A Comparison of the Uptake of $^{75}\text{Se} \text{Selenite}$, $^{75}\text{Se} \text{Selenomethionine}$ and $^{35}\text{S} \text{Methionine}$ by Tissues of Ewes and Lambs

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Abstract
The fate of selenium, given as Na$_2$$^{75}$SeO$_3$, or $^{75}$Se selenomethionine, and of $^{35}$S methionine administered intravenously to ewes and lambs, has been examined. The main intention was to follow the incorporation of selenium into protein in a number of tissues, including liver and kidney, and to measure the extent of that incorporation of selenoamino acid, particularly with respect to the administration of selenite. The ewes chosen were lactating ewes with lambs at foot, and the lambs were animals which had been weaned on to fodder low in selenium and were recovering from white muscle disease with selenium therapy. These two experimental situations were chosen as they offered conditions under which selenium incorporation might be considered to be maximal.

Entry of isotope into milk was rapid and was greater when $^{75}$Se was given as the selenoamino acid than as selenite. In both ewes and lambs greater amounts of activity, derived from selenite, were bound to plasma proteins than to the proteins of milk. This was particularly evident in samples taken some hours after administration. This ability of the plasma to bind selenium was demonstrated by alkaline dialysis.

Small, though significant amounts of selenium, derived from Na$_2$$^{75}$SeO$_3$, were incorporated as selenoamino acids into the proteins of liver, kidney and pancreas, as well as into the proteins of milk and plasma. In ewes, both selenomethionine and selenocystine were identified chromatographically in enzyme digests of defatted liver and kidney. Some differences occurred in the distribution of labelled compounds in organs from lactating ewes and recovering lambs.

The incorporation of selenium into protein is discussed briefly in relation to the recent findings of an association between selenium and the enzyme glutathione peroxidase.

Introduction
The entry of inorganic selenium into animal proteins in vivo was under investigation in 1950 (McConnell and Cooper 1950), almost a decade before its importance as a trace element in nutrition was recognized (Schwarz and Foltz 1957). This last report led immediately to the demonstration of the effectiveness of selenium against white muscle disease in sheep (Muth et al. 1958).

McConnell's early work was followed by a series of papers in which the general conclusion was reached that part of the administered inorganic selenium is incorporated as seleno-analogues of the sulphur-containing amino acids (S-amino acids). These findings were criticized by Cummins and Martin (1967) and by Jenkins (1968) on the grounds that evidence for selenoamino acids was the presence of $^{75}$Se activity in the vicinity of S-amino acids in certain chromatographic procedures. Their criticism was supported by the observation that inorganic selenium ions could bind to S-amino acids (Schwarz and Sweeny 1964).

In an attempt to elucidate these differences we gave Na$_2$$^{75}$SeO$_3$ to lactating ewes and showed the presence of $^{75}$Se selenomethionine in enzyme digests of milk proteins.
by using a combination of gel–liquid and gas–liquid chromatography (Godwin et al. 1971). More recently we have presented evidence for the conversion of inorganic selenium to selenoamino acid in the rabbit (Godwin and Fuss 1972).

The present experiments were a continuation of the enquiry into the fate of inorganic selenium in the lactating ewe, and the experiments include observations made on lambs recovering from white muscle disease by means of sodium selenite administration.

The questions asked were (i) could selenoamino acids, derived from the intravenous administration of selenite, be found in different tissues; (ii) how does the entry and distribution of labelled selenite compare with that of labelled selenomethionine and methionine; (iii) are there differences in behaviour of labelled selenite in lactating ewes when compared with growing lambs recovering from selenium deficiency; and (iv) how much of the administered selenite becomes incorporated into selenoamino acid?

Materials and Methods

(a) Animals

(i) Three lactating Merino ewes, with lambs at foot, were selected. For the duration of the experimental period each lamb was housed next to its dam but did not have access to it. By this means the dam remained quiescent and milk flow did not fall off. The ewes were fed a standard ration (2 parts wheaten-hay chaff and 1 part lucerne) and water was supplied.

(ii) Three Merino lambs, which had been weaned onto pelleted fodder low in selenium from a selenium-responsive area on Kangaroo Island, S.A., were used. When clinically selenium-deficient, each lamb was given 3 mg selenium as sodium selenite by drench a few days prior to the administration of isotope.

(b) Treatment and Sampling

(i) Lactating ewes. The three ewes were given respectively 3 mCi $^{75}$Se activity as Na$_2$$^{75}$SeO$_3$*, 0.45 mCi $^{75}$Se activity as selenomethionine and 0.45 mCi $^{88}$S activity as methionine. All isotopes were given intravenously. No carrier was used and the amounts of metabolites were between 0.01 and 0.1 mg.

Blood and milk samples were taken at 2, 4, 8 and thereafter every 4 h throughout the 48-h experimental period. A few minutes before milking 0.5 ml oxytocin (Oxytocin-S, Organon Labs, Ltd, Surrey, U.K.) was given intravenously, and this allowed easy emptying of the mammary glands each time. Blood and milk were centrifuged immediately and the radioactivity of plasma and skimmed milk determined.

At the end of the 48-h period each ewe was anaesthetized and bled to death from a cannula inserted in the carotid artery. Serum was separated and stored at $-20^\circ$C, together with the liver, pancreas and kidneys of each animal. The organs were removed rapidly and frozen in dry ice and acetone.

(ii) Recovering lambs. Two lambs were each given approximately 1 mCi $^{75}$Se activity as Na$_2$$^{75}$SeO$_3$, and a third lamb was given almost 1 mCi $^{75}$Se activity as selenomethionine. No carrier was used and the isotopes were administered intravenously in normal saline. Blood samples were taken at 10 min, 1 h and hourly till 8 h, then at 10, 12, 16 h and 4-hourly thereafter, through the 28-h period. Faeces and urine were collected at the above times, so that an estimate of activity retained in the animal at slaughter could be made.

The lambs were anaesthetized and bled to death in the same way as the ewes. Serum was separated and stored at $-20^\circ$C and the livers and kidneys, frozen in acetone and dry ice, were stored at $-20^\circ$C.

(c) Analytical Procedures

(i) Dialysis. Two different systems were used. The first was a conventional system using Visking tubing and was described in an earlier study (Godwin et al. 1971). The second was a negative

* All isotopes were supplied by Radiochemicals Centre, Amersham, U.K.
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pressure system where dialysis takes place through a thick collodion bag (Sartorius Membranfilter, Göttingen). The pore size is rated at 8 nm and the retentive capacity is quoted at a molecular weight of 25000. The conditions under which dialysis was carried out are specified later in the text.

(ii) Digestion of samples. All digestions were carried out enzymically using the preparation Pronase (Koch-Light, Colnbrook, U.K.). The reasons for this are discussed in an earlier paper (Godwin et al. 1971). Serum and skimmed milk were diluted 1:1 with phosphate buffer (0.1M, pH 7-4) and Pronase was added to give a final concentration of 0.1%. Chloramphenicol was added to give 0.01% concentration. The mixtures were incubated at 38°C for 2-3 days. Nitrogen was bubbled through and the mixtures constantly stirred.

Liver and kidney were rendered fat-free by Soxhlet extraction with ether and ethanol, and then suspended in phosphate buffer as a 10% suspension, together with Pronase and chloramphenicol. In the lamb experiment the fat-free tissues were subjected to phosphate buffer and trichloroacetic acid extractions prior to enzyme digestion.

Undigested protein, following incubation, was precipitated with ethanol and after rotary evaporation the crude amino acid fraction was taken up in 0.1M HCl.

(iii) Column chromatography. The systems employed have been described already (Godwin et al. 1971). Sephadex chromatography, using grade G10, with water as eluent to avoid loss of seleno-compounds, was carried out with a column 100 cm long and 5 cm diameter.

In the experiments with ewes, amino acid analyses were performed manually, using columns of fine-mesh, sulphonated, cross-linked polystyrene resins. The buffer system of Moore et al. (1958) was used, as described earlier by Godwin and Fuss (1972).

Amino acid analyses in the experiment with lambs were made on a Jeol amino acid analyser using Aminex resin, fraction B. The buffer was sodium citrate, 0.2M with respect to sodium, and its pH was 3.26 for application and 4.25 for elution. The operating temperature was 52°C.

Gas-liquid chromatography was conducted using a Pye Panchromatograph which was adapted for the collection of effluent fractions. A stream-splitter was fitted and open-ended tubes filled with glass wool were positioned for condensation of the non-combusted fraction.

The column packing was Chromosorb G, 80-100 mesh coated with OV-17 (1·35 g per 28·65 g solid phase). The injection port was held at 240°C and a temperature program was used with a 7-min hold at 75°C, and a 6°C/min rise to 235°C. Essentially the method of Gehrke et al. (1969) was followed.

(iv) Absorption of amino acids onto Dowex-50. As an independent measure of the proportion of radioactivity likely to be present in a digest as selenoamino acids, a system of purification was set up using Dowex-50, 200-400 mesh. The preparation of the two columns used is described in detail by Burchfield and Storrs (1962). The two columns are connected in tandem with the Dowex in the ammonium form in the upper column, and in the acid form in the lower. Neutral and acidic amino acids can be eluted from the acid column with 8-10 ml 2M NH₄OH if the dimensions of the column are kept small enough.

Results

Fig. 1 shows the levels of activity found in plasma and milk in the three ewes. Fig. 2 shows the plasma radioactivity levels in one of the [75Se]selenite-treated lambs and in the lamb given [75Se]selenomethionine.

The samples upon which Fig. 1 is based were subjected to dialysis against normal saline for 24 h, followed by water for 24 h and then 0.2M NaOH. Visking tubing was used and the dialysis continued for 12 or more days. In Table 1 are given the percentages of radioactivity retained when plasma samples from each of the three ewes were dialysed. Data are presented in Table 2 for the similar dialysis of milk samples. The complete sets of data on activity after dialysis are presented in the graphs shown in Fig. 3.

Dialysis of plasma and milk samples under alkaline conditions using the Sartorius membrane system gave essentially the same results as those obtained using Visking tubing. When the Sartorius membrane system was used, 2 ml samples were dialysed against 60 ml of 0.2M NaOH, pH 11·5, with reduced pressure applied.
Fig. 1. Three lactating ewes were injected (intravenously) with (a) 3 mCi $^{75}$Se as Na$_2$$^{75}$SeO$_3$, (b) 0.45 mCi $^{75}$Se as selenomethionine and (c) 0.45 mCi $^{85}$S as methionine at zero time. Activities in milk (-----) and plasma (----) were plotted for samples taken at 2 and 4 h and thereafter 4-hourly over a period prior to slaughter of the ewes.

Fig. 2. Two lambs recovering from white muscle disease were injected (intravenously) with approximately 1 mCi of either Na$_2$$^{75}$SeO$_3$ (-----) or $[^{75}$Se]selenomethionine (----). Activities in plasma were plotted in samples taken over the 27-h period following injection.
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Table 1. Percentage of activity retained during dialysis of plasma samples through Visking tubing over a period of 12 days

<table>
<thead>
<tr>
<th>Sampling time (h)</th>
<th>Dialysis with NaCl, followed by water, 24 h each</th>
<th>Dialysis with 0·2M NaOH for 2 days</th>
<th>Dialysis with 0·2M NaOH for 10 days</th>
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<tr>
<td></td>
<td>S</td>
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<tr>
<td>2</td>
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</tr>
<tr>
<td>Average</td>
<td>93</td>
<td>96</td>
<td>96</td>
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Table 2. Percentage of activity retained during dialysis of milk samples through Visking tubing over a period of 9–11 days

<table>
<thead>
<tr>
<th>Sampling time (h)</th>
<th>Dialysis with 0·9% NaCl, followed by water, 24 h each</th>
<th>Dialysis with 0·2M NaOH for 9–11 days</th>
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<tr>
<td></td>
<td>S</td>
<td>S-M</td>
</tr>
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<td>69</td>
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<td>48</td>
<td>87</td>
<td>73</td>
</tr>
<tr>
<td>Average</td>
<td>76</td>
<td>74</td>
</tr>
</tbody>
</table>

At the end of the dialysis period, both types of dialysis membrane were checked for permeability to small molecular moieties. When either labelled selenomethionine or part of an enzyme digest of kidney or liver was dialysed, both systems allowed over 70% of the activity to pass through in the first hour.
Pronase digests of serum, milk, liver and kidney from the ewe which was given $^{75}\text{Se}$selenomethionine were examined. Using Sephadex G10 chromatography, followed by amino acid analysis on manually operated columns and gas–liquid chromatography on the peaks of activity from Sephadex columns, the presence of selenomethionine in each of these tissues was confirmed. Similarly, the presence of methionine was demonstrated in the milk and kidney obtained from the ewe injected with $^{35}\text{S}$methionine. These analyses provided data against which the results from the ewe given selenite could be evaluated.

Serum from the ewe given $^{75}\text{Se}$selenite was enzymically digested and the resulting crude amino acid fraction was subjected to column chromatography. Using the Sephadex G10 column, a peak corresponding to the position of selenocystine was eluted. Subsequent amino acid analysis of this Sephadex fraction showed a peak of $^{75}\text{Se}$ activity in the position of selenocystine.

The acid-soluble fraction obtained after enzymic digestion of skimmed milk from the $^{75}\text{Se}$selenite-treated ewe gave three major peaks of $^{75}\text{Se}$ activity on Sephadex G10. Amino acid analysis of the second and third peaks from the Sephadex column showed peaks corresponding to selenocystine and selenomethionine respectively. When the liver from this ewe was examined it was shown to have a Sephadex peak in the position of selenomethionine and both amino acid analysis and gas–liquid chromatography confirmed the presence of selenomethionine in this peak. There was also a peak in the position of selenocystine on Sephadex (see Fig. 4).

Similarly, the kidney was shown to contain selenomethionine by the three chromatographic procedures. A selenocystine peak was shown by Sephadex chromatography followed by amino acid analysis (Fig. 4). As yet, the $N$-trimethylsilyl ester of selenocystine has not been successfully prepared and recovered from the gas–liquid chromatography column.
Table 3 shows the estimated percentage of selenoamino acid present in various tissues of the ewes given either $[^{75}\text{Se}]$selenite or $[^{75}\text{Se}]$selenomethionine. These percentages are calculated on the basis of the amount of $^{75}\text{Se}$ activity found in the corresponding Sephadex and subsequent amino acid analyser peaks.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Selenite-treated Seleno-</th>
<th>Selenomethionine-treated Seleno-</th>
<th>Seleno-</th>
<th>Seleno-</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>methionine</td>
<td>cystine</td>
<td></td>
<td>cystine</td>
</tr>
<tr>
<td>Serum</td>
<td>——</td>
<td>1·2</td>
<td>14·8</td>
<td>Nil</td>
</tr>
<tr>
<td>Milk</td>
<td>0·31</td>
<td>——</td>
<td>21·0</td>
<td>5·0</td>
</tr>
<tr>
<td>Kidney</td>
<td>0·24</td>
<td>0·33</td>
<td>4·7</td>
<td>Nil</td>
</tr>
<tr>
<td>Liver</td>
<td>0·51</td>
<td>0·62</td>
<td>13·8</td>
<td>Nil</td>
</tr>
</tbody>
</table>

Corresponding peaks were eluted from Sephadex columns but could not be confirmed on polystyrene columns.

The results from the lamb experiment were similar, though not identical. The kidney from the lamb given $[^{75}\text{Se}]$selenite gave the elution pattern on Sephadex G10 shown in Fig. 5. The presence of selenomethionine in peak II was confirmed using the Jeol amino acid analyser, when $^{75}\text{Se}$ activity was shown in the position of selenomethionine, which is eluted at 62 min under the buffer system described earlier. The position of peak I corresponds to that of selenocystine, but there was insufficient $^{75}\text{Se}$ activity available for the effluent from the Jeol amino acid analyser to show a radioactive peak in the position of selenocystine. The latter elutes at 51 min.
Fig. 6. (a) Chromatographic separation of $^{75}$Se-labeled selenomethionine and Na$_2^{75}$SeO$_3$ standards (1 mg each) on a Dowex-50 column using the eluents as shown. Chromatographic separation of active peaks from enzyme digests of (b) defatted liver and (c) pancreas, taken from an ewe receiving 3 mCi Na$_2^{75}$SeO$_3$ 48 h before slaughter. Comparing the position of the peaks with those in a, as is discussed in the text, a considerable proportion of the activity elutes with the amino acid fraction.

— Ammonium column. —— Acid column.
The liver from the lamb given \([^{75}\text{Se}]\text{selenite}\) differed from that of the corresponding ewe, in that Sephadex chromatography showed the presence of selenocystine and not selenomethionine (see Fig. 5).

As an independent check of the amount of activity present in the enzyme digests as selenoamino acid-like material, a number of the digests prepared from the \([^{75}\text{Se}]\text{selenite}\)-treated ewe were applied to the dual column system described by Burchfield and Storrs (1962).

Test runs were made using authentic \([^{75}\text{Se}]\text{selenomethionine}\), \([^{75}\text{Se}]\text{selenite}\) and a combination of these. Also, \([^{75}\text{Se}]\text{selenite}\) was added to a mixture of cystine and methionine before being applied to the column. These results showed that \([^{75}\text{Se}]\text{selenomethionine}\) eluted at 235 ml from the acid column. Some selenite was eluted off the basic column during the water washing, and the remainder came off with the ethanol (see Fig. 6a).

Pronase digests of liver and pancreas were applied to the Dowex-50 columns and the results are shown in Figs 6b and 6c. In the case of the liver, 4–5% of the total activity was absorbed and subsequently eluted from the acid column in the narrow fraction corresponding to selenomethionine. The corresponding value for pancreas was 2–3%.

**Discussion**

Evidence is presented in this paper that selenium, given intravenously as Na\(^{75}\text{SeO}_3\) to a lactating ewe or to a lamb recovering from white muscle disease, is incorporated partially into various proteins as selenoamino acids. By comparing in different animals the behaviour of \([^{75}\text{Se}]\text{selenite}\) with that of \([^{75}\text{Se}]\text{selenomethionine}\) and \([^{75}\text{S}]\text{methionine}\), it has been possible to gain some idea of the extent of the conversion of inorganic selenium to organic form. Other workers have studied the extent of labelling of certain protein fractions, for example, plasma and milk proteins (Jenkins and Hidiroglou 1971), but the primary objective of the experiments reported here has been to identify some of the \(^{75}\text{Se}\)-labelled fractions forming part of the protein. Fractions have been separated from different organs and examined in order to extend the earlier findings reported on ewe’s milk (Godwin et al. 1971).

Pronase digests of serum, milk, liver and kidney of the ewe were shown to contain both selenomethionine and selenocystine following the administration of Na\(^{75}\text{SeO}_3\), whilst liver from lamb contained selenocystine, and kidney contained both selenomethionine and selenocystine. Evidence for selenocystine came from Sephadex chromatography and amino acid analysis, since no characteristic derivative could be obtained for gas–liquid chromatographic separation. Other workers (Caldwell and Tappel 1968) have claimed to have prepared the silylated derivative of selenocystine, but in our experience, using synthetic \(^{75}\text{Se}\)-labelled selenocystine, it was not possible to detect radioactivity associated with effluent fractions, using the technique reported earlier (Godwin et al. 1971) which had been used to identify \([^{75}\text{Se}]\text{selenomethionine}\).

One of the more obvious differences in behaviour between labelled selenite and labelled selenomethionine in the ewes was the apparently selective uptake of the amino acid by the mammary gland. This was also true for labelled methionine. Fig. 1 shows the curves of radioactivity in milk for both selenomethionine and methionine being above that of plasma; the position is reversed for selenite.
From Fig. 2 it is apparent that less activity was lost overall from the plasma of the lamb given selenomethionine when compared with that given selenite. A similar result was apparent with the ewe.

Dialysis of plasma showed that little radioactivity was lost when samples were dialysed for 24 h against 0·9% NaCl. This was followed by a further 24 h against water (see Table 1). The dialysis against NaOH at pH 11·5 led to the loss of $^{75}$Se activity, but it also led to the loss of $^{35}$S activity as Table 1 shows. There is slightly more activity retained after dialysis in the ewes given amino acid than in that receiving selenite. Furthermore, in the case of the selenite-treated ewe, more of the radioactivity was dialysable at the beginning of the 48-h period than at the end.

These observations are in agreement with those of Jenkins and Hidiroglou (1971) who found greater retention of $^{75}$Se derived from selenomethionine than from selenite, in ewe and lamb serum proteins. After prolonged dialysis there was, in each case, an appreciable quantity of non-dialysable activity retained. Our results show that plasma drawn 48 h after administration of the labelled compounds yields about 50% of the activity on prolonged dialysis against 0·2M NaOH, irrespective of whether the compound given was selenomethionine, methionine or selenite.

Millar (1972) found that when $^{75}$Se, given as neutralized selenous acid, was injected intrafemorally into rats, there was more trichloroacetic acid-precipitable $^{75}$Se in serum 72 h after injection than after 3 h.

Dialysis of milk, reported in our experiments, gave similar results to those obtained with plasma. Perhaps the most striking difference, shown in Table 2, is the comparatively large percentage of $^{75}$Se activity that was dialysable from the milk of the ewe given $[^{75}\text{Se}]$selenite. This percentage fell during the 48 h following administration of the isotope (Table 2).

The averaged $^{75}$Se or $^{35}$S activities retained in all samples for each ewe for plasma and milk are shown in Fig. 3. The figure shows that under these conditions there is comparable behaviour in plasma amongst the three radioactive compounds. On the other hand there is a marked difference in behaviour in milk between the selenite-treated and amino acid-treated ewes. The proportion therefore of unbound selenium in milk, following the administration of selenite selenium, is much lower than is found after selenomethionine administration. Plasma appears to contain more selenium-binding protein than does milk. Millar (1972) found that the binding of selenium by rat plasma became more complete with time; this was found to be so with ewe milk in these experiments (Fig. 3).

When the Sartorius membrane system was used for dialysis of plasma and milk samples the results were essentially the same. Selenite-treated ewe milk behaved differently from plasma again, and early samples contained significant amounts of dialysable material compared with later ones.

These experiments demonstrate a similarity of behaviour in the distribution of three somewhat dissimilar compounds, namely selenite, selenomethionine and methionine. Protein binding by all three is widespread, and in a variety of tissue protein fractions some of the selenite is incorporated into the protein as selenoamino acid.

The importance and extent of this incorporation, and the exact nature of the binding of selenium in the tissues, is not yet fully understood. However, recent work by Rottruck et al. (1973) and Flohé et al. (1973) indicates that at least one specific
enzyme, glutathione peroxidase, requires molecular amounts of selenium. Earlier work by Rotruck et al. (1972) introduced the term ‘dietary selenium’, the implication being that under the conditions in which its effectiveness was being observed selenium had to be added to the diet. When selenium was added to the test system in vitro it was ineffective. The particular observations were in relation to autohaemolysis in red cells from selenium-deficient rats.

In general terms, however, it is clear that more understanding is needed of the nature of selenium binding in animal tissues.

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References


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