THE ENTRY OF SELENIUM INTO RABBIT PROTEIN FOLLOWING THE ADMINISTRATION OF $Na_2{}^{75}SeO_3$*

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Recently evidence was offered that selenium supplied orally to lactating ewes, as $Na_2{}^{75}SeO_3$, was partly incorporated into milk proteins as selenomethionine (Godwin, Handreck, and Fuss 1971). Previous studies, claiming similar conversion of inorganic selenium in animal tissues, have been based on the demonstration of ^{75}Se activity in the vicinity of certain sulphur analogues during chromatographic procedures (McConnell and Wabnitz 1957; Rosenfeld 1962).

Cummins and Martin (1967) failed to show the presence of selenoamino acids in rabbit liver protein, and Jenkins (1968), working with the chick, concluded that inorganic selenium was not incorporated into plasma proteins as selenoamino acid. Jenkins suggested that most of the protein-bound selenium was present in covalent selenium disulphide binding. However, neither Jenkins nor Cummins and Martin accounted for all of the ^{75}Se activity bound to protein.

By using similar isolation techniques to those used in the study with ewes (Godwin, Handreck, and Fuss 1971), evidence has now been obtained that some of the inorganic selenium administered to rabbits is present after a time as selenoamino acid.

Two rabbits were used, the first was given ^{75}Se-labelled sodium selenite only, and the second was given ^{75}Se-labelled sodium selenite and ^{35}S-labelled methionine simultaneously. The object of this combined administration was to be able to show whether ^{75}Se and ^{35}S compounds were being convincingly separated, and also to ensure that the methods of separation used were applicable to amino acids of the methionine type.

Experimental

The first rabbit was given ^{75}Se (1 mCi) as $Na_2{}^{75}SeO_3$‡ by stomach tube; it was mixed with 0·5 mg of carrier. The animal was killed after 48 hr. The second rabbit received intravenously ^{75}Se (0·25 mCi) as $Na_2{}^{75}SeO_3$ and in the same injection ^{35}S (0·17 mCi) as methionine. This second rabbit was killed after 24 hr. Following administration of the isotopes, blood samples (5 ml) were taken every 2 or 4 hr throughout the experimental periods. Samples were centrifuged immediately and plasma radioactivity measured.

At slaughter anaesthesia was induced using paraldehyde (1·7 ml/kg) and the carotid artery was cannulated so that as much blood could be collected as possible. Serum was stored at −20°C to await analysis. Liver and kidney were quickly plunged into dry ice–acetone mixture and stored at −20°C to await analysis.

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‡ Obtained from Radiochemical Centre, Amersham, U.K.

As in the previous study on the ewe (Godwin, Handreck, and Fuss 1971) all digestions were carried out enzymically using Pronase,* an extract of *Streptomyces griseus*, since acid hydrolysis is known to destroy both selenocystine and selenomethionine (Painter 1941; Blau 1961).

Serum was incubated with one volume of 0·1M phosphate buffer at pH 7·4 in the presence of 0·1% Pronase and 0·01% chloramphenicol. Digestion was allowed to proceed for 2–3 days and was carried out under nitrogen at 40°C.

Liver and kidney were homogenized, the fat extracted using ethanol and ether, and the extracted homogenate subsequently incubated under similar conditions to the serum.

Following Pronase digestion undigested protein was precipitated with ethanol or acetone and the soluble fraction reduced by rotary evaporation at 40°C. The residue was taken up in 0·1N HCl and the resultant solution used for column chromatography.

Firstly a large fraction of the digest solution was put through a large-capacity Sephadex G10 column (100 cm long, 5 cm diam.), using water which was free of dissolved oxygen as eluent. Nitrogen was bubbled through the system continuously after passing through ascorbic acid. Elution diagrams were drawn showing peaks of radioactivity and from these fractions were selected for pooling and further separation on manually operated amino acid analyser columns. Again, as in the previous study (Godwin, Handreck, and Fuss 1971), commercial-type amino acid analysers were not used because all of the ^{75}Se activity could not be eluted from the column. Two manual columns were operated, the first contained a resin EEL 120/CG, supplied by Electroselenium Ltd., and the other Aminex MS, fraction D, supplied by Bio-Rad; both were fine-mesh, sulphonated, cross-linked, polystyrene resins. Elution buffers, pH 3·25 and 4·25, were made up according to Moore, Spackman, and Stein (1958).

Some gas–liquid chromatography (GLC) was carried out; the system has already been described (Godwin, Handreck, and Fuss 1971).

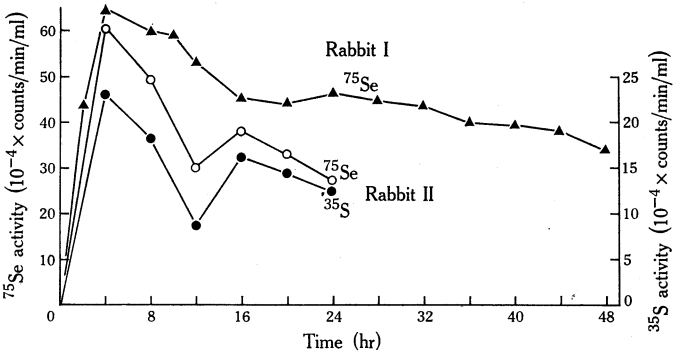


Fig. 1.—Rabbit I received by stomach tube 1 mCi ^{75}Se as $Na_2{}^{75}SeO_3$. Rabbit II received intravenously 0·25 mCi ^{75}Se as $Na_2{}^{75}SeO_3$ and 0·17 mCi ^{35}S as [^{35}S]methionine simultaneously. ^{75}Se and ^{35}S activities were measured on the same sample.

Results

The rise in radioactivity in rabbit plasma, following oral and intravenous administration of $Na_2{}^{75}SeO_3$, is shown in Figure 1. On the same figure is shown the radioactivity derived from the simultaneous intravenous injection of ^{35}S-labelled methionine.

A portion of the Pronase digest of serum taken from the first rabbit, containing 49,000 counts/min, was evaporated to dryness and redissolved in 0·2 ml citrate

* A protease supplied by Koch-Light Ltd., Colnbrook, Bucks, England.

buffer, pH 2·1. This was injected on to the manually operated amino acid analyser column and it gave the elution diagram shown in Figure 2.

The majority of the activity was associated with a peak which corresponded to the position of selenite ions; this was checked by running selenite alone, or mixed with selenoamino acids, through the column. There were other small peaks of ^{75}Se activity in the chromatogram as can be seen from Figure 2. Most of the fractions had some activity associated with them.

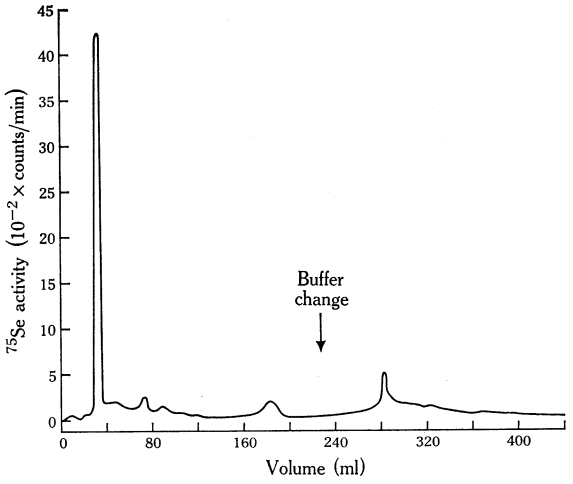


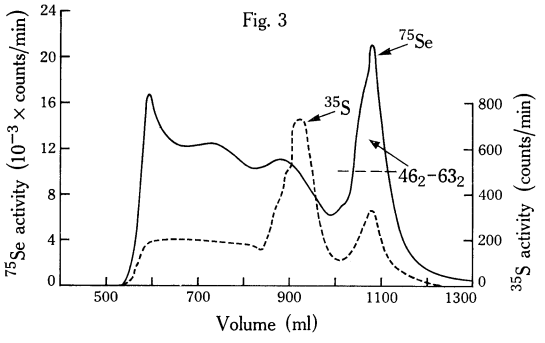
Fig. 2.—A sample of a whole serum Pronase digest was injected on to a manually operated amino acid analyser column. The column was maintained at room temperature and buffers of pH 3·25 and 4·25 were used for elution. The buffer change was effected at 240 ml.

Serum from the second rabbit (26 ml taken at slaughter) was enzymically digested before being passed through the large Sephadex G10 column. The activity in the 26-ml sample was 7×10^6 counts/min; 14% was precipitated with the undigested protein fraction. Of the remaining counts in the soluble "crude amino acid" fraction $2{\cdot}26 \times 10^6$ counts/min were placed on the G10 column. The elution diagram obtained is shown in Figure 3. None of the peaks eluted at the position of either selenocystine or selenomethionine. The major ^{75}Se peak (fractions 46_2–63_2 in Fig. 3) was examined by GLC but no active fractions could be detected in the effluent.

Liver from the first rabbit was homogenized in phosphate buffer and digested with Pronase, and a chromatographed sample showed that the majority of the activity was present as selenite.

Liver from the second rabbit from which fat was extracted was used for Sephadex chromatography as follows. The total weight of the liver was 56 g, with a ^{75}Se activity of 84×10^6 counts/min. The ethanol–ether fat extraction removed material with an activity of 5×10^6 counts/min. The dried and defatted liver was digested for 48 hr with Pronase; less than 5% of the remaining activity was precipitated with the undigested protein fraction. A sample of the soluble "crude amino acid" fraction containing 6×10^6 counts/min of ^{75}Se activity was separated on the large Sephadex G10 column. The elution diagram is shown in Figure 4. Although the elution pattern is similar to that of serum (Fig. 3) the major difference is in the position of the last ^{75}Se peak (fractions 26_2–42_2). It is

separated, though not completely, from the methionine peak. The fractions comprising this peak were combined and used for amino acid analysis. The result is shown in Figure 5. The important differences from the chromatogram of whole liver digest (Fig. 2) are that the majority of the activity was not in the position of inorganic selenite, and that two unidentified peaks of ^{75}Se activity are present, well separated from the methionine peak. The latter appeared in the expected position and agreed well with authentic standard methionine.



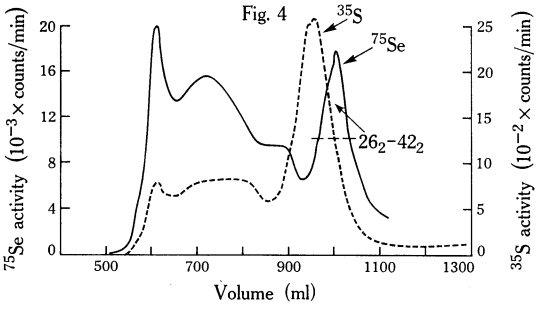


Fig. 3.—Serum Pronase digest contained both ^{75}Se and ^{35}S radioactivity. The diagram is based on measurement of both isotopes in all fractions. 4 ml digest was placed on the column and water was used as eluent to minimize degradation of ^{75}Secom pounds. The numbers (46_2–63_2) are fraction numbers comprising the peak.

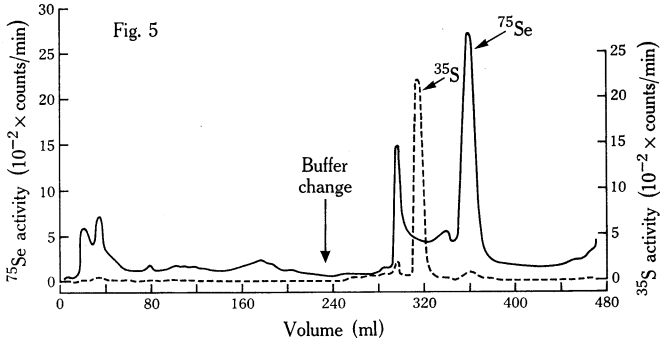
Fig. 4.—Similar elution diagram to Figure 3. 4 ml of a liver Pronase digest was used.

Finally, kidney from the second rabbit from which fat was extracted was enzymically digested. The weight of the combined kidneys was 17 g, and the total ^{75}Se activity was 23×10^6 counts/min. Of these, $1{\cdot}5 \times 10^6$ counts/min were removed by the ethanol–ether extraction. Following Pronase digestion, over 50% of the ^{75}Se activity was precipitated as undigested protein (cf. $< 5\%$ for liver). Of the soluble "crude amino acid" fraction 1×10^6 counts/min were separated on the Sephadex G10 column. The elution diagram is shown in Figure 6.

The first ^{75}Se peak (fractions 94_1–19_2) corresponds to the position of selenocystine, as will be seen by comparing the elution pattern with that of a standard run (see Fig. 7). The second peak (fractions 67_2–83_2), well separated from both "seleno-

cystine" and methionine (the ^{35}S peak) is an unidentified peak which elutes somewhat later than selenomethionine (cf. Fig. 7). The fractions comprising the first peak (94_1–19_2), eluting at the position of selenocystine, were combined, the volume reduced by rotary evaporation, and a sample used for amino acid analysis. The presence of a peak in the position of selenocystine was confirmed.

Attempts were made to further confirm the presence of selenocystine in the Sephadex fraction by GLC, after forming the bis(trimethylsilyl)trifluoroacetamide



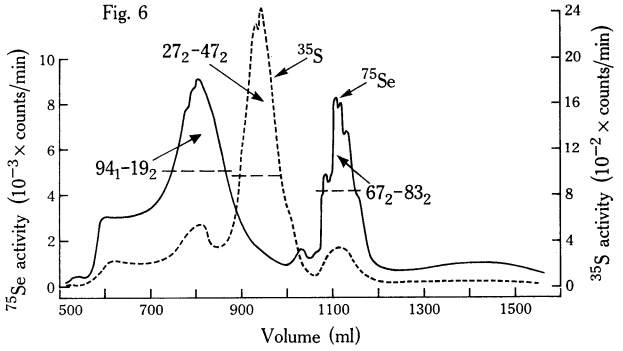


Fig. 5.—Elution pattern derived from placing a sample of fractions 26_2–42_2 (see Fig. 4) on the amino acid analyser column. Conditions were the same as for Figure 2.

Fig. 6.—Elution diagram, based on radioactivity of samples, for kidney Pronase digest, on a Sephadex G10 column. 4 ml digest was used and water was used as eluent.

derivative. This proved unsuccessful as when authentic ^{75}Se-labelled selenocystine was derivatized not only did no characteristic peak appear but radioactivity could not be recovered in any of the effluent fractions.

The second peak derived from the ^{75}Se-labelled kidney Pronase digest (fractions 67_2–83_2, Fig. 6) gave two peaks on the amino acid analyser. One, the smaller, eluted near methionine, the other eluted later at 384 ml, and accounted for most of the ^{75}Se in the fractions. Neither peak has been identified.

Discussion

The evidence presented suggests that, following the administration of inorganic selenium as $Na_2{}^{75}SeO_3$ to the rabbit, whilst after 24 or 48 hr much of the selenium can only be recovered as selenite selenium, some is incorporated into protein as selenoamino acid. On the basis of a two-stage column chromatographic separation, involving Sephadex chromatography and amino acid analyses on sulphonated polystyrene columns, it is provisionally concluded that selenocystine is present in rabbit kidney. A number of other amino acid-like peaks were present in liver, kidney, and plasma, but further work must be done to identify them.

An empirical estimation of the amount of the activity incorporated into the kidney which is actually present as selenocystine indicates that it is a very small percentage, approximately 0·5%. It is impossible to say, at this stage, whether this conversion is of physiological significance to the animal. Dickson and Tappel (1969) in an *in vitro* study using selenocystine and selenomethionine have shown that these amino acids have marked effects on certain enzyme systems at very low concentrations.

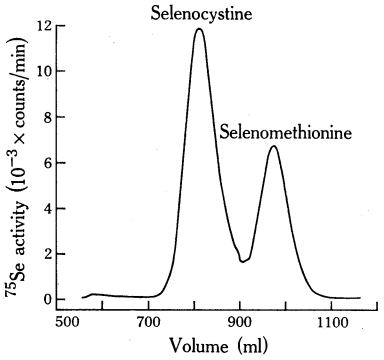


Fig. 7.—Separation of authentic ^{75}Se-labelled selenocystine and selenomethionine on a Sephadex G10 column, using water as eluent. It will be seen that the elution position of the peak "94_1–19_2" in Figure 6 corresponds to the position of selenocystine in this figure.

The occurrence of a number of "unknown" ^{75}Se-active peaks was a feature of a similar study of plant material made by Peterson and Butler (1962). They did have the advantage, because of the higher concentrations of selenium in plant material, of being able to isolate some selenoamino acid in crystalline form. Even so, they reported a number of unidentified peaks and some for which only provisional identification was offered, e.g. selenocysteic acid, selenocysteine seleninic acid, *Se*-methylselenomethionine selenonium salt. Selenocystathionine has been isolated from the selenium accumulator plant *Astragalus pectinatus* by Horn and Jones (1941), and *Se*-methylselenocysteine has been found in *A. bisulcatus* by Trelease, di Somma, and Jacobs (1960).

It is essential to consider the findings reported in this paper in the light of the conclusion drawn by Cummins and Martin (1967) that "in the rabbit there is no biosynthetic pathway by which selenium as selenite can replace sulphur in cystine and methionine". There appear to be two important differences in the experiments. The use of Sephadex chromatography has allowed subsequent amino acid analysis to be done on a greatly enriched "selenoamino acid" fraction. Although, contrary to the findings of Cummins and Martin we did find, even with amino acid analysis of a sample of the whole liver Pronase digest, that whilst the majority of the selenium

eluted at the position of selenite there was some evidence of peaks eluting much later.

The second difference is that all of the observations made by Cummins and Martin that suggested in any way the presence of selenoamino acids could be satisfactorily explained by the observed phenomenon of binding between certain sulphur compounds and selenite (Schwarz and Sweeney 1964). In this study Sephadex chromatography and elution under neutral aqueous conditions have allowed separation of ^{75}Se and ^{35}S peaks. When authentic labelled selenite was added to a number of mixtures separated in the course of this work, it was always possible to recover virtually all the selenite as a separate peak.

It is concluded that whilst the majority of a given dose of inorganic selenium is not converted to selenoamino acid within the rabbit after 48 hr, a small proportion of it is. One of the amino acids that can be separated from kidney Pronase digest is selenocystine. Other amino acid-like selenium compounds exist, but they have not yet been identified.

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