IDENTIFICATION OF [^{75}Se]SELENOMETHIONINE IN EWE MILK PROTEIN FOLLOWING THE INTRARUMINAL ADMINISTRATION OF $Na^{75}SeO_3$ AS A SINGLE ORAL DOSE

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Abstract

Inorganic selenium, as $Na_2{}^{75}SeO_3$, was given intraruminally to lactating ewes, and for 48 hr afterwards samples of blood and milk were taken regularly. The animals were then slaughtered and tissues taken for further analysis. Evidence is presented, based on isolation techniques using Sephadex chromatography and gas–liquid chromatography, that ^{75}Se is incorporated into milk proteins as selenomethionine. It is estimated that at least 3% of the selenium that enters the milk, from this inorganic source, is present as bound selenomethionine. This is a minimal value, since it has been shown that selenomethionine is easily degraded during a number of the isolation procedures.

The steps followed in arriving at the conclusion were: (1) a large proportion of the ^{75}Se activity in the milk was non-dialysable; (2) activity emerges with the milk protein on Sephadex and can be recovered as "soluble casein" by precipitation with ammonium sulphate; (3) Pronase digestion of the labelled protein produces fragments that behave like methionine and cysteine in column chromatography; and (4) gas–liquid chromatography, including collection of the effluent in fractions, has enabled positive identification of a [^{75}Se]selenomethionine peak.

Other selenoamino acid-like fractions are present but have not yet been identified.

I. Introduction

The importance of trace amounts of selenium in the diet of ruminant and non-ruminant animals is now well established. In 1958, Muth *et al.* administered inorganic selenium to ewes grazing dystrophogenic pasture and reported that lambs born to treated ewes did not develop white muscle disease. Other maladies, such as certain types of ill-thrift in weaners and infertility in ewes, have responded to selenium administration (Hartley and Grant 1961; Hartley 1963; Godwin, Kuchel, and Buckley 1970). Over a decade before the work of Muth *et al.* (1958), Poley *et al.* (1941) had reported a growth response in chicks fed a low level of seleniferous grain.

Despite these findings there is little known of either the function of selenium, or even the forms in which it occurs chemically in animal tissues, particularly with respect to the ruminant. Cummins and Martin (1967) working with the rabbit and Jenkins (1968) with the chick have presented evidence that selenium, administered as $Na_2{}^{75}SeO_3$, is incorporated into protein covalently bound between the sulphurs of half-cystine residues. The conclusion, in each case, was that inorganic selenium is not

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incorporated into the protein of the monogastric animal as selenoamino acid. The former authors reviewed what little evidence there was for the formation of selenoamino acids from inorganic sources in animal tissues, and pointed out that the evidence rested mainly on showing ^{75}Se activity to be associated with the sulphur analogues of the selenoamino acids during certain chromatographic procedures. Pertinent to this problem is the observation of Schwarz and Sweeney (1964) that a number of sulphur compounds can bind the selenite ion.

Shrift (1967) has drawn attention to some of the problems associated with the identification of the selenium analogues of the sulphur-containing amino acids, and suggests that "until more is known" identification based on one or two solvent systems must be taken with reservation.

This paper reports the identification of ^{75}Se-labelled selenomethionine in the milk protein of ewes, following the intraruminal administration of sodium[^{75}Se] selenite. The two systems employed, and upon which the evidence offered is based, were Sephadex chromatography and gas–liquid chromatography. The latter is of particular value in this field for two reasons: (1) its specificity, as it involves the preparation of a discrete derivative; and (2) its sensitivity, which is of the order of 1×10^{-12} mole; the level of selenium in proteins is usually less than 1 μg/g.

II. Materials and Methods

(*a*) *Animals*

Two lactating ewes were housed in metabolism cages for 48 hr. Their lambs were kept in adjacent pens but were not allowed access to the dams.

(*b*) *Treatment and Sampling*

Whole blood samples were taken initially to determine the selenium status of the animals; selenium analysis was done by Watkinson's method (1966). ^{75}Se (3 mCi), as $Na_2{}^{75}SeO_3$, was given intraruminally in 50 ml normal saline; 3–4 mg selenium as unlabelled sodium selenite was given as carrier.

Blood samples (20 ml), heparinized using one drop of Pularin [Evans Medical Australia (Pty.) Ltd.], were collected every 4 hr and milk samples obtained at the same time; the size of the milk samples ranged from 50 to 100 ml. Blood was centrifuged immediately and 1-ml aliquots of plasma and milk used for the determination of radioactivity; a Packard 3375 gamma counter was employed. The remainder of the plasma and milk was stored at −20°C to await further analysis.

After 48 hr the ewes were anaesthetized under Nembutal and bled to death by cannulation of the carotid artery. Blood was collected and together with liver, pancreas, heart, kidneys, and some skeletal muscle stored at −20°C.

(*c*) *Analytical Systems*

(i) *Plasma and Milk*

Samples of plasma and milk were dialysed against normal saline to remove free or loosely bound selenium. Further plasma and milk samples were diluted 1:1 with water, adjusted daily with concentrated NaOH to pH 11·5, and dialysed at 1–2°C for 4–5 days against water. Similar dialysis was carried out on a sample of soluble casein prepared by precipitation from labelled ewe milk with ammonium sulphate.

(ii) *Thin-layer Chromatography*

Attempts to obtain unequivocal evidence for the presence of selenoamino acids in milk digests by thin-layer chromatography proved unsuccessful. Authentic selenomethionine and methionine separated, as reported by Millar (1965), and by a modification of Haworth's and

Heathcote's method (1969), namely using a more alkaline solvent system, good separation was achieved between authentic selenocystine and cystine. When these methods were applied to milk digests radioactivity was too scattered to allow definitive conclusions to be drawn. In 10–20 μl of such a digest a theoretical assessment of the amount of selenoamino acid that could be present places the amount well below the level of ninhydrin detection.

(iii) *Column Chromatography*

A Dowex-50 column (200–400 mesh) was used to separate ^{75}Se-labelled fractions present in digests of milk. The elution system was similar to, though not identical with, that of Shrift and Virupaksha (1965), who separated ^{75}Se fractions from plant digests. The elution system adopted was 1·5N HCl, followed by water, and then 2N NaOH.

A few amino acid analyses were done on a Beckman–Spinco amino acid analyser, model 120C, using a 4-hr cycle and a flow rate of 68 ml/hr. Fractions were collected for measurement of radioactivity in the gamma counter.

Because all of the ^{75}Se activity could not be eluted from the amino acid analyser column, two manually operated columns were set up. The first contained a resin, Eel 120/CG, supplied by Electroselenium Ltd., the other Aminex MS, fraction D, supplied by Bio-Rad; both were fine-mesh, sulphonated, cross-linked polystyrene resins. Eluting buffers were prepared according to Moore, Spackman, and Stein (1958), Triton X-100 being used instead of Brij-35. The first buffer was pH 3·25, the second 4·25, and the buffer change was effected at 240 ml; flow rate was 15–20 ml/hr.

(iv) *Gas–Liquid Chromatography (GLC)*

Two systems were employed: that of Coulter and Hann (1968), in which the n-propyl-*N*-acetyl esters of the amino acids are formed, and that of Gehrke, Nakamoto, and Zumwalt (1969), in which the *N*-trimethylsilyl esters are prepared using bis(trimethylsilyl)trifluoroacetamide (BSTFA). Some modifications which favoured the separation of certain selenoamino acids were made from the method of Gehrke, Nakamoto, and Zumwalt (1969).

The first system* was operated using a Perkin–Elmer gas chromatograph, model 881. The instrument was modified to allow collection of the effluent from one column, in order to check that radioactivity was present in the peak under consideration. A teflon tube was connected to the end of the column, and led out of the oven so that tubes containing glass wool could be positioned for collection of the effluent fractions. The teflon tube was surrounded by a heating coil wound around copper tube, the temperature inside the tube being set at 250°C by means of a thermocouple. The collecting tubes were cooled in dry ice to allow maximum condensation of the n-propyl-*N*-acetyl esters to occur. Several collection systems were tried before success was achieved; even so, only 50% of the activity was recovered by the method described when authentic ^{75}Se-selenomethionine was used.

The second system was carried out on a Pye Panchromatograph. A heating coil was inserted around the injection port to enable injections to be made at >230°C, and all metal tubing was eliminated. A spiral column was constructed, 1·75 m in length and with an internal diameter of 0·4 cm, and column packing was HP Chromosorb G (80–100 mesh) coated with OV-17 (phenyl methyl siloxane).

Stringent precautions were needed to eliminate traces of water vapour at every stage.

(v) *Sephadex Chromatography*

Two columns were employed, a Sephadex G25 grade to separate milk proteins prior to enzymatic hydrolysis, and a Sephadex G10 grade to separate the components of the digest.

The Sephadex columns offered major advantages when it was found that authentic selenoamino acids were being destroyed during the comparatively mild conditions of rotary evaporation in the presence of buffers. Such advantages were the use of water as eluent, and the possibility of handling large samples. The G10 column was comparatively large, 100 cm long and 5 cm diameter, and the total hydrolysate from 100 ml ewe milk could be placed on the column.

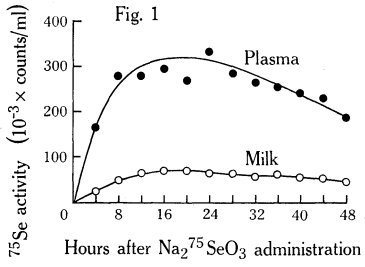
* This phase of the work was done in collaboration with Dr. J. Coulter and Mr. C. Hann.

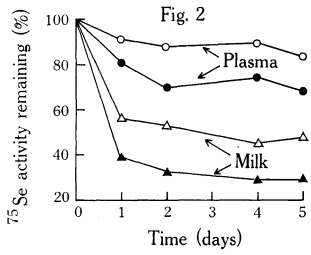
Pooled milk protein fractions from the G25 column were rotary-evaporated to reduce the total volume to less than 100 ml for hydrolysis. The hydrolysate, after treatment outlined in the next section, was passed through the G10 column, using boiled distilled water, with nitrogen bubbling through continuously.

(vi) *Enzymatic Hydrolysis*

Pronase* was used for all hydrolyses after it was found that hydrolysis with HCl led to all the ^{75}Se activity being associated with the charred portion of the digest. Nomoto, Narahashi, and Murakami (1960) have supplied evidence that the protease of *Streptomyces griseus* has a very broad specificity, hydrolysing almost all peptide bonds and releasing 70–90% of the constituent amino acids. After rotary evaporation the pooled milk protein samples from the G25 column were mixed with an equal volume of phosphate buffer, pH 7·4. Pronase was added at a concentration of 0·2%, chloramphenicol at 0·02%, and the mixture incubated under nitrogen at 37°C for 2–3 days.

After incubation any undigested protein was precipitated by the addition of ethanol or acetone, centrifuged, and the supernatant rotary-evaporated at 40°C. The residue was dissolved in acidified 10% aqueous isopropanol and used for the isolation of ^{75}Se compounds.





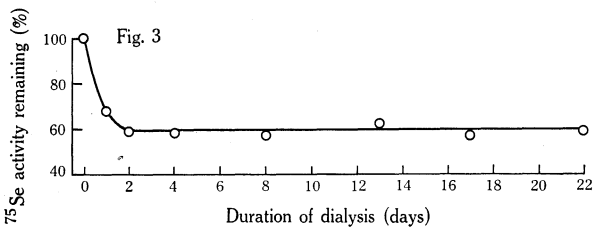


Fig. 1.—^{75}Selenium activity in 4-hourly samples of milk and plasma of the ewe, following the intraruminal administration of a single dose of $Na_2{}^{75}SeO_3$.

Fig. 2.—Alkaline dialysis (pH 11·5) of plasma and milk from ewe given $Na_2{}^{75}SeO_3$ intraruminally. The upper curve of each pair is the 48-hr sample, the lower the 4-hr sample. Intermediate 4-hourly samples gave curves that fell within these limits.

Fig. 3.—Alkaline dialysis (pH 11·5) of soluble casein prepared from labelled ewe milk, following the intraruminal administration of $Na_2{}^{75}SeO_3$.

III. Results

Infusion of the $Na_2{}^{75}SeO_3$ led to the rapid appearance of ^{75}Se activity in the blood and milk of the ewe. The peak of activity occurred 20–24 hr after infusion, although within 4 hr appreciable amounts were present in both fluids (Fig. 1).

* A protease from *Streptomyces griseus*, supplied by Koch-Light Ltd.

Amongst the few tissues collected at slaughter of a lactating ewe 48 hr after the administration of $Na_2{}^{75}SeO_3$ there was considerable variation in the level of ^{75}Se present as indicated in the following tabulation:

Tissue	$10^{-4}\times$Counts/g (wet wt. basis)	Tissue	$10^{-4}\times$Counts/g (wet wt. basis)
Kidney	121·0	Cardiac muscle	7·7
Liver	26·8	Skeletal muscle	1·6
Pancreas	13·8		

Dialysis, against normal saline, of the 4-hourly samples of plasma and milk, collected throughout the 48-hr period, showed that a decreasing proportion of the ^{75}Se was dialysable (Table 1). Dialysis of aliquots of the same samples, under alkaline

TABLE 1

PERCENTAGE OF ^{75}Se IN EWE PLASMA AND MILK DIALYSABLE AGAINST NORMAL SALINE

Sampling Time*	Percentage Dialysed after 5 Days		Sampling Time*	Percentage Dialysed after 5 Days	
	Plasma	Milk		Plasma	Milk
2	21·9	—	28	1·4	2·9
4	14·5	6·7	32	1·0	2·7
8	8·6		36	0·8	2·5
12	3·8	5·0	40	0·9	2·8
16	2·4	4·1	44	0·8	2·7
20	1·6	2·9	48	0·8	2·2
24	1·3	2·5			

* Hours after intraruminal infusion.

conditions, resulted in a greater release of dialysable ^{75}Se. The results for both plasma and milk are shown in Figure 2. Similar dialysis, carried out on the soluble casein fraction, gave the result shown in Figure 3.

Skimmed milk, passed through the Sephadex G25 column, gave a single peak of ^{75}Se activity, corresponding to the milk protein fraction; there was no evidence that free ^{75}Se was present.

When an aliquot of the Pronase digest of milk was injected on to the Dowex-50 column, a number of active peaks emerged (Fig. 4). The addition of ^{14}C-labelled methionine showed that partial separation from ^{75}Se compounds could be achieved; this suggested the presence of discrete selenoamino acid-like fractions.

An aliquot of the same type of Pronase digest of milk was injected on to the Beckman–Spinco amino acid analyser. The aliquot contained 8118 counts/min, and the elution diagram, based on the presence of ^{75}Se in the fractions, is shown in Figure 5. Peak A corresponds to the position of selenite; peak B on GLC gave a small peak, having a retention time corresponding to selenomethionine, when the fraction was used for the formation of the n-propyl-*N*-acetyl ester and injected on to the Perkin–Elmer machine. Other peaks are apparent but have not yet been identified.

A run, using the manually operated column, was conducted to check whether the selenite ion binds to sulphur amino acids under these conditions. Authentic [^{75}Se]sodium selenite, [^{14}C]methionine, and [^{14}C]cysteine, were mixed with carrier

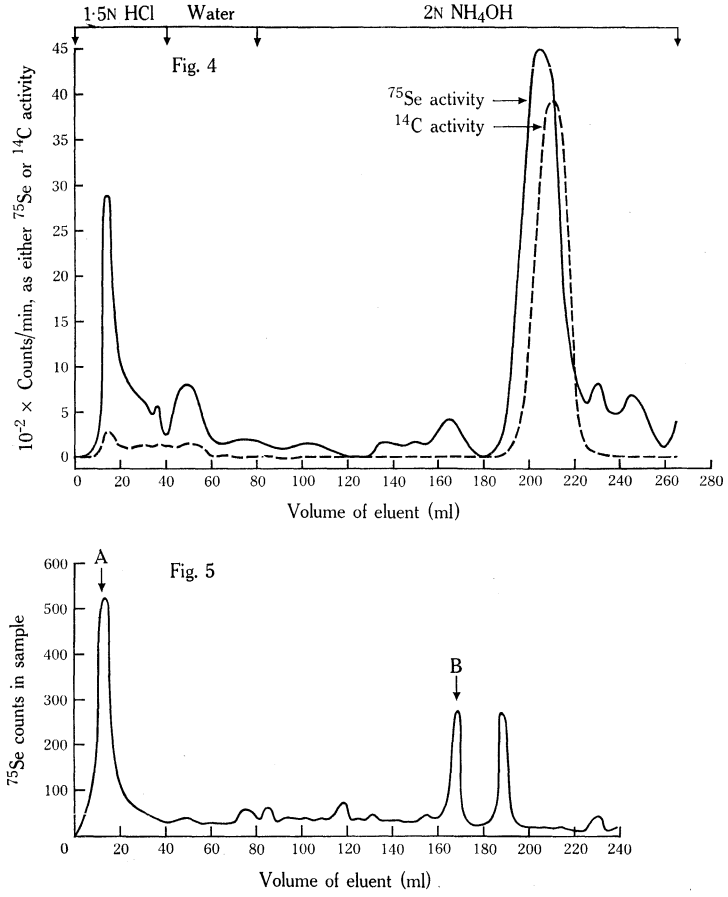


Fig. 4.—Separation of ^{75}Se peaks on a 100-cm Dowex-50 column, after injection on the column of a Pronase digest of labelled ewe milk. [^{14}C]Methionine was added to the sample before it was placed on the column.

Fig. 5.—Elution diagram obtained by plotting ^{75}Se activity in 3-ml fractions emerging from the Beckman–Spinco amino acid analyser, following the injection on to the column of a Pronase digest of labelled ewe milk. A, position of authentic selenite. Fractions pooled from peak B gave a small peak on GLC corresponding to selenomethionine.

so that a total of 3 mg amino acid was injected on the column. There was complete separation of the ^{75}Se, this emerging with the solvent front, from the ^{14}C-labelled amino acids; no binding of the selenite ion occurred.

The presence of a small peak on GLC, derived from the amino acid analyser fraction corresponding to selenomethionine, was not considered sufficient evidence by itself. Figure 6 shows the GLC read-out, together with the corresponding position for

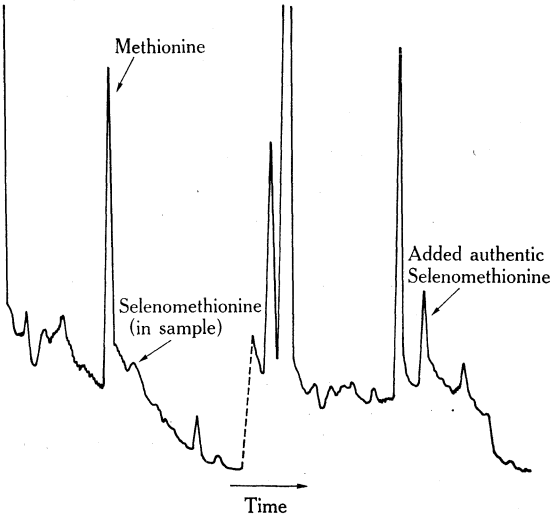


Fig. 6.—GLC pattern obtained from a peak containing ^{75}Se activity eluted from a Sephadex G10 column (100 cm long, 5 cm int. diam.). The original Pronase digest of milk was run through the column, and the ^{75}Se-peak corresponding to selenomethionine was re-run through the column before being subjected to GLC analysis. The retention time of the peak in question was 11 min and the temperature for elution 196°C. This agrees with authentic selenomethionine supplied by Sigma Chemical Co.

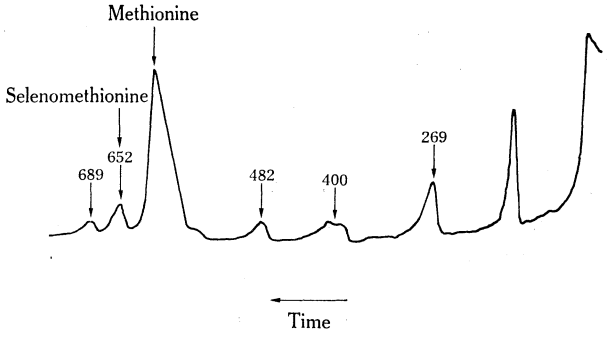


Fig. 7.—GLC pattern showing position of selenomethionine. The labelled peak was identified by its retention time, and the presence of ^{75}Se activity in the effluent fraction corresponding to selenomethionine. The numbers are retention times in seconds.

authentic solenomethionine. With Coulter's and Hann's system selenomethionine and methionine are well separated, a difference of 50 sec and nearly 6°C, so the peak was

not considered to be methionine. However, with such a small peak the presence of some other impurity could not be overlooked.

Ewe milk (100 ml) was treated as indicated in Section II(*c*)(vi). When the total Pronase hydrolysate was passed through the large Sephadex G10 column a number of radioactive peaks emerged, one prominent one was present at the position of selenomethionine. A synthetic mixture of nine ^{14}C-labelled amino acids (proline, valine, tyrosine, phenylalanine, methionine, aspartic, glutamic, glycine, and threonine) together with authentic [^{75}Se]selenomethionine* was passed through the G10 column and fractions identified by GLC. The fractions containing ^{75}Se, eluting at 950–1000 ml, were pooled and found to contain selenomethionine and some methionine.

The active fractions, corresponding to authentic selenomethionine, derived from the milk, were re-run through the G10 column, when a sharp peak occurred at the position of selenomethionine. After rotary evaporation of the sample it was injected on to the Pye Panchromatograph as the trimethylsilyl derivative, then a further injection, combining authentic selenomethionine, was made. The results are shown in Figure 6.

An aliquot of the same sample from the Sephadex column was used for the formation of the n-propyl-*N*-acetyl ester. This was injected on to the Perkin–Elmer gas chromatograph and effluent fractions collected and counted for radioactivity. A small peak emerged that corresponded to authentic selenomethionine; approximately one-tenth of the ^{75}Se injected on the column as the ester emerged with this peak. Separate GLC runs using authentic [^{75}Se]selenomethionine indicated that maximum recovery of injected ^{75}Se was about 30–40% (Fig. 7).

IV. Discussion

This paper provides evidence that inorganic selenium, given intraruminally to lactating ewes as $Na_2{}^{75}SeO_3$, is readily converted to selenoamino acid and incorporated into milk proteins. Although the main purpose of the work was to establish the qualitative, rather than the quantitative aspects of the problem, it is possible to gain some idea of the amount of selenium appearing as selenomethionine in the milk under the conditions of these experiments.

Starting with 100 ml milk, having a total number of counts per minute of $1{\cdot}8 \times 10^6$, 100,000 counts/min were found in the precipitate formed by adding ethanol, following Pronase digestion. One-twentieth, appeared therefore as "undigested protein". A total of 170,000 counts/min were re-run through the G10 column the second time—this represents a "crude" selenomethionine fraction. Finally 60,000 counts/min appeared in the fraction taken for GLC as being largely selenomethionine. It was this fraction that gave a peak in the position of selenomethionine, and with which was associated ^{75}Se in the GLC effluent. It is possible, therefore, that as much as 3% of the selenium that enters the milk from inorganic sources is present as the amino acid selenomethionine. The presence of other radioactive peaks in both

* Obtained from Radiochemical Centre, Amersham, England. A small amount was tested for chemical purity (as advocated by Shrift 1967) by GLC, and found to contain only one fraction, and this corresponded to selenomethionine.

amino acid analyser chromatograms, and the elution diagrams from the Sephadex columns, suggests that other selenoamino acids may be present.

The peak corresponding to selenomethionine on GLC (for example in Fig. 6) would presumably contain both labelled and unlabelled amino acid. The fact that both the trimethylsilyl derivative and the n-propyl-*N*-acetyl ester contain appreciable ^{75}Se activity, combined with the fact that no activity is lost in the preparation of the ester even though an extraction with ethyl acetate is involved, is good evidence that the ^{75}Se is present as selenomethionine and not as a contaminant. Furthermore, the association of the peak corresponding to selenomethionine with radioactivity in the effluent at that point greatly strengthens the case, since selenium binding to another amino acid would affect its retention time and it is highly unlikely that it would correspond exactly with selenomethionine.

Dialysis of both plasma and milk suggests that the ^{75}Se incorporated into the protein consists of an easily dialysed component, and one that resists dialysis, even under alkaline conditions. The non-dialysable component accounted for the larger fraction of plasma ^{75}Se (68–87%) and an appreciable fraction of the milk ^{75}Se (24–49%). These results contrast markedly with those of Cummins and Martin (1967) and of Jenkins (1968); the former authors used rabbit liver protein and the latter author chick plasma and the amount of non-dialysable ^{75}Se in these tissues was 20 and 10% respectively. It was concluded, in each of these papers, that there was no evidence that monogastric animals could utilize sodium selenite for the synthesis of selenoamino acids. This does not appear to be the case for the ruminant.

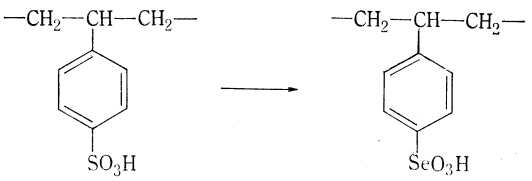
McConnell has studied extensively the subject of incorporation of ^{75}Se, supplied in inorganic form, into the proteins of the dog. He and his colleagues have shown that ^{75}Se activity is associated with a large number of protein fractions, and ^{75}Se has been detected in the position of certain sulphur analogues. However, there has not been an unequivocal demonstration of the presence of selenoamino acids derived from inorganic selenium in these studies (McConnell and Wabnitz 1957; McConnell 1959, 1963; McConnell and Roth 1968).

There are two reasons at least why it has been difficult in the past to demonstrate the presence of selenoamino acids in animal tissues. Firstly, they are very easily degraded, and secondly, they are present in only microgram quantities. A third reason could be added—it has been recognized by some workers (Peterson and Butler 1962), namely, the similarity of chemical behaviour between the sulphur and selenium analogues.

In relation to the first point it was reported by Blau (1961) that selenomethionine was destroyed by acid hydrolysis; this was confirmed in the preliminary stages of this work, and was the reason given earlier for the exclusive use of Pronase digestion. The degradation of selenoamino acids was investigated further in the course of this work, however, and the findings are briefly reported here.

It became apparent that whenever either authentic [^{75}Se]selenomethionine or ^{75}Se-labelled compounds in a milk digest were injected on to sulphonated polystyrene columns, a proportion of the counts were always lost. Elution with strong alkali did not bring them off the column. After such runs, dismantling of the column and measurement of the radioactivity of the resin showed repeatedly that activity was held tightly at every level of the column. The ^{75}Se could not even be removed by

boiling the resin in 4N NH_4OH. It is suggested that the selenium may replace the sulphur in the following manner:



When authentic unlabelled selenomethionine was rotary-evaporated in citrate buffer at pH 3·25 and 4·25, most of the selenomethionine was destroyed as was evident when the residue was used for GLC analysis.

The second difficulty mentioned was the minute quantities of the selenoamino acids that are present, on theoretical grounds, even if all the selenium was present in this form. If for example the concentration of selenium in a protein digest was 1 μg/g (for example, milk from sheep has a concentration of 0·005–0·02 μg Se/ml—unpublished observations) there would be in 4 mg of amino acid approximately 0·0004 μg Se, equivalent to about 0·001 μg "selenoamino acid". Considering the very small amount of material, and the fact that the compound is easily degraded, it is evident that rigid precautions would be necessary to avoid its destruction, or even loss. Such an amount is below the level of ninhydrin detection but would be detectable by GLC since the order of sensitivity is 1×10^{-12} mole.

On the other hand, however, the highly reactive nature of selenoamino acids has attracted attention (Olcott, Brown, and van der Veen 1961) and should be considered in the light of the significance of the role of selenium to the ruminant. It is well established that minute amounts of sodium selenite given to the ewe will completely prevent white muscle disease in the lamb, even when the ewe is fed dystrophogenic hay (Oldfield, Schubert, and Muth 1963). Comparatively small doses of selenium lead to noticeable rises in milk selenium in ewes (Godwin, unpublished observations). The present work shows that some of that rise is due to the presence of seleno-methionine. Dickson and Tappel (1969) have recently demonstrated, in *in vitro* studies, the activating effect of both selenomethionine and selenocystine on certain sulphydryl enzymes, e.g. papain. Now that it has been established that in the ruminant seleno-methionine is passed to the lamb, following the administration of inorganic selenite, the work of Dickson and Tappel may be significant in relation to the role of selenium in the ruminant.

Two mechanisms at least could explain the conversion of inorganic selenium to selenoamino acids. One is by the microbial population of the rumen. There is evidence that microorganisms can effect this conversion (Blau 1961; Tuve and Williams 1961), however, Shrift's criticisms apply to the data presented in that work. The other possibility is that suggested by McConnell and Roth (1966), that selenoamino acids might be formed by reduction of oxidized forms of selenium to selenide and a –SH $\rightleftharpoons$ –SeH exchange reaction involving the sulphur amino acids. Microorganisms are known to be able to synthesize methionine in a multistep process from cysteine and homoserine (Fling and Horowitz 1951) and, by analogy, selenomethionine might

be formed similarly. The extreme reactivity of the selenoamino acids, as supported by the work reported in this paper, lends support to this concept.

The advantage of GLC for the identification of compounds occurring in the tissues at the level at which selenomethionine was found in ewe milk, are brought out clearly in this paper. Other authors, in order to demonstrate a technique, have separated selenoamino acids by GLC (Caldwell and Tappel 1968) but this is the first unequivocal demonstration of the presence of selenomethionine in a protein hydrolysate of animal tissue. Furthermore, it is the first unequivocal demonstration of inorganic selenium being converted to selenomethionine in the animal body.

V. Acknowledgments

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