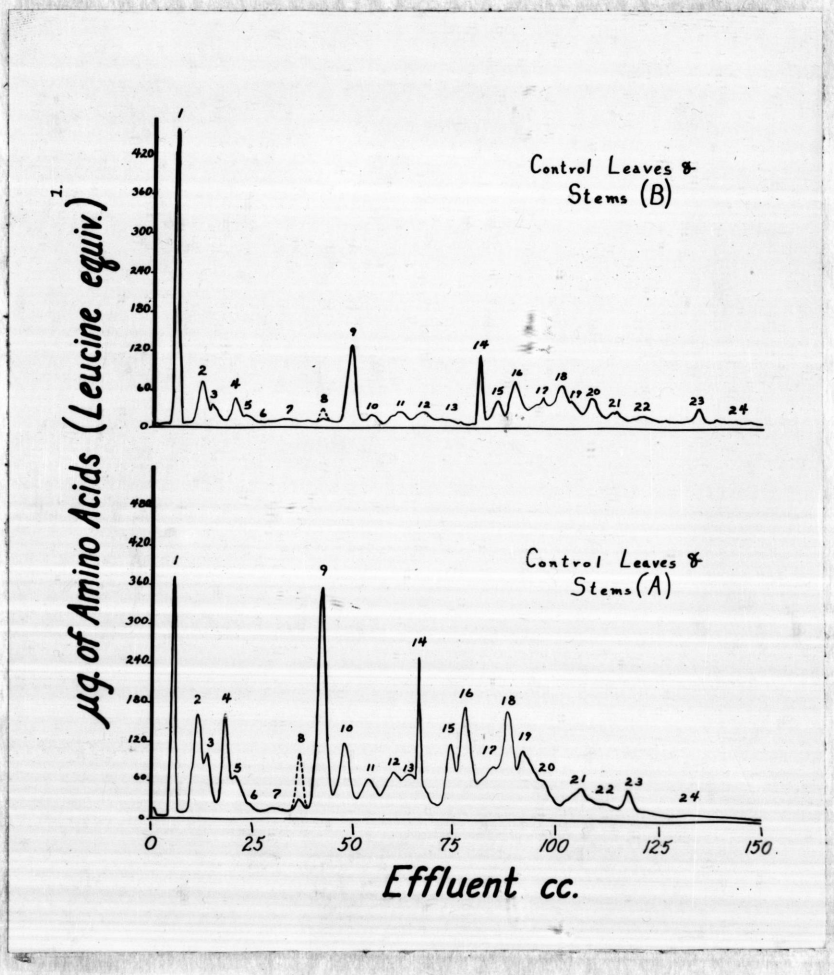

Figure 9. Graphs of amino acids in water extracts of the first (A) and second (B) harvests of control wheat leaves and stems grown at Reed's Ranch, Lyman County, South Dakota. [1]Amino acid data are given on the basis of leucine equivalents per gram of air-dry sample.
(For key to identify the amino acid peaks see Figure 8).

Table 7 presents data expressing the concentrations of the various amino acids in the water extracts of the five samples of wheat plants represented in Figures 8,9,10 and 11. The values for the two greenhouse

Table 7

SOLUBLE AMINO ACIDS PRESENT IN WHEAT PLANTS
Micrograms of Amino Acid Expressed as Leucine Equivalents* per Gram of Dry Plant Tissue

Peptide or Amino Acid	Peak No.	Wheat stems 2 p.p.m.	Wheat stems 8 p.p.m.	Field stems Control 1st Hvst.	Field stems Radioactive 1st Hvst	Field stems Control 2nd Hvst.	Ave. for greenhouse wheat (x)	Ave. for field grown wheat stems (y) 1st Hvst.	Ratio x:y (Approx.)
Leucine group	2	1720	2360	740	760	280	2040	750	3:1
Phenylalanine + Tryptophane	3	560	760	390	340	140	660	365	2:1
Valine	4	1000	1400	600	530	170	1220	565	2:1
Tyrosine + Methionine	5	480	400	440	390	70	460	415	1:1
Gsh?	7	-	--	110	130	120	-	-	-
Proline	8	240	480	1560	1330	400	360	1445	1:4
Glutamic Acid + Alanine	9	3960	5320	1640	1420	540	4640	1530	3:1
Unidentified "Amino Acid" I	10	2160	3240	730	700	90	2700	715	4:1
Thereonine	11	760	1240	460	380	160	1000	420	2:1
Aspartic Acid	12	1120	920	580	500	140	1020	540	2:1
Unidentified "Amino Acid" II	13	-	-	870	760	110	-	-	-
Artifact**	14	-	---	-	-	-	-	-	-
Serine + Glutamine	15	1240	2120	550	530	140	1680	540	3:1
Glycine	16	920	1120	1010	830	340	1020	920	1:1
Ammonium	18	1630	2200	1040	1190	360	1915	1115	2:1
Asparagine	19	1680	2290	640	680	160	1985	660	3:1
Glucosamine	20	920	760	580	660	210	840	620	4:3
Arginine	21	1080	1200	610	270	100	1140	440	3:1
Lysine	22	1480	1360	330	150	80	1420	240	6:1
Histidine	23	440	720	170	90	30	580	130	4:1

* Proline is expressed as proline equivalents.
**An artifact produced by exchanging 1:2:1 (propanol-butanol-0.1 N HCl) solvent for 2:1 (propanol-0.5 N HCl) solvent upon emergence of the aspartic peak.

grown samples were averaged and were compared with the averages of the values for two samples of field grown plants (1st harvest). It was noted that with respect to almost every amino acid, the greenhouse grown plants contained more than the field grown wheat stems. The ratios of the micrograms of amino acid per gram of greenhouse grown plant tissue to micrograms of amino acid per gram of field grown plant tissue ranged mostly

between 2:1 and 3:1. There were three significant exceptions to this generalization, however, i.e., with tyrosine + methionine and glycine where the ratios were about 1:1, and with proline where the ratio was about 1:4. It has been previously found that starving corn plants contain less proline and more peptide than normal plants. The greenhouse wheat plants, which were under a condition of nutrient deficiency most of the time, could follow a similar pattern, while the field grown plants were not crowded and grew under more nearly normal conditions. The generalization that the greenhouse plants, as a rule, contain more water extractable amino acids than the field plants might be explained on the same condition. Since amino acids are the building blocks used in the synthesis of plant proteins, it is conceivable if the plants were unable to absorb enough of specific factors, such as certain minor elements, required for protein synthesis, that amino acid concentrations would build up within the plant tissues. The concentrations of the amino acids in the final harvests of the field stems were much lower than those for the first harvest. This would indicate that most of the free amino acids were utilized in protein synthesis during the filling and maturation of the kernels. (Caution should be used when applying data from greenhouse grown plants to field conditions.)

Figure 10 presents the amino acid chromatogram obtained for the first harvest of the radioactive field grown leaves and stems and effluent positions of radioactivity.* In Figure 11, similar curves and count data are given for the same sample of leaves and stems (A), as well as the

---

*Methods for obtaining count data for samples appearing in Figures 10 and 11 were discussed previously.

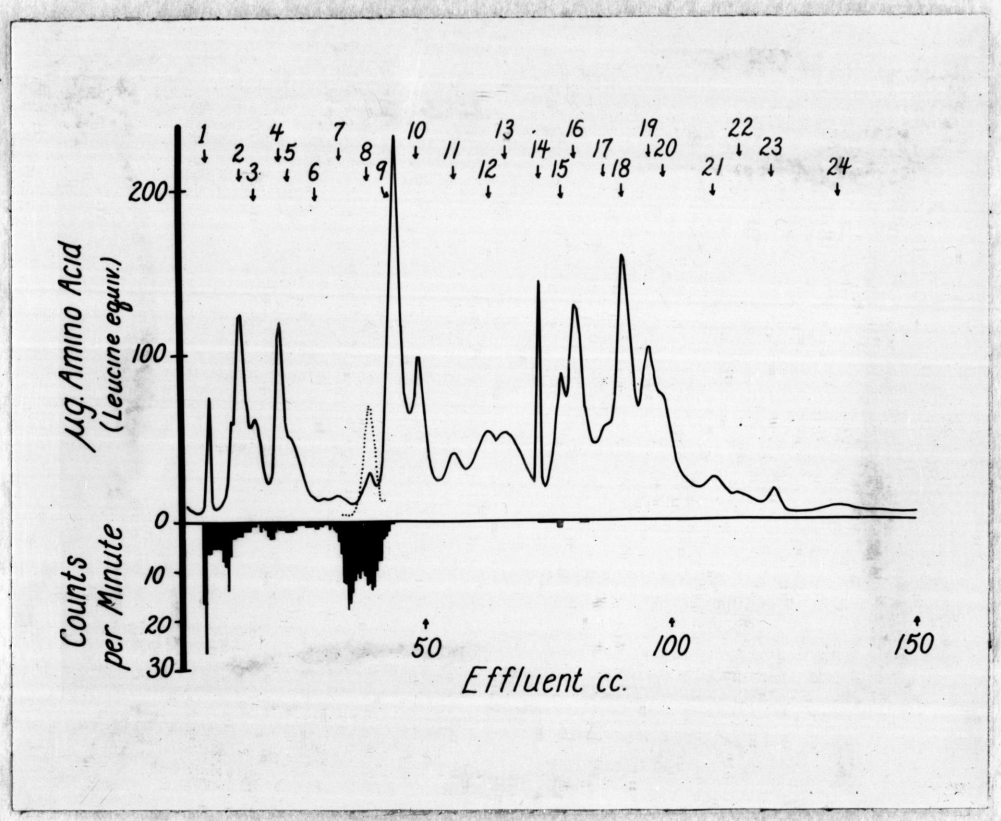

Figure 10. A graph of amino acids and selenium 75 activity in a water extract of the first harvest of radioactive wheat leaves and stems grown at Reed's Ranch, Lyman County, South Dakota. Amino acid data are given on the basis of leucine equivalents per gram of air-dry tissue, while counts per minute are given for the activity per 0.1 gram of sample.
(For key to identify the amino acid peaks see Figure 8).

kernels of the first harvest, and for the leaves and stems, and the kernels of the last harvest. Figure 12 illustrates the position of selenite and selenate selenium, on similar chromatograms, relative to the valine and alanine peaks. The first area of activity in the chromatogram appeared to be associated with the supposed peptide peak (No. 1). Selenite emerges in this region, also. Therefore, the true identity of this activity remains doubtful. Another band of activity was found associated with the leucine peak (No. 2) and the selenium compound responsible for it is un-

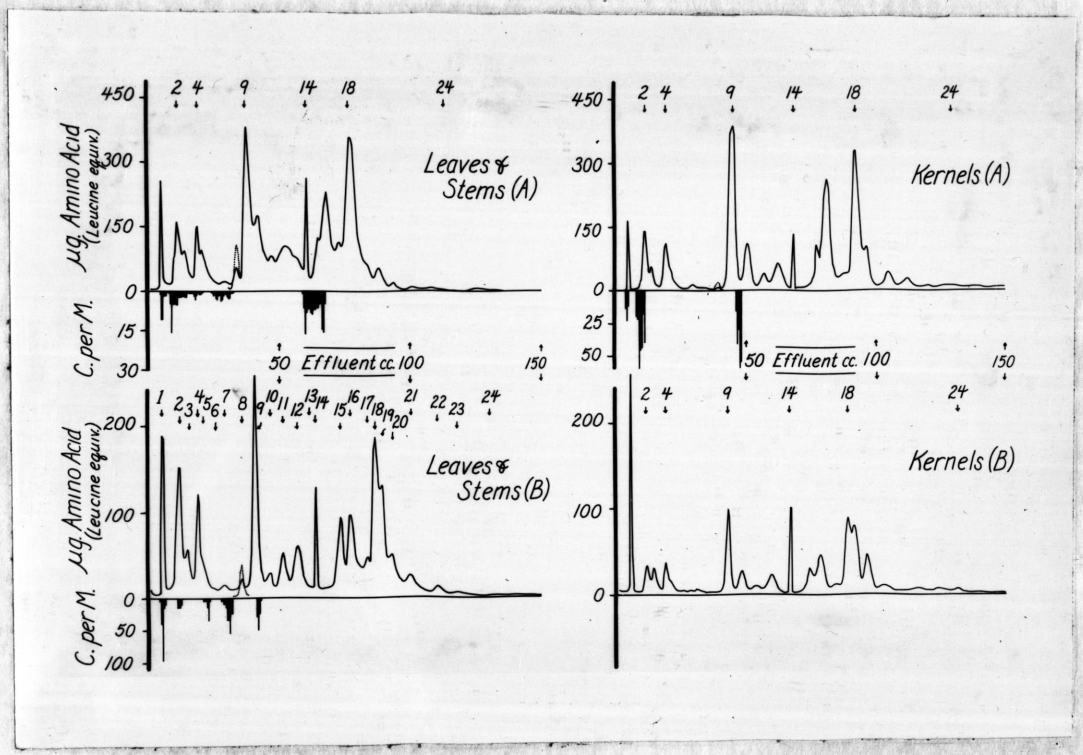

Figure 11. Graphs of amino acids and selenium activity in water extracts of first (A) and second (B) harvests of radioactive wheat leaves and stems and radioactive wheat kernels grown at Reed's Ranch, Lyman County, South Dakota. Amino acid data are given on the basis of leucine equivalents per gram of air-dry sample, while counts per minute are given for the activity per 0.1 gram of sample.
(For key to identify the amino acid peaks see Figure 8).

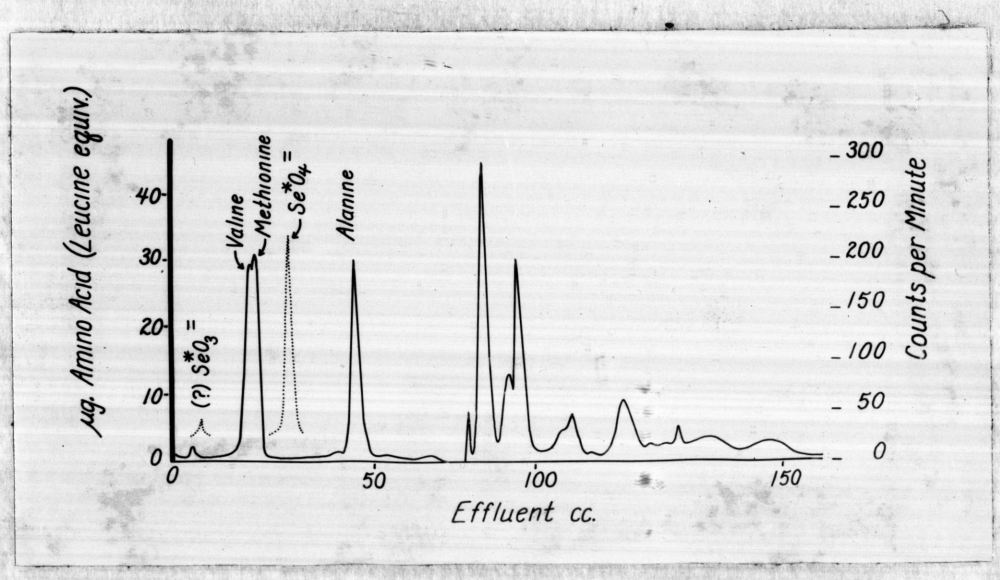

Figure 12. A graph showing the emergence of peaks of selenium 75 activity in selenite and selenate relative to the position of the valine and alanine peaks.

known. Only in the leaves and stems (B) in Figure 11 did any significant activity appear in the methionine peak (No. 5). In all the graphs representing leaves and stems, some activity appeared under peak No. 7, which is thought to represent glutathione. Unfortunately, selenate is known to appear in this same area and could account for a large proportion of this activity. Separations of the inorganic and organic forms of radioactive selenium found in these chromatograms have not been accomplished as yet. In Figure 10, a large proportion of the activity appeared under the 7th peak and under the proline peak, No. 8. However, in the duplicate of the same sample in Figure 11, little activity appeared under the same peaks but a new area of activity emerged with and following the artifact peak (No. 14). The only other area of activity appearing was that emerging between peaks 9 and 10 in kernels (A) and leaves and stems (B) in Figure 11. Kernels (B) in Figure 11 did not give sufficient counts to be considered significant enough to plot. Comparisons of Figure 10 and leaves and stems (A) of Figure 11 (duplicates) showed a clear loss in counting rate due to decay of the selenium 75 during the time interval between runs (143 days). The half-life of selenium 75 is 127 days.

Too much significance should not be placed on the counting data accompanying these amino acid curves because of the low counting rates obtained. It does clearly show, however, that the activity separates into discrete bands indicating that the selenium 75 was probably associated with several definite compounds. Further work is in progress involving similar experiments but using selenate of much higher activity. These studies must be continued, since the data thus far obtained is inadequate for determining the metabolic pathways of selenium in plants.

SUMMARY

Growth rates, as measured by plant dry-weight, of wheat plants grown on seleniferous soils showed that selenite selenium is not markedly toxic until present in the soil in amounts exceeding 4 p.p.m. When plant dry-weights are used as an index of selenium toxicity to wheat plants, five or more replications of a given soil selenium level were found to be desirable. With additions of potassium selenite to the soil, it was shown that selenium uptake by wheat plants is greatest during head and kernel formation.

The selenium content of wheat plants grown in soil containing varying amounts of selenium 75 (as selenate) compared favorably with earlier findings that selenium uptake increased greatly with head formation. Agreement among the count data for the different levels of selenium 75 applied to the soil was satisfactory. Values for selenium content as determined by counting the selenium 75 activity compared favorably with the values obtained by the Klein method (A.O.A.C.) Samples yielding higher count rates should increase the accuracy of the counting method.

In general, greenhouse-grown wheat plants contained higher concentrations of amino acids than field grown plants. Results showed that caution should be used when applying data for greenhouse grown wheat plants to field conditions. Chromatographic separation of the amino acids in a sample containing selenium 75 showed low counting values but the activity was separated into discrete bands. This indicated that the selenium 75 was probably associated with several definite compounds of unknown identity.

The need for plant material containing higher activity, which could

be obtained with some of the selenium 75 preparations now available, is apparent, and further experiments of this type are planned.

REFERENCES CITED

1. Dent, C.E. A study of the behavior of some sixty amino acids and other ninhydrin-reacting substances on phenol-'collidine' filter paper chromatograms, with notes as to the occurrence of some of them in biological fluids. Biochem. J., 43: 169-180 (1948).

2. Franke, Kurt W. Report on a preliminary field survey of the so-called "alkali disease" of livestock. U.S.D.A. Circular No. 320 (1934).

3. Franke, Kurt W. A new toxicant occurring naturally in certain samples of plant foodstuffs. I. Results obtained in preliminary feeding trials. J. Nutrition, 8: 597-607 (1934).

4. Franke, Kurt W. A new toxicant occurring naturally in certain samples of plant foodstuffs. II. The occurrence of the toxicant in the protein fractions. J. Nutrition, 8: 604-613 (1934).

5. Franke, Kurt W. and E. Page Painter. Selenium in proteins from toxic foodstuffs. I. Remarks on the occurrence and nature of the selenium present in a number of foodstuffs or their derived products. Cereal Chem., 13: 67-70 (1936).

6. Franke, Kurt W. and E. Page Painter. Selenium in proteins from toxic foodstuffs. IV. The effect of feeding toxic proteins, toxic protein hydrolysates, and toxic protein hydrolysates from which the selenium has been removed. J. Nutrition, 10: 599-611 (1935).

7. Franke, Kurt W. and E. Page Painter. Effect of sulfur additions on seleniferous soils - - binding of selenium by soil. Ind. Eng. Chem., 29: 591-595 (1937).

8. Franke, Kurt W. and Van R. Potter. A new toxicant occurring naturally in certain samples of plant foodstuffs. IX. Toxic effects of orally ingested selenium. J. Nutrition, 10: 213-221 (1935).

9. Horn, Millard J. and D. Breese Jones. Isolation from Astragalus pectinatus of a crystalline amino acid complex containing selenium and sulfur. J. Biol. Chem., 139: 649-660 (1941).

10. Horn, Millard J., E.N. Nelson, and D.Breese Jones. Studies on toxic wheat grown on soils containing selenium. Cereal Chem., 13: 126-139 (1936).

11. Hurd-Karrer, Annie M. Selenium injury to wheat plants and its inhibition by sulfur. J. Agr. Research., 49: 343-357 (1934).

12. Hurd-Karrer, Annie M. Selenium absorption by plants and their resulting toxicity to animals. Smithsonian Report, 289-301 (1935).

13. Hurd-Karrer, Annie M. Comparative toxicity of selenates and selenites to wheat. Am. J. Botany, 24: 720-728 (1937).

14. Hurd-Karrer, Annie M. Selenium absorptions by crop plants as related to their sulfur requirement. J.Agr. Research, 54: 601-608 (1937).

15. Klein, A. K. Report on selenium. J. Assoc. Official Agr. Chem., 24: 363-380 (1941).

16. Levine, V. E. The reducing properties of microorganisms with special references to selenium compounds. J. Bact., 10: 217 (1925).

17. Martin, Alan L., Toxicity of selenium to plants and animals. Am. J. Botany, 23: 471-483 (1936).

18. Moore, Stanford, and William H. Stein. Photometric ninhydrin methods for use in chromatography of amino acids. J. Biol. Chem., 176: 367- (1948).

19. Moore,Stanford, and William H. Stein. Chromatography of amino acids on starch columns. Solvent mixture for fractionation of protein hydrolysates. J. Biol. Chem., 178: 53 (1949).

20. Moxon, Alvin L., Oscar E. Olson, and Walter V. Searight. Selenium in rocks, soils, and plants. S. Dakota Agr. Expt. Sta. Tech. Bull. No. 2 (1939).

21. Olson, Oscar E. The adsorption of selenium by certain inorganic colloids. Proc. S. Dakota Acad. Sci., 19: 22-24 (1939).

22. Olson, Oscar E., and Curtis W. Jensen. The adsorption of selenate and selenite selenium by colloidal ferric hydroxide. Proc. S. Dakota Acad. Sci., 20: 115-121 (1940).

23. Painter, E. Page, and Kurt W. Franke. Selenium in proteins from toxic foodstuffs III. The removal of selenium from toxic protein hydrolysates. J. Biol. Chem. 111: 643-651(1935).

24. Painter, E. Page, and Kurt W. Franke. Selenium in proteins from toxic foodstuffs. II. The effect of acid hydrolysis. Cereal Chem., 13: 172-179 (1936).

25. Painter, E. Page, and Kurt W. Franke. On the relationship of selenium to sulfur and nitrogen deposition in cereals. Am. J. Botany, 27: 336-339 (1940).

26. Pucher, G. W., and H.B. Vickery. A method for determining glutamine in plant tissues. Ind. Eng. Chem., Anal. Ed., 12: 29 (1940).

27. Stanford, George W., and Oscar E. Olson. The effect of low concentrations of selenium upon the growth of wheat, corn, oats and sorghum. Proc. S. Dakota Acad. Sci. 19: 25-32 (1939).

28. Stein, William H., and Stanford Moore. Chromatography of amino acids on starch columns. Separations of phenylalanine, leucine, isoleucine, methionine, tyrosine, and valine. J. Biol. Chem., 176: 337 (1948).

29. Stein, William H., and Stanford Moore. Amino acid composition of β-lactoglobulin and bovine serum albumin. J. Biol. Chem., 178: 79 (1949).

30. Trelease, Sam F., and Helen M. Trelease. Physiologically balanced culture solutions with stable hydrogen-ion concentrations. Science, 78: 438-439 (1933).

31. Trelease, Sam F., and Orville A. Beath. Selenium. Published by the Authors (1949).