THE UPTAKE AND ASSIMILATION OF SELENITE BY HIGHER PLANTS

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Summary

Plants of wheat, ryegrass, red clover, white clover, and *Neptunia amplexicaulis* (a selenium accumulator) were grown in nutrient solutions containing ^{75}Se-selenite ion. The uptake of ^{75}Se was measured and the root and shoot tissues fractionated to examine the products of selenite assimilation.

Over a period of 10 days the uptake of ^{75}Se-selenite from culture solutions was of a similar order (expressed per gram fresh weight) for all five species.

Characteristic differences were observed between species in the distribution of ^{75}Se in different fractions. The highest ethanol-soluble concentration of ^{75}Se was found in *Neptunia*, in which at least two unidentified seleno-compounds were present in appreciable amounts. Selenite was predominant in the ethanol extracts of wheat, whereas those from the pasture species contained selenium analogues of several common sulphur-containing metabolites. An appreciable unidentified fraction was present in ryegrass extracts.

Aqueous extracts made after alcohol extraction contained selenite, as well as material which was immobile in chromatographic and electrophoretic systems and was probably elemental Se.

Extensive protein incorporation of ^{75}Se in seleno-amino acids was observed in ryegrass, wheat, red clover, and white clover. Protein incorporation of ^{75}Se occurred to a much smaller extent in *Neptunia*.

I. INTRODUCTION

The uptake of selenium by plants has received considerable attention in the past, because of the toxic effects on animals of forages containing amounts of selenium greater than 1 p.p.m. on a dry matter basis (Trelease and Beath 1949). A wider interest in selenium has recently been stimulated by the discovery that trace amounts (0·01 p.p.m. Se) have important nutritional and metabolic functions in ruminants and non-ruminants (Schwarz and Foltz 1957; Patterson, Milstrey, and Stokstad 1957; Drake, Grant, and Hartley 1960; reviews by Schultze 1960, Sharman 1960, and Tagwerker 1960). The factors governing the uptake and state of combination of selenium in plants with a low selenium content have therefore also acquired nutritional significance.

In this paper, a comparative study is reported of the uptake and assimilation of ^{75}Se-selenite by five plant species, which were chosen to be representative of the three groups of plants defined by Miller and Byers (1937) on the basis of total selenium contents. These groups and the plants chosen were:

(1) Selenium accumulators—up to *c.* 5000 p.p.m. Se on a dry matter basis. *Neptunia amplexicaulis*, an endemic Australian legume which is confined to seleniferous soils in central Queensland, was chosen. This plant exhibits

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a growth response to selenium when grown in nutrient culture (McCray, personal communication).

(2) Plants which absorb moderate amounts—up to *c.* 30 p.p.m. Se—without growth retardation. Wheat (*Triticum vulgare*) was chosen.

(3) Plants which absorb up to *c.* 5 p.p.m. Se without growth retardation. White clover (*Trifolium repens*), red clover (*T. pratense*), and perennial ryegrass (*Lolium perenne*) were chosen.

The states of chemical combination of selenium in higher plant tissues are very incompletely understood, because of the difficulty of isolating and characterizing compounds present in such low concentration. Only a limited number of compounds have been recognized, all from plants belonging to the first and second groups in the above classification. By the use of ^{75}Se-selenite ion of high specific activity in these studies it was possible to construct for the five species a balance sheet for the distribution of selenium in various fractions and to identify a number of the seleno-compounds present. The nutritional implications of the findings are discussed.

II. Methods

(*a*) *Materials*

Seed of *Neptunia amplexicaulis*, collected near Richmond, Qld., was kindly supplied by Mr. W. McCray, Animal Research Institute, Brisbane. Wheat seed, cv. Aotea, was supplied by the Crop Research Division, D.S.I.R. Seed of New Zealand certified strains of white clover, red clover, and perennial ryegrass was used.

Radioactive selenium was obtained from Oak Ridge National Laboratory, U.S.A., as ^{75}Se-selenious acid in 1–2N HCl, specific activity 10,000 mc/g Se. This material was chromatographically and electrophoretically homogeneous.

The enzyme preparations used were obtained from the following sources: trypsin (crystallized, substantially salt-free), α-chymotrypsin (crystallized, salt-free), and pepsin (crystallized three times) from Sigma Chemical Company, St. Louis, U.S.A.; ribonuclease from Light & Co., London; mould pectinase from Mann Research Laboratories, New York.

For seedling culture Hoagland–Arnon nutrient solution (Hoagland and Arnon 1938) was used except that ferric tartrate was replaced by the more stable ferric ethylenediaminetetraacetate (Jacobsen 1951). A.R. grade reagents were used throughout.

(*b*) *Growth of Plants and Exposure to ^{75}Se-Selenite*

Preliminary experiments were carried out with wheat seedlings which had germinated on glass beads moistened with water in the bottom of 1-l. beakers. Aliquots (50 μc) of ^{75}Se-selenious acid were dispensed into the beakers at sowing, using both hypodermic syringes with steel needles and glass micropipettes. The seedlings were harvested after 10 days when it was found that the radioactivity taken up by the leaves was 30 times greater when the ^{75}Se-selenious acid was dispensed using glass micropipettes than when hypodermic syringes with steel needles were employed. Chromatographic examination of the solution showed that this difference was a reflec-

tion of the amount of soluble selenite ion present. After passing the ^{75}Se-selenious acid solution through steel needles, at least 90% of the total radioactivity was immobile in the chromatographic or electrophoretic systems (outlined below) and was probably present as an insoluble heavy metal selenite in suspension. Glass micropipettes were therefore used for dispensing ^{75}Se-selenite in subsequent experiments.

The following experimental procedures were used:

(i) Neptunia *and Wheat Seeds.*—These were pretreated with conc. sulphuric acid for 10 min (McCray, personal communication), washed, and sown in vermiculite. Germination and subsequent seedling growth (17 days from sowing) was carried out in a controlled-environment cabinet (18±1°C; 12-hr light period; 2000 f.c. light intensity). Wheat seeds were similarly sown in vermiculite but without the seed pretreatment. Nutrient solution was applied in daily alternation with distilled water.

Neptunia and wheat seedlings were transferred to aerated nutrient solutions contained in 2-l. polythene pots wrapped in aluminium foil. Each pot contained four seedlings of the one species supported with the aid of cotton-wool pellets on opaque "Perspex" covers. There were three treatments, carrier selenite being present in the nutrient solutions at levels of 0, 0·1, and 1·0 p.p.m. Two pots of plants were used for each treatment in each experiment. After 17 days' growth in these solutions, the nutrient concentrations were reduced to one-tenth full strength and ^{75}Se-selenious acid added at the rate of 50 μc/l. Growth was continued for 10 days in these radioactive solutions.

Neptunia seedlings not required for the above experiments were transferred to 8-in. glazed crocks containing a Manawatu silt loam/sand mixture (4 : 1) and grown on in the glasshouse to the flower-setting stage (4 months). Six plants were then removed from the soil and placed singly in full-strength nutrient solution. After 3 days, the nutrient strength was decreased to one-tenth with replacement of sulphate by chloride and ^{75}Se-selenite added as above. The plants were harvested after a further 10 days.

(ii) *White Clover, Red Clover, and Ryegrass Seeds.*—These were sown in washed river sand and germinated under glasshouse conditions. The seedlings were grown for 42 days and watered on alternate days with either nutrient solutions or distilled water. These plants were transferred to culture pots (two pots for each species, four plants per pot) containing full-strength nutrient solution. The day and night temperatures were 25±1°C and 17±1°C, respectively, and the day length and light intensity were as previously used. After 3 days the nutrient solution strength was reduced to one-tenth, ^{75}Se-selenious acid added (50 μc/l), and the plants grown for 10 days. Since plant growth was rapid, supplementary amounts of the macronutrients were added, equivalent to one-tenth strength nutrient solution after 5 days' growth.

(iii) *Wheat for Protein Preparation.*—Seeds were germinated and grown for 7 days in washed river sand under glasshouse conditions with addition of nutrient solution or water as required. The endosperms were then removed from 150 seedlings which were transferred to drilled "Perspex" plates supported inside each of three

2-l. beakers each containing 1 l. full-strength nutrient solution aerated continuously. Environmental conditions were the same as for the pasture plants above. ^{75}Se-selenious acid (50 μc/l) was added and after 5 days the nutrient solutions were renewed with a further addition of ^{75}Se. The plants were harvested after a further 5 days, and used for the soluble protein preparation only.

(c) *Extraction Procedures and Examination of Residues*

Roots were excised from the shoots, blotted, weighed, cut finely, added to 100 ml hot 80% (v/v) aqueous ethanol, and boiled for 15 min. This extraction procedure was repeated four times for each of the tissues and followed by three extractions (each for 15 min) with boiling water. Leaves plus stems were similarly treated. Ethanolic and aqueous solutions were concentrated under reduced pressure at less than 40°C. The insoluble residues remaining after the above extractions were freeze-dried. The residues were examined by the following procedures.

(i) *Acid Hydrolysis.*—Samples (50 mg) of material were hydrolysed with 5–6N HCl for varying times from 2–50 hr.

(ii) *Digestion with Enzymes.*—Enzyme digestion was based on the procedures of Stange, Moses, and Calvin (1960). The standard procedure was to incubate finely divided 40-mg portions with 20 mg of trypsin dissolved in 2 ml 0·01M potassium phosphate buffer, pH 7·6, for 39 hr at 37°C. Chloramphenicol (100 μg) was also present to prevent bacterial contamination. The samples were placed in a wrist-action shaker during incubation. The residues were filtered off, washed with water, and incubated with 20 mg chymotrypsin in 2 ml 0·01M potassium phosphate buffer containing 100 μg chloramphenicol, pH 7·6, for 39 hr at 37°C. The residues were filtered off and re-incubated with trypsin followed by chymotrypsin. (Depending on the particular samples, the procedure was sometimes repeated a third time.) All filtrates were concentrated under reduced pressure and examined for the presence of ^{75}Se-labelled compounds (see below). The radioactivity retained on filters was determined so that a balance sheet of the recovery of radioactivity could be constructed.

In some experiments incubation with 20 mg pepsin in 2 ml 0·01M citrate buffer, pH 4·0, for 39 hr at 37°C was carried out following the chymotrypsin treatment.

In initial experiments, the ribonuclease treatment described by Stange, Moses, and Calvin (1960) was carried out prior to the treatments with proteolytic enzymes. In one experiment, the residues were incubated with 25 mg pectinase in 2 ml 0·04M acetate buffer, pH 5·0, at 37°C for 39 hr, after they had been treated with proteolytic enzymes.

(iii) *Fractionation with Various Reagents.*—Finely divided 40-mg portions of several of the "residue" fractions were shaken with the following reagents in attempts to bring various selenium fractions into solution:

(1) Potassium phosphate buffer, 0·01M, pH 7·6, containing 50 μg/ml chloramphenicol, for 39 hr at 37°C.

(2) Potassium cyanide (1% w/v) for 20 hr at 37°C.

(3) Bromine water at room temperature for 20 min.

(4) DL-Methionine (0·01M), pH 7·0, containing 50 μg/ml chloramphenicol, for 20 hr at 37°C.

(iv) *Soluble Protein Preparations.*—These were made from the roots and shoots of young wheat plants grown and exposed to ^{75}Se-selenite by the procedure described under Section II(*b*)(iii). Prior to extraction the plants were placed in nutrient solutions containing no radioactive selenite for 1 hr. Roots were excised from shoots, blotted, weighed, and a portion taken for radioactivity determination The cell sap was expressed from the remainder (14 g) into 50 ml chilled 0·02M phosphate buffer, pH 7·0, using a stainless steel press (Pirie 1956), and centrifuged for 30 min at 25,000 *g* and 1°C. The supernatant (65 ml) was dialysed for 16 hr against 1 l. 0·01M phosphate buffer, pH 7·0, and for a further 16 hr against 1 l. 0·005M phosphate buffer, pH 7·0. The dialysate was freeze-dried and made to a final volume of 10 ml with water. Leaves (17 g fresh wt.) were similarly treated.

(*d*) *Separation and Identification of ^{75}Se-labelled Compounds*

Since the selenium compounds were present in very small amounts their isolation was impracticable. Identifications were made by comparison of the distribution of ^{75}Se-radioactivity in various chromatographic and electrophoretic systems with the localization of pure seleno- and sulphur-reference compounds. The separation procedures used are summarized below; their relative merits for various identifications are discussed in detail in a separate publication (Peterson and Butler 1962).

(i) *Paper Chromatography.*—One- and two-dimensional paper chromatography was carried out using Whatman No. 3 MM paper and the following solvents:

No.	Solvent
1	n-Butanol–pyridine–water (1 : 1 : 1 v/v)
2	n-Butanol–acetic acid–water (25 : 6 : 25 v/v, upper phase)
3	n-Butanol–ethanol–water (2 : 2 : 1 v/v)
4	t-Butanol–formic acid–water (14 : 3 : 3 v/v)
5	Methyl ethyl ketone–acetone–water (3 : 1 : 0·6 v/v)

One-dimensional chromatograms were run on diethylaminoethyl (DEAE)-cellulose paper (Whatman No. DE 20) using 0·01M acetate buffer, pH 4·7, containing 0·001M disodium ethylenediaminetetraacetate as the developing solvent, as a modification of the method of Knight (1960).

(ii) *Ion Exchange.*—The ion-exchange resin "Dowex 50-X8" (200–400 mesh) was washed and prepared after the method of Wang (1960) and used for a preliminary fractionation of the ethanol extracts of *Neptunia*, with elution with 1N ammonium hydroxide.

(iii) *Paper Electrophoresis.*—Paper electrophoresis of aliquots of protein-free extracts was carried out for 3–4 hr in the apparatus of Markham and Smith (1952) using Whatman No. 3 MM paper and a potential gradient of 20 V/cm. Neutral, basic, and acidic fractions were separated at pH 6·0 in a pyridine–acetate buffer (Dose and Caputo 1956), while separation of seleno- and sulphur-amino acids was carried out at pH 2·7 in a phosphate–citrate buffer (Ostrowski, Skarzynski, and Szczepkowski 1958).

A 3-mg aliquot of a soluble protein preparation from wheat leaves was subjected to paper electrophoresis in phthalate buffer for 4 hr at a potential gradient of 10 V/cm (Moustafa 1959).

(*e*) *Detection and Determination of Radioactivity*

Radiation from ^{75}Se compounds in liquid and solid samples was determined with constant geometry using a scintillation probe (Phillips PW 4111) coupled to the necessary Phillips scaling gear. Sufficient counts were recorded to give a standard error of $\pm 3\%$.

One-dimensional chromatograms and electrophoresis strips were scanned automatically by drawing the strips at the rate of 2·5 cm/hr under the scintillation probe, which was coupled to a rate-meter (Phillips PW 4042) and automatic recorder (Phillips PR 2210 A 21). A 5-mm thick lead plate with a slit 5 mm wide was situated between the probe and the paper for collimation.

Radioautographs of one- and two-dimensional chromatograms were prepared by placing the chromatograms in close contact with sheets of "Gevaert" screen X-ray film for 2 weeks. Radioautography was only used on samples of high activity since film emulsion is relatively insensitive to gamma-radiation (Boyd 1955). The rate-metering procedure described above was considerably more sensitive.

(*f*) *Selenium Analyses*

Analyses for total selenium in tissues of *Neptunia* and pasture species were made by Mr. J. Watkinson, Rukuhia Soil Research Station, Hamilton, using his published method (Watkinson 1960).

III. Results

(*a*) *Uptake of ^{75}Se-selenite*

Table 1 shows the uptake of ^{75}Se-selenite from nutrient solutions to the roots and shoots of the five plant species. It will be seen that both for young *Neptunia* and wheat the proportions of radioactivity taken up at the three carrier selenite levels were not very different. Also there was little difference between the uptake of ^{75}Se-selenite by wheat and young *Neptunia*. With mature *Neptunia* plants, most of the ^{75}Se taken up was confined to the roots; although no sulphate was present in the nutrient solutions during the 10-day uptake period, very little ^{75}Se was transported to the shoots and the quantity present in the root systems was comparable with that observed per gram fresh weight for young *Neptunia* plants.

For the experiments with pasture plants, no additions of carrier selenite were made in view of the overall similarity in distribution with different carrier levels observed in the experiments with wheat and young *Neptunia*, and also because of the low tolerance of this group of plants for selenium. The amounts of ^{75}Se taken up were somewhat greater than the quantities taken up by wheat and *Neptunia*. This was associated with a greater rate of growth observed in the experiments with pasture plants.

TABLE 1

UPTAKE OF ^{75}SE-SELENITE BY FIVE PLANT SPECIES GROWN IN NUTRIENT SOLUTIONS

Plants were grown for 10 days in solutions containing an initial activity of 50 μc/l

Species	Carrier Se Level (p.p.m.)	Selenite Uptake per Gram Fresh Weight (counts/min $\times 10^{-3}$)		Selenite Uptake per Plant (counts/min $\times 10^{-3}$)		Percentage Distribution	
		Shoots	Roots	Shoots	Roots	Shoots	Roots
Neptunia							
Young	0·0	104	220	68	175	28	72
Young	0·1	57*	200	51	179	22	78
Young	1·0	125	171	79	138	36	64
Mature	0·0	2·9	182	28	2550	1	99
Wheat	0·0	55	186	90	323	22	78
Wheat	0·1	57	135	98	273	26	74
Wheat	1·0	51	165	69	217	24	76
Red clover	0·0	185	1030	867	6040	13	87
White clover	0·0	216	907	1020	4030	20	80
Ryegrass	0·0	193	601	421	2050	17	83

* Plants contained a higher proportion of stem than in other treatments.

TABLE 2

PERCENTAGE DISTRIBUTION OF ^{75}SE IN 80% ETHANOL, WATER, AND "RESIDUE" FRACTIONS OF ROOTS AND SHOOTS OF FIVE PLANT SPECIES

Species	Carrier Se Level (p.p.m.)	Shoots			Roots		
		80% Ethanol	Water	Residue	80% Ethanol	Water	Residue
Neptunia							
Young	0·0	93·4	4·1	2·5	62·0	11·4	26·6
Young	0·1	89·6	4·1	6·3	57·5	10·7	31·8
Young	1·0	94·4	2·5	3·1	62·8	14·9	22·3
Mature	0·0	60·3	13·7	26·0	25·7	11·0	63·3
Wheat	0·0	19·8	4·5	75·7	20·2	20·5	59·3
Wheat	0·1	18·7	4·7	76·6	27·9	26·9	45·2
Wheat	1·0	15·3	5·3	79·4	22·2	22·8	55·0
Red clover	0·0	34·1	4·4	61·5	17·3	6·0	76·7
White clover	0·0	27·5	4·1	68·4	17·4	6·4	76·2
Ryegrass	0·0	19·1	3·2	77·7	28·0	7·6	64·4

(*b*) *Distribution of* ^{75}Se *in Various Fractions*

Preliminary experiments with *Neptunia* showed a similar distribution of ^{75}Se in ethanol, water, and residue fractions of stems and leaves. These were bulked in the main investigation. Table 2 shows the distribution of ^{75}Se in the 80% ethanol, aqueous, and residue fractions of roots and shoots of the five species. Whereas approximately 90% of the total radioactivity in young *Neptunia* shoots was extracted by 80% aqueous ethanol, much lower proportions were extracted from shoots of the other species by this solvent. Conversely, most of the radioactivity in shoots of the latter species was not extracted by ethanol or water, whereas the amount of activity not extracted from young *Neptunia* shoots was only 5%. A small proportion of the total radioactivity was extracted by water from the shoots of all species.

A considerably higher percentage of the radioactivity remained in the residue fraction of *Neptunia* roots than was the case for shoots. The proportion of ^{75}Se in the residue fraction of mature *Neptunia* roots was particularly high. For all species the percentage of radioactivity in the aqueous extracts was greater for the roots than the shoots.

For each of the wheat and young *Neptunia* tissues examined, the distributions of radioactivity for the three selenium levels were strikingly similar.

The two clovers showed a particularly similar distribution whereas the results for ryegrass shoots were very similar to those observed for wheat shoots (Table 2).

(*c*) *Fractionation of Alcohol Extracts*

The percentage distribution of the principal substances observed in these extracts are tabulated in Table 3 and details of their chromatographic behaviour are given in the sections below. No differences in the types of compounds present were noted for plants grown at different selenium concentrations.

(i) Neptunia.—Considerable difficulty was experienced in examining ethanol extracts of *Neptunia* because the compounds containing ^{75}Se were poorly resolved in all the solvents tried. When chromatograms were prepared using solvents 1 and 2, peaks corresponding to selenite were seen; this was confirmed by electrophoresis at pH 2·7. Approximately 25% of the radioactivity in the root and shoot extracts was present as selenite. For both roots and shoots, the rate-meter tracings and radioautographs showed an appreciable amount of immobile material (approx. 15%) and a general streaking of the remainder of the radioactivity.

On chromatography of aliquots of the ethanol extracts in solvent 4, it was observed that there was general degradation of the ^{75}Se-compounds present (with the exception of selenite) to give immobile material.

A further attempt at fractionation was made by applying an aliquot of the root extract, corresponding to 1·8 g fresh weight of roots, to a 6 by 1 cm "Dowex 50-X8" column. Since a preliminary experiment had shown that only 8% of the radioactivity retained by the column was eluted by 1-6N HCl, elution was carried out with 0·01–1N NH_4OH. Most of this radioactivity was eluted by 1N NH_4OH; the total recovery of the activity which had been retained on the column was 75%.

TABLE 3

PERCENTAGE DISTRIBUTION OF ^{75}SE-LABELLED COMPOUNDS PRESENT IN THE 80% ETHANOL EXTRACTS OF FIVE PLANT SPECIES

Compound No.	Compound	*Neptunia*		Wheat		Red Clover		White Clover		Ryegrass	
		Shoot	Root	Shoot	Root	Shoot	Root	Shoot	Root	Shoot	Root
1	Immobile	14	18	10	5	Trace	4·4	Trace	5·3	Trace	4·9
2	Selenocystine	—	—	—	—	17	41·5	16	41·1	13	2·5
3	Unknown (selenoglutathione?)	—	—	—	—	—	18·3	—	20·5	—	4·8
4	Se-methylselenomethionine selenonium salt (?)	—	—	—	—	—	2·4	—	2·4	—	5·9
5	Selenocysteic acid	—	—	—	—	40	10·2	20	7·6	10	6·0
6	Selenocysteine seleninic acid (?)	—	—	—	—	Trace	Trace	Trace	Trace	—	6·8
7	Selenite ion	27	23	90	93	8	4·4	11	7·7	12	38·8
8	Unknown	—	—	—	—	—	3·0	—	5·7	—	—
9	Selenomethionine selenoxide	—	—	—	—	10	5·0	14	Trace	Trace	Trace
10–11	Unknowns	—	—	—	—	—	Trace	—	Trace	—	1·3
12	Selenomethionine	—	—	—	—	18	4·7	17	4·6	15	1·3
13	Unknown	—	—	—	—	4	Trace	5	Trace	Trace	Trace
14	Unknown	—	—	—	—	3	6·1	17	5·1	50	3·1
15–19	Unknowns	—	—	—	—	—	—	—	—		24·6
20	Unknowns (3)	—	—	—	2	—	—	—	—	—	—
21	Unknown streaks	59	59	—	—	—	—	—	—	—	—

The eluate was concentrated and chromatographed in solvents 1 and 2, when 3 peaks of approximately equal size were observed at the following R_F values:

Solvent 1	0·0, 0·12, 0·29
Solvent 2	0·0, 0·43, 0·50

The presence of two smaller peaks was also indicated. It was surprising that there was immobile material present in the eluates from the "Dowex" column since most

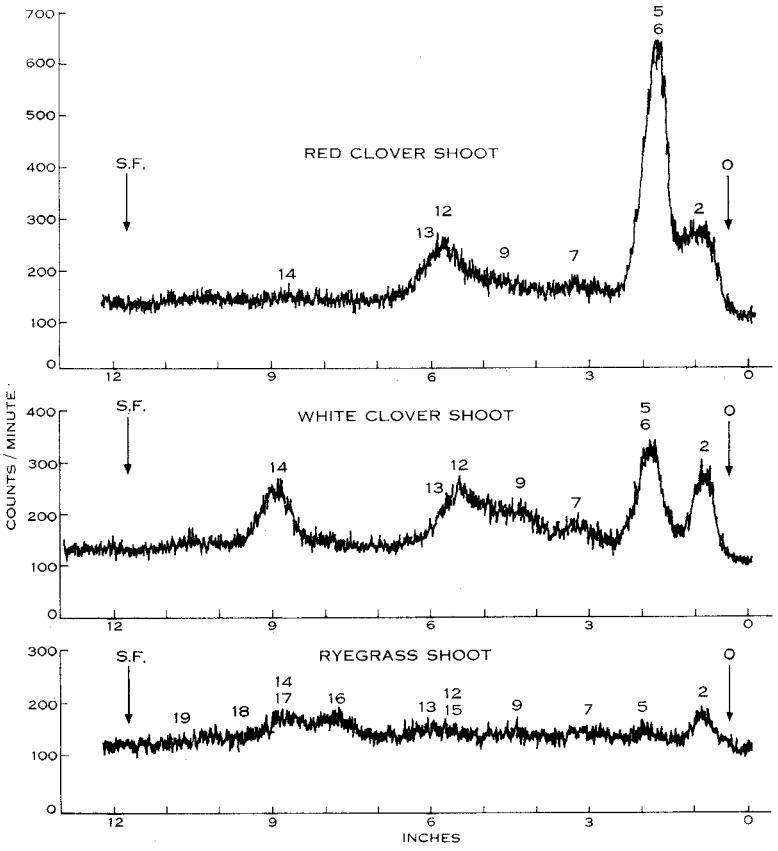

Fig. 1.—Rate-meter tracings recording the distribution of ^{75}Se radioactivity on one-dimensional chromatograms, developed with solvent 2, of 80% ethanol extracts from shoots of red clover, white clover, and ryegrass. The identity and relative concentration of the numbered ^{75}Se compounds are shown in Table 3. Arrows indicate origin (O) and solvent front (S.F.).

of the immobile material present in the original ethanol extract was washed off the column with water. It would appear that the ^{75}Se-compound(s) were unstable during this procedure. The above R_F values do not correspond to known common seleno-amino acids, with the possible exception of seleno-taurine. The R_F values of taurine were observed to be 0·14 and 0·41 in solvents 1 and 2 respectively.

(ii) *Wheat.*—90% of the radioactivity in these extracts was present as selenite, as shown by chromatography in solvents 1 and 2 and electrophoresis at pH 2·7. A

small quantity (5–10%) was immobile in these three systems and in the root extract traces of radioactivity were present in three unidentified peaks, totalling 2% of the total radioactivity.

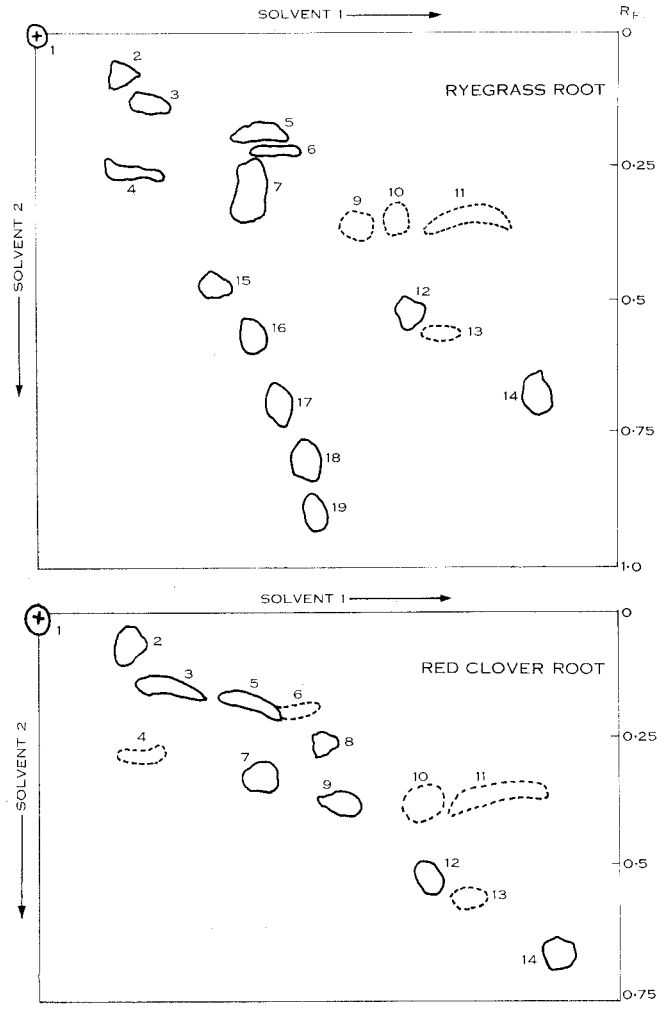

Fig. 2.—Tracings from radioautographs of two-dimensional chromatograms of 80% ethanol extracts from roots of red clover and ryegrass. The identities and relative concentrations of the numbered ^{75}Se compounds are shown in Table 3.

(iii) *Red and White Clovers.*—Good resolution of the ^{75}Se-seleno-compounds present was obtained by paper chromatography and electrophoresis. A close similarity was observed between white and red clover in the types of compounds present, although the relative amounts of some of these differed. More compounds were observed in the root than in the shoot extracts (Table 3); this may have been due to the higher activity of the root preparations.

Examples of the separations achieved of substances present in the ethanol extracts of clovers are shown in Figures 1 and 2 as rate-meter and radioautograph tracings. In Figure 2 the radioactive areas have been outlined since the more faintly labelled spots could not be reproduced with clarity. Details of the identification procedures are as follows:

Compound 1: Immobile in chromatographic and electrophoretic systems. Much of this material was probably elemental selenium or insoluble heavy metal complexes. High molecular weight organic seleno-compounds could also be immobile, but it is unlikely that these would have been soluble in boiling aqueous ethanol during extraction. The radioactivity was largely soluble in bromine water at room temperature in 20 min and the dissolved ^{75}Se chromatographed as selenite. This is consistent with the immobile material being elemental selenium.

Compound 2, *Selenocystine*: The ^{75}Se-labelled spot was eluted from a chromatogram run in solvent 1. Aliquots moved identically with marker selenocystine in solvents 2 and 3 and upon electrophoresis at pH 2·7.

Compound 3: Unknown. This substance was present in the root extracts only, where it comprised one-fifth of the total radioactivity. Its chromatographic behaviour in solvents 1 and 2 suggested that it was selenoglutathione.

Compound 4, *Se-methyl Selenomethionine, Selenonium Salt* [$(CH_3)_2Se^+CH_2CH_2CHNH_2COOH$]: A well-separated peak with a high mobility on a paper electrophoresis strip, pH 2·7, was eluted and run in solvent 1, when its position corresponded with marker S-methylmethionine, sulphonium chloride. Peaks were also observed in the expected positions on chromatograms of the ethanol extracts run in solvents 1 and 2 and, more characteristically, on DEAE-cellulose paper at pH 4·7.

Compound 5, *Selenocysteic Acid*: The suspected substance was eluted from a chromatogram run in solvent 1 and suitable aliquots ran identically with cysteic acid in solvents 2 and 3. Upon electrophoresis at pH 2·7 most of the radioactivity ran similarly to cysteic acid, but a small peak was observed slightly behind it (compound 6).

Compound 6, *Selenocysteine Seleninic Acid* ($HO_2SeCH_2CHNH_2COOH$): This substance chromatographed identically with cysteine sulphinic acid in solvents 1 and 2. It also ran similarly to cysteine sulphinic acid upon electrophoresis at pH 2·7.

Compound 7, *Selenite Ion*: This substance ran identically with marker selenite in all systems.

Compounds 8, 10, *and* 11: Unknowns (see Fig. 2).

Compounds 9 *and* 12, *Selenomethionine Selenoxide* ($CH_3Se{:}OCH_2CH_2CHNH_2COOH$) *and Selenomethionine*: Suspected selenomethionine was eluted from a chromatogram run in solvent 1. The eluate was purified by re-running in solvent 1 and the appropriate zone eluted and run in solvent 2. The radioactive zone was again eluted, oxidized by 3% (w/v) H_2O_2 for 5 min at room temperature, and run in solvent 2, followed by solvent 1. Before and after oxidation the radioactive zones ran identically in both solvents with marker selenomethionine and selenomethionine selenoxide respectively. The peaks observed after electrophoresis of the ethanol extracts were also consistent with this interpretation.

Compounds 13 *and* 14: Unknowns—see Figure 2 and Table 3. In the clover shoot extract, unknown 14 contained 17% of the total radioactivity. Unknown 13 was unstable, breaking down during elution and re-running in solvents 1 and 2.

(iv) *Ryegrass.*—Compounds 1–7 and 9–14 were observed in the ryegrass extracts, and identified as described for the clovers. The concentration of selenite in the root extract was particularly high, comprising 39% of the total radioactivity. In addition, unknown compounds 15–19 were observed to contain 24·6% of the radioactivity in the root extracts and approximately 50% in the shoots. Of these, unknowns 17 and 18 were present in the greatest amount in the root extracts. From their chromatographic behaviour (Fig. 2), it would appear likely that these unknowns are members of an homologous series.

(*d*) *Fractionation of Aqueous Extracts*

In general, a simple labelling pattern was observed for the aqueous extracts as shown in Table 4. Most of the radioactivity present was either in immobile

TABLE 4

PERCENTAGE DISTRIBUTION OF ^{75}SE-LABELLED COMPOUNDS PRESENT IN AQUEOUS EXTRACTS OF FIVE PLANT SPECIES

Compound	*Neptunia*		Wheat		Red Clover		White Clover		Ryegrass	
	Shoot	Root	Shoot	Root	Shoot	Root	Shoot	Root	Shoot	Root
Immobile	70	4	Trace	18	100	34	100	28	Trace	40
Selenite ion	30	96	—	75	—	50	—	56	—	42
Unknowns (2)	—	—	—	—	—	16	—	16	—	18
Unknowns (3)	—	—	—	7	—	—	—	—	—	—

TABLE 5

EXTRACTION OF RADIOACTIVITY FROM "RESIDUE" FRACTIONS OF ROOTS AND SHOOTS OF WHEAT, AND ROOTS OF NEPTUNIA, USING VARIOUS ENZYMES AND CHEMICAL REAGENTS

Values are expressed as percentage of total radioactivity at commencement. Details of the treatments are given in the text

Extraction Treatments (in order)	Wheat		*Neptunia*	
	Roots*	Shoots*	Young Roots*	Mature Roots†
Water	2·8	7·4	4·4	9·1
Ribonuclease	2·3	2·1	—	—
Trypsin	33·2	46·8	3·7	4·8
Chymotrypsin	19·3	20·5	3·8	4·1
Pepsin	7·8	3·1	—	—
Trypsin	1·5	0·7	—	—
Chymotrypsin	1·8	1·7	—	—
Pepsin	1·0	0·5	—	—
Potassium cyanide	9·9	2·2	—	—
Bromine water	—	—	68·9	65·0
Residual activity	9·9	2·6	19·2	17·2
Percentage recovered‡	105·8	98·3	100	103·5

* Plants grown in nutrient solutions containing 0·1 p.p.m. selenite.

† Plants grown in nutrient solutions containing no carrier selenite.

‡ Includes counts retained on filters.

material (probably elemental selenium or as a heavy metal complex (see Section III(*c*)(iii) above) or as selenite. Whereas immobile material predominated in the shoot fractions, selenite usually predominated in the roots. Three unknown compounds

observed in the wheat root aqueous extract had similar R_F values in solvents 1 and 2 to those found in the corresponding ethanolic extract (see Section III(*c*)(ii) above).

(*e*) *Fractionation of Plant Residues*

(i) *Acid Hydrolysis.*—Hydrolysis of plant residues was carried out by refluxing with 5–6N HCl at 110°C for times varying from 2 to 50 hr. The recoveries of ^{75}Se in the hydrolysates were unsatisfactory and decreased with increasing hydrolysis times. In some cases deposition of red elemental selenium was observed on the walls of the reflux condenser. Chromatography of these extracts in solvent 2 showed one peak at R_F 0·90; this was believed to be a decomposition product formed during hydrolysis. When ^{75}Se-selenocystine was hydrolysed with 6N HCl for 1 hr and the hydrolysate chromatographed in solvent 2 a peak was also observed at R_F 0·90.

TABLE 6

EXTRACTION OF RADIOACTIVITY FROM "RESIDUE" FRACTIONS OF ROOTS AND SHOOTS OF RYEGRASS, WHITE CLOVER, AND RED CLOVER, USING TRYPSIN, CHYMOTRYPSIN, AND BROMINE WATER

Values are expressed as percentage of total radioactivity at commencement

Extraction Treatments (in order)	Red Clover		White Clover		Ryegrass	
	Roots	Shoots	Roots	Shoots	Roots	Shoots
Water	2·1	0·3	2·2	0·4	2·4	1·6
Chymotrypsin	64·1	74·5	71·3	82·4	32·1	79·5
Trypsin	6·4	7·3	3·7	7·1	8·4	6·3
Chymotrypsin	2·8	3·4	2·4	1·7	4·0	1·2
Trypsin	1·0	1·0	0·7	1·0	2·1	1·0
Bromine water	5·6	0·8	1·2	0·2	7·2	0·8
Residual activity	10·0	13·5	8·3	3·8	20·0	6·5
Percentage recovered*	95·7	110·0	95·5	103·0	92·7	100·2

* Includes counts retained on filters.

(ii) *Enzymic Digestion and Miscellaneous Treatments.*—In Table 5 are shown the percentages of ^{75}Se-radioactivity extracted by enzymes and by chemical reagents from the residues after ethanol and water extractions of roots and shoots of wheat plants and roots of *Neptunia* plants. The amount of radioactivity remaining in the residues of *Neptunia* shoots after solvent extraction was too small for further fractionation to be practicable.

It will be seen that the major portion of the radioactivity in the residues from both roots and shoots of wheat was extracted by treatment with proteolytic enzymes. With the *Neptunia* root tissue, however, only a small proportion of the total radioactivity was extracted by treatments with trypsin and chymotrypsin, whereas large amounts were soluble in bromine water. This indicates that a high proportion of the selenium was in insoluble inorganic form.

The percentages of ^{75}Se-radioactivity extracted by proteolytic enzymes and by bromine water from the residues after ethanol and water extractions of roots and shoots of ryegrass and red and white clovers are shown in Table 6. In all cases the

major portion of the total radioactivity was extracted by treatment with proteolytic enzymes. The percentage thus extracted was markedly lower for ryegrass roots than for the other tissues.

Extraction of wheat root and shoot residues was carried out with several other reagents. Negligible radioactivity was brought into solution by treatment with ribonuclease (Table 5) or pectinase.

During the course of this work it was observed that ^{75}Se compounds continued to be slowly but steadily released into solution when residue fractions (40 mg) of wheat root and shoot tissues were shaken at 37°C for 39 hr with four successive quantities of 2 ml 0·01M phosphate buffer, pH 7·6, containing 50 μg/ml chloramphenicol. The rate of release of ^{75}Se compounds was very much greater when proteolytic enzymes were also present. This is clearly shown in the experiment summarized in Table 7.

TABLE 7

COMPARISON OF THE AMOUNT OF ^{75}SE RELEASED FROM WHEAT ROOT AND SHOOT "RESIDUES" IN 0·01M PHOSPHATE BUFFER, pH 7·6, IN THE PRESENCE AND ABSENCE OF PROTEOLYTIC ENZYMES

Tissue	Treatment*	^{75}Se Extracted (%)	Treatment*	^{75}Se Extracted (%)
Roots	Buffer	4·6	Buffer + trypsin	45·5
	Buffer	3·1	Buffer + chymotrypsin	20·7
Shoots	Buffer	5·8	Buffer + trypsin	64·0
	Buffer	3·5	Buffer + chymotrypsin	19·7

* Each tissue sample shaken successively with two buffer solutions or two buffer + enzyme solutions.

(iii) *Identity of Selenium Compounds Extracted.*—The nature of the selenium compounds brought into solution by treatment with phosphate buffer, pH 7·6, and by proteolytic enzymes was examined chromatographically and electrophoretically. For residues from wheat shoots and roots, the radioactivity extracted by phosphate was present in two compounds which behaved identically with selenomethionine and selenomethionine selenoxide when chromatographed in solvents 1, 3, and 5. In solvent 2, two peaks were again obtained, corresponding to selenomethionine and selenomethionine selenoxide. In addition a third peak was observed at R_F 0·90, accounting for up to 40% of the total radioactivity. This was believed to be a decomposition product; a similar peak was observed in acid hydrolysates (Section III(*e*)(i)). Electrophoresis of these extracts at pH 2·7 in citrate–phosphate buffer gave two incompletely resolved peaks which were located in the same positions as selenomethionine and selenomethionine selenoxide. After oxidation of extracts with 3% (w/v) H_2O_2 at room temperature for 5 min, the peak corresponding to selenomethionine was absent and the peak corresponding to selenomethionine selenoxide was augmented.

Rate-meter tracings of chromatograms in solvents 1 and 2 of extracts from treatment of wheat residues of roots and shoots with proteolytic enzymes showed, in addition to the two peaks mentioned above, a considerable amount of poorly resolved and immobile material which could not be identified. This was probably peptide in nature.

Trypsin and chymotrypsin digests of root residues of the three pasture species were chromatographed in solvent 1 and also on DEAE-cellulose paper at pH 4·7. More complex rate-meter tracings were obtained than in the case of the wheat-tissue residues, particularly for the clovers. In the latter cases, the tracings bore a general similarity to those obtained for the corresponding ethanol extracts. In all cases a large peak was observed at the R_F corresponding to selenomethionine; peaks were also observed in the selenocystine zones of the chromatograms.

When a 40-mg portion of finely divided red clover root tissue was shaken with 0·01M DL-methionine, only 2·5% of the total radioactivity was brought into solution. This indicates that the selenomethionine released by proteolytic enzymes from this tissue was probably incorporated in protein and not adsorbed. The assumption is made that methionine and selenomethionine show similar adsorption behaviour.

(*f*) *Soluble Protein Preparations*

Two preparations were made from roots and shoots of wheat respectively; 3·2% and 9·9% of the total counts were recovered in the soluble protein preparations. Several tests were made to establish whether the ^{75}Se present was all incorporated into protein:

(1) When chromatograms were made using solvent 1, 95% of the radioactivity was found in immobile material; this is consistent with the selenium being contained in protein.

(2) When a suitable aliquot of the leaf preparation was subjected to paper electrophoresis in phthalate buffer, pH 7·5, and the protein stained with "Amido-Schwarz 10B" it was found that all the protein had moved slightly towards the positive terminal without further resolution. Rate-meter tracings showed that all the radioactivity coincided with the protein-containing zone.

(3) The protein preparations underwent partial denaturation during storage at 2°C. When denatured leaf protein was digested with trypsin and chymotrypsin using techniques described earlier, 95% of the radioactivity was brought into solution. The rate-meter tracing of a chromatogram of the digests run in solvent 1 showed the typical pattern found previously, with a particularly sharp peak in the selenomethionine position.

(*g*) *Total Selenium Analyses*

Seeds obtained near Richmond, Qld., were freeze-dried and analysed for total selenium, as were seeds from the next generation of plants grown locally on Manawatu silt loam. The values obtained were 123 μg Se per seed for *Neptunia* seeds from Queensland and 0·45 μg Se per seed for New Zealand seeds.

Plants from the Queensland seeds, which had grown to the seed-setting stage on Manawatu silt loam, were harvested, freeze-dried, ground, and the roots and shoots analysed for selenium. Clover and perennial ryegrass herbage from plants which had grown on the same soil were also analysed for comparison. The results were:

Sample	Se Content (p.p.m.)	Se per Plant (μg)
Neptunia shoots	2·5	32
Neptunia roots	4·0	63
Clover shoots	0·018	—
Perennial ryegrass herbage	0·016	—

If the selenium present in the Queensland seed had been mobilized by the plants grown from this seed, all of the selenium found in the plants could have arisen from the seed.

IV. Discussion

(*a*) *Selenium Uptake and Distribution*

It is surprising that the amount of ^{75}Se-selenite taken up over a period of 10 days by young *Neptunia* plants was not very much greater than the amounts taken up by the other species studied. The selenium analyses, however, indicate that mobilization of selenium from the seeds was probably important both when the *Neptunia* plants grew in nutrient solutions and in a soil of low selenium content.

It is known that species differ in their relative capacities for absorbing selenite ion, selenate ion, and selenium in other forms of combination (Trelease and Beath 1949). Whereas selenite is less readily absorbed than selenate by several crop plants (Moxon, Olson, and Searight 1950), it is taken up to the same extent as selenate by some *Astragalus* species which accumulate selenium (Trelease and Trelease 1938; Ganje and Whitehead 1958). Because of the difficulty of obtaining ^{75}Se-selenate of high specific activity, no comparisons of selenate and selenite uptake by *Neptunia* were made in these studies. It appears likely, however, that selenite ion is not absorbed by this species to the same extent as some other forms of selenium.

It has been observed by several workers (Trelease and Trelease 1938; Martin and Trelease 1938) that high sulphate to selenite ratios depress selenium uptake by crop plants and *Astragalus* species. The effect is not large, particularly at low selenite concentrations, and does not appear to have been important in the experiments reported here.

Lakin, Williams, and Byers (1938) and Moxon, Olson, and Searight (1950) have reported the low availability to crop plants of selenite in heavy metal combination. The very low absorption by wheat seedlings of ^{75}Se-selenite which had been dispensed using a steel needle is consistent with these findings.

The similarity of the distribution of ^{75}Se in *Neptunia* and in wheat tissues when carrier selenite had been added to the nutrient solutions at 0, 0·1, and 1 p.p.m. respectively (Tables 1 and 2) is probably associated with ready replacement of sulphur by selenium in various plant constituents at all the selenium levels used. The sulphate-S concentration of the nutrient solution was 64 p.p.m. during the preliminary establishment phase and 6·4 p.p.m. during the 10-day absorption period.

It is of interest that the selenium-accumulating species of *Astragalus* contain a large fraction of readily soluble selenium compounds of low molecular weight (Horn and Jones 1941; Trelease and Beath 1949), as has been observed for *Neptunia* in this work.

(*b*) *Aspects of Methodology*

Two points were observed to be essential to avoid extensive modification of seleno-compounds in plant extracts. These were:

(1) Recognition of the possibility for chemical reaction of selenite ion with sulphydryl and selenohydryl groups during extraction and subsequent storage of extracts. There is disagreement in the literature on the nature of the reaction products (Stekol 1942; Klug and Petersen 1949; Petersen 1951). Selenite should be separated from other seleno-compounds at an early stage after extraction, e.g. by electrophoresis.

(2) Care in the choice of chromatographic solvents. Phenol–water and t-butanol–formic acid–water were observed to be particularly destructive, although they have been used extensively by other workers in the selenium field. Acid hydrolysis also resulted in extensive destruction, as has been noted by earlier workers (Shrift 1958).

(*c*) *Fractionation*

(i) *Low Molecular Weight Substances.*—It is noteworthy that selenate ion was not detected in any of the extracts from plants which had absorbed ^{75}Se-selenite.

Only one soluble seleno-organic compound has been isolated from tissues of higher plants and rigidly identified, namely Se-methylselenocysteine from *Astragalus bisulcatus* (Trelease, Di Somma, and Jacobs 1960). On the basis of chromatographic and electrophoretic data, the present study shows that seleno-analogues of several common sulphur-containing metabolites are present in small quantities in the tissues of ryegrass, red clover, and white clover. It appears probable that some unusual soluble seleno-compounds are present in *Neptunia amplexicaulis*. Also five unknown compounds, which from their chromatographic behaviour appear to be members of a homologous series, were observed to comprise a substantial proportion of the total soluble selenium in ryegrass tissues.

The release of some selenomethionine by incubation of the residue fractions of wheat with phosphate buffer at pH 7·6 suggests that adsorbed seleno-amino acids may also be present under some circumstances.

(ii) *Insoluble Inorganic Material.*—Labelling of this fraction with ^{75}Se was particularly pronounced in the root tissues of *Neptunia*. The extent to which labelling was due to reduction of selenite ion followed by deposition of elemental selenium or immobilization of selenite by precipitation with heavy metals or both of these factors is a matter for further investigation.

(iii) *Selenium Incorporated in Protein.*—Evidence for extensive incorporation of selenium was obtained in the experiments with enzymic hydrolysates of wheat, ryegrass, red clover, and white clover. The bulk of the selenium thus incorporated was present as constituent seleno-amino acids of the proteins, bound by peptide links.

Previous workers examined the association of selenium with proteins in toxic wheat, corn, and barley grain (Painter 1941; Trelease and Beath 1949). Smith (1949) and Johnson (1952) inferred the presence of protein-containing selenomethionine and selenocysteine from studies of acid hydrolysates of wheat and corn gluten, but Whitehead, Hendrick, and Moyer (1955) found no evidence for the presence of these compounds in acid hydrolysates of various protein preparations from wheat plants.

The selenomethionine selenoxide observed in the trypsin and chymotrypsin digests could have arisen from selenomethionine during extraction and manipulation. Neumann (1960) states "it would appear as though sulphoxide residues are not normal constituents of proteins."

The proportion of ^{75}Se bound to protein was very much lower in *Neptunia* tissues. This may be due to high carrier concentrations of various selenium compounds in the tissues, because of the relatively high selenium content. It is possible, however, that a mechanism may be present in this selenium-accumulating plant for discriminating between seleno- and sulphur-amino acids in the synthesis of protein.

(*d*) *Implications for Ruminant Nutrition*

These experiments suggest that the greatest proportion of the selenium present in herbage of pasture plants growing under conditions of low selenium nutrition, as in New Zealand, will be present as seleno-amino acids in proteins or in the free form. Selenite is unlikely to be present in more than minor amounts; the possibility of selenate accumulation has not been covered by these experiments. Work with forages growing on seleniferous soils has shown that "naturally-occurring organic selenium" was more readily available to ruminants than selenium in inorganic form (Sharman 1960).

The relatively large amount of unidentified selenium compounds in ethanol extracts from ryegrass herbage may have nutritional significance, since the identity of the organic selenium-containing "factor 3", which prevents dietary necrotic liver degeneration in rats (Schwarz and Foltz 1957) is not known (Schwarz 1960).

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VI. References

Boyd, G. A. (1955).—"Autoradiography in Biology and Medicine." (Academic Press Inc.: New York.)

Dose, K., and Caputo, A. (1956).—Die Anwendung der Hochspannungspherographie bei der quantitativen Totalanalyse von Proteinhydrolysaten. *Biochem. Z.* **328**: 376–85.

DRAKE, C., GRANT, A. B., and HARTLEY, W. J. (1960).—Selenium and animal health. Pts. I and II. *N.Z. Vet. J.* **8**: 4–10.

GANJE, T. J., and WHITEHEAD, E. I. (1958).—Selenium uptake by plants as affected by the forms of selenium in the soil. *Proc. S. Dak. Acad. Sci.* **37**: 85–8.

HOAGLAND, D. R., and ARNON, D. I. (1938).—The water culture method for growing plants without soil. Circ. Calif. Agric. Exp. Sta. No. 347.

HORN, M. J., and JONES, D. B. (1941).—Isolation from *Astragalus pectinatus* of a crystalline amino acid complex containing selenium and sulphur. *J. Biol. Chem.* **139**: 649–60.

JACOBSEN, L. (1951).—Iron complexes in nutrient solutions. *Plant Physiol.* **26**: 411–13.

JOHNSON, R. R. (1952).—Experiments with selenium-79 and selenium-75 preparatory to studies of the metabolic pathways of selenium in plants using radioisotopes. M.Sc. Thesis, S. Dakota State College of Agriculture and Mechanical Arts.

KLUG, H. L., and PETERSEN, D. F. (1949).—The reaction of selenous acid with cysteine. *Proc. S. Dak. Acad. Sci.* **28**: 87–91.

KNIGHT, C. S. (1960).—A new two-dimensional paper chromatographic method applied to the separation of amino acids. *Nature* **184**: 1486–7.

LAKIN, H. W., WILLIAMS, K. T., and BYERS, H. G. (1938).—"Nontoxic" seleniferous soils. *Ind. Eng. Chem.* **30**: 599–600.

MARKHAM, R., and SMITH, J. D. (1952).—The structure of ribonucleotides. I. Cyclic nucleotides produced by ribonuclease and by alkaline hydrolysis. *Biochem. J.* **52**: 552–7.

MARTIN, A. L., and TRELEASE, S. F. (1938).—Absorption of selenium by tobacco and soy beans in sand cultures. *Amer. J. Bot.* **25**: 380–5.

MILLER, J. T., and BYERS, H. G. (1937).—Selenium in plants in relation to its occurrence in soils. *J. Agric. Res.* **55**: 59–68.

MOUSTAFA, E. M. (1959).—Paper electrophoretic separation of the proteins of some leguminous seeds. *Nature* **183**: 199.

MOXON, A. L., OLSON, O. E., and SEARIGHT, W. V. (1950).—Selenium in rocks, soils, and plants. Tech. Bull. S. Dak. Agric. Exp. Sta. No. 2.

NEUMANN, N. (1960).—In discussion of paper by Ray, W. J., and Koshland, D. E.—Comparative structural studies of phosphoglucomutase and chymotrypsin. In "Protein Structure and Function." Brookhaven Symposia in Biology. Vol. 13. pp. 135–50.

OSTROWSKI, W., SKARZYNSKI, B., and SZCZEPKOWSKI, T. W. (1958).—Identification of organic sulphur compounds formed during thiosulphate oxidation by *Thiobacillus thioparus*. Proc. 2nd U.N. Conf. on Peaceful Uses of Atomic Energy, Geneva. Vol. 24. Pt. 1. pp. 43–6.

PAINTER, E. P. (1941).—The chemistry and toxicity of selenium compounds with special reference to the selenium problem. *Chem. Rev.* **28**: 179–213.

PATTERSON, E. L., MILSTREY, R., and STOKSTAD, E. L. R. (1957).—Effect of selenium in preventing exudative diathesis in chicks. *Proc. Soc. Exp. Biol. Med.* **95**: 617–20.

PETERSEN, D. F. (1951).—The reaction of selenious acid with glutathione. *Proc. S. Dak. Acad. Sci.* **30**: 53–6.

PETERSON, P. J., and BUTLER, G. W. (1962).—Paper chromatographic and electrophoretic systems for the identification of sulphur and selenium amino acids. *J. Chromatogr.* (In press.)

PIRIE, N. W. (1956).—A disintegrator for use with small quantities of fresh leaves. *J. Agric. Engng. Res.* **1**: 81–2.

SCHULTZE, M. O. (1960).—Nutrition. *Annu. Rev. Biochem.* **29**: 391–412.

SCHWARZ, K. (1960).—Factor 3, selenium and vitamin E. *Nutr. Rev.* **18**: 193–7.

SCHWARZ, K., and FOLTZ, C. M. (1957).—Selenium as an integral part of factor 3 against dietary necrotic liver degeneration. *J. Amer. Chem. Soc.* **79**: 3292.

SHARMAN, G. A. M. (1960).—Selenium in animal health. *Proc. Nutr. Soc.* **19**: 169–72.

SHRIFT, A. (1958).—Biological activities of selenium compounds. *Bot. Rev.* **24**: 550–83.

SMITH, A. L. (1949).—Separation of the selenium compounds in seleniferous plant protein hydrolysates by paper partition chromatography. M.Sc. Thesis, S. Dakota State College of Agriculture and Mechanical Arts.

STANGE, L., MOSES, V., and CALVIN, M. (1960).—Fractionation of the insoluble material of *Chlorella* cells. *Biochim. Biophys. Acta* **42**: 272–82.

STEKOL, J. A. (1942).—Selenium tetracysteine. *J. Amer. Chem. Soc.* **64**: 1742.

TAGWERKER, F. J. (1960).—Vitamin E and selenium in animal and poultry nutrition. Pts. I and II. *Agric. Vet. Chem.* **1**: 23–6, 78–84.

TRELEASE, S. F., and BEATH, O. A. (1949).—"Selenium." (Published by the authors: New York.)

TRELEASE, S. F., DI SOMMA, A. A., and JACOBS, A. L. (1960).—Seleno-amino acid found in *Astragalus bisulcatus*. *Science* **132**: 3427.

TRELEASE, S. F., and TRELEASE, H. M. (1938).—Selenium as a stimulating and possibly essential element for indicator plants. *Amer. J. Bot.* **25**: 372–80.

WANG, D. (1960).—An ion-exchange resin method for the fractionation of alcoholic plant extracts. *Nature* **186**: 326–7.

WATKINSON, J. H. (1960).—Fluorometric determination of traces of selenium. *Analyt. Chem.* **32**: 981–3.

WHITEHEAD, E. I., HENDRICK, C. M., and MOYER, F. M. (1955).—Studies with selenium 75. II. Comparison of selenium and sulphur metabolism in wheat. *Proc. S. Dak. Acad. Sci.* **34**: 52–7.