Are Selenocystine and Selenomethionine Synthesized *in Vivo* from Sodium Selenite in Mammals?*

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ABSTRACT: These experiments suggest that there is not a pathway for the *in vivo* synthesis of selenocystine nor for selenomethionine from selenite in the rabbit. This conclusion is based on the evidence that neither amino acid is apparently present in liver protein or urine from a rabbit on a radioselenite-supplemented diet. Results do show that fractions isolated from urine could have been mistaken for these selenium amino acids, but such fractions appear to be nothing other than selenite bound to sulfur compounds. Liver protein was purified by dialysis and the dialysates were concentrated and analyzed for selenite. More than 90% of the selenium in the dialysates was recovered as selenite. Following dialysis, in which approximately 93% of the initial radioactivity was removed, the protein was enzymically hydrolyzed. The hydrolysate was chromatographed by ion exchange, and all the radioactivity appeared at an elution volume which coincided with that of selenite. In a second experiment, a urine sample from a 24-hr collection was chromatographed by ion exchange, and two distinct radioactive fractions were detected which suggested the presence of selenocystine and selenomethionine. Radioactive fractions having the same elution volume were also detected in a control urine sample and in a sulfur compound mixture spiked with radioselenite and chromatographed under the same conditions. Upon chemical analysis of these fractions, it was determined that the major portion of the selenium existed as selenite. An analytical method capable of detecting sulfur-bound selenite is discussed, along with the phenomenon of sulfur compound–selenite binding.

Since selenium is both a toxicant and an essential micronutrient, it presents one of the most intriguing of the apparently minor problems in biochemistry. The exact nature of how it functions in either of these roles is not fully understood. Even though Schwarz (1961; Schwarz and Foltz, 1958) has maintained that the selenium amino acids are not the naturally occurring form of selenium in biological material, it is accepted by many other investigators that in biological systems selenium replaces sulfur in sulfur-containing compounds. This generalization is based on the fact that several investigators have reported the isolation and identification of several selenium analogs of well-known sulfur compounds. In mammals, the two most commonly reported organic selenium metabolites arising from inorganic selenium are selenocystine and selenomethionine. In all the reported studies the identification of these selenium amino acids was based upon the criterion that ^{75}Se was detected on chromatograms in the vicinity where their corresponding sulfur analogs are known to appear. The reason for this sole reliance on radioisotope content is that due to the extreme toxicity of selenium, it is not possible to feed animals large enough quantities to induce the *in vivo* synthesis of measurable quantities of selenium metabolites.

McConnell and Wabnitz (1957) first reported that these two selenium amino acids were present in dog liver protein. Rosenfeld (1962) also reported their existence in wool hydrolysates. In both experiments the evidence presented for the presence of the two selenium amino acids was based on the presence of radioactivity in areas of a chromatogram similar to those of the corresponding sulfur compounds. However, as a result of the discovery by Schwarz and Sweeney (1964) that various sulfur compounds can bind the selenite ion, this criterion of identification may not be sufficient.

The results from this investigation strongly suggest that the rabbit does not synthesize either selenocystine or selenomethionine from selenite. This conclusion is based upon the failure of finding either amino acid in hydrolysates of purified liver protein or in the urine of rabbits administered $^{75}SeO_3^{2-}$. In support of the work by McConnell and Wabnitz and that of Rosenfeld the initial results of this experiment would also lead one to believe that the selenium amino acids might be present, but by use of recently developed

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analytical techniques, compounds which previously could have been mistaken for selenium amino acids now appear to be radioselenite bound to sulfur compounds.

Experimental Methods

Isolation and Detection of Radioactive Fractions. All chromatographic separations were performed by utilizing the ion-exchange columns of a Beckman–Spinco Model 120B amino acid analyzer, according to the procedures described by Spackman *et al.* (1958) and modified by Martin and Cummins (1966). Buffers employed in the ion-exchange procedures were prepared according to the Beckman Model 120B Handbook, with the exception that thiodiglycol was omitted.

Radioactivity was detected with a Nuclear-Chicago liquid-flow scintillation counter. With this system the eluate from the ion-exchange column passed through the scintillation counter prior to reaching the ninhydrin-reaction bath. Therefore, a lag between the radioactive peaks (descending peaks on chromatograms) and the ninhydrin-positive peaks (ascending peaks on chromatograms) was found to be 9.0 ml or 18 min. All radioactive fractions were collected manually by diverting the column eluates into a collection flask immediately upon leaving the scintillation crystal.

Radioselenite Determination with and without Carrier Selenite. The pH of each sample was initially adjusted to between 10 and 11 with 3.0 N NaOH. Then 150 μg of sodium selenite was added as carrier, and the samples were allowed to stand at room temperature for 2 hr. The sample volume was adjusted to 8.0 ml with water, and the initial radioactivity was determined in a γ-well counter. The selenite content was determined by a modification of the procedures described by Cummins *et al.* (1965). In this modification the initial digestion was omitted, and the samples were washed directly into a 250-ml beaker with approximately 10 ml of distilled water, followed by the addition of 5.0 ml of 12 N HCl. Then proceeding with the addition of the 0.1% *m*-cresol purple indicator, the procedure was followed *verbatim* (except NH_2OH was omitted) until the final extraction. Here, 6.0 ml of toluene was used instead of 4.0 ml. The toluene extract was rinsed into a tube with 2.0 ml of acetone, thus yielding the same volume as the initial sample, and the radioactivity was again determined in a γ-well counter. The above-described method will be referred to as the "carrier technique." Radioselenite was also determined without the addition of carrier selenite, following the above-described procedures.

Selenite Determination in the Presence of Sulfur Compounds. A sulfur compound mixture containing 5.0 μmoles each of methionine, cysteine, cystine, taurine, homocysteine, cystathionine, GSSG,[1] and GSH was prepared by dissolving the compounds in 9.0 ml of 0.2 N sodium citrate buffer (pH 2.2). To this solution 15 μc of ^{75}Se as $Na_2{}^{75}SeO_3$ was added and the volume was adjusted to 10 ml with the citrate buffer. The mixture was incubated at 38° for 14 hr and will be referred to as "sulfur compound mixture." Aliquots of this mixture were analyzed with and without carrier selenite, and the results of recovery of the radioselenite appear in Table I.

TABLE I: Per Cent Recovery of Radioselenite from Sulfur Compound Mixture.

	Av % Recov[a] without Carrier Selenite	Av % Recov[a] with Carrier Selenite
	19.1	60.5
	19.0	53.6
	20.2	55.4
Av recov	20.9	56.5

[a] All values are corrected to 100% recovery.

Radioselenite Incorporation and Protein Isolation. One 10-month-old male Californian rabbit weighing 3.84 kg was fed a commercial diet[2] and given tap water *ad libitum*. For the first 35 days this rabbit was fed both radioactive and nonradioactive selenite. A gelatin capsule containing both 7.0 μc of ^{75}Se as $Na_2{}^{75}SeO_3$ (sp act. 0.46 μc/μg of selenium) and 1.0 mg of selenium as Na_2SeO_3 was given daily by oral administration. During the 35-day period the rabbit exhibited no symptoms of selenium toxicity. Furthermore, a control rabbit which was carried through the entire study weighed slightly less at the end of the study than did the experimental animal. Twenty-four hours prior to the termination of the experiment 250 μc of ^{75}Se as $Na_2{}^{75}SeO_3$ (sp act. 63 μc/μg of selenium in a sterile isotonic saline solution at a concentration of 500 μc/ml) was injected into the marginal ear vein. At the time of the injection, 250 μc of ^{75}Se in this same solution was administered orally.

After fasting for 15 hr, the rabbit was sacrificed after anesthetizing with ether. The liver, upon removal, was immediately frozen in a Dry Ice–acetone solution and stored at −20° until analyzed.

A 40-g portion of the liver was homogenized with 400 ml of 0.01 N NaOH, first with a Virtis blender, and later treated with a Teflon Tri-R tissue homogenizer. Cellular debris was removed by centrifuging for 20 min at 600*g*. Half of the supernatant containing the soluble liver proteins was purified by dialysis (using Visking dialysis tubing) against distilled water at 2°

[1] Abbreviations used: GSSG and GSH, oxidized and reduced glutathione; DAB, 3,3′-diaminobenzidine.

[2] Purina Rabbit Laboratory Chow, Ralston Purina Co., St. Louis, Mo.

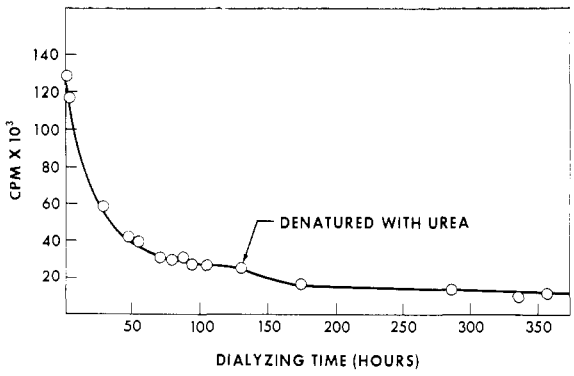

FIGURE 1: Rate of ^{75}Se removal from liver proteins by dialysis.

for 9 days. The dialysates were changed every 24 hr and individually concentrated on a rotary evaporator to about 100 ml and analyzed for selenite as described previously (Table II). The protein solution was maintained at a pH of approximately 11 throughout the dialysis by the addition of saturated NaOH solution.

Aliquots (5 ml) of the protein solution were taken at intervals starting with time zero. The radioactivity of the aliquots was determined, and a graph of activity *vs.* dialyzing time was constructed to determine when all selenite not bound to protein was removed (Figure 1). After 6 days the proteins in solution were further denatured by adding urea until a 6.0 M urea concentration was obtained. The dialysis was continued for an additional 8 days.

A portion of the protein solution was frozen and later enzymically hydrolyzed. The protein in the remainder of the solution was precipitated by adjusting the pH to 1.0 with 12 N HCl, isolated by centrifuging at 600*g* for 10 min, washed twice with distilled water, twice with acetone, and dried in a vacuum oven. The activity of the purified protein was determined using a γ-well counter.

Enzymic Hydrolysis of the Purified Liver Protein. The pH of 10 ml of the purified protein solution (containing approximately 70 mg of protein and 20,800 cpm of radioactivity) was lowered to 7.5 with 12 N HCl. The solution was mixed with 10 ml of 0.28 M phosphate buffer (pH 7.7), and after adding 3.0 mg of Pronase,[3] the solution was incubated at 38° for 30 hr in a water bath. Following incubation the enzyme and all nonhydrolyzed protein were precipitated with 20 ml of 1.0% picric acid and removed by centrifuging for 10 min at 600*g*.

The supernatant was prepared for analysis on the amino acid analyzer according to the ion-exchange procedure set forth by Stein and Moore (1954) for picrate removal. The final volume of the protein hydrolysate was 8.0 ml (containing 11,200 cpm), of which 2.0 ml was analyzed on the amino acid analyzer (Figure 2).

TABLE II: Per Cent Recovery of Radioselenite from Protein Hydrolysates.

Dialysate Taken after (days)	% of Total Radioselenium Removed and Contained in Dialysate	% of Total Radioselenium Recovered from Dialysate as Selenite without Carrier[a]	% of Total Radioselenium Recovered from Dialysate as Selenite with Carrier[a]
1	58.5	33.7	53.5
2	22.1	11.5	20.7
3	6.2	4.5	5.5
4	3.4	2.2	2.9
5	3.4	2.8	3.3
6	3.6	3.1	3.2
7	1.0	0.8	0.7
8	0.9	0.8	0.8
9	0.9	0.6	0.6
% of total radioselenium removed by dialysis recovered as selenite		60.0	91.2

[a] Each value represents the average of duplicate analyses which were corrected to 100% recovery.

Animal Treatment and Urine Collection. One 5-month-old male Californian rabbit weighing 3.14 kg was maintained under conditions similar to the rabbit used for the protein study. After a 24-hr control urine sample was obtained, 350 μc of ^{75}Se as $Na_2{}^{75}SeO_3$

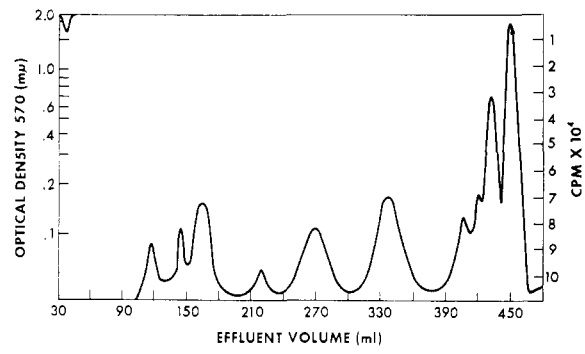

FIGURE 2: Ion-exchange chromatogram of enzyme-hydrolyzed rabbit liver protein. The protein was isolated from a [^{75}Se]selenite-treated rabbit. It was purified by exhaustive dialysis against distilled water and enzymically hydrolyzed with Pronase. Ascending peaks represent ninhydrin-positive compounds, and the descending peak represents radioactivity.

[3] Obtained from Sigma Chemical Co., St. Louis, Mo.

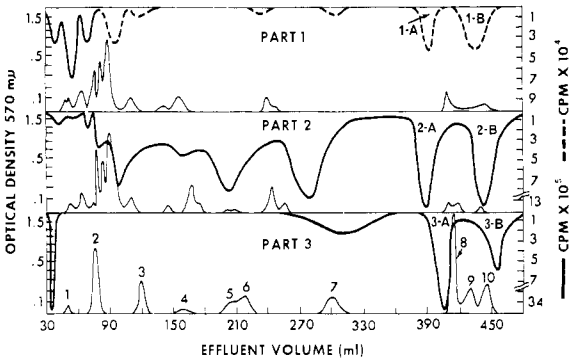

FIGURE 3: Treatment with [^{75}Se]selenite. (Part 1) Rabbit urine taken 24 hr after [^{75}Se]selenite treatment. (Part 2) Control rabbit urine treated with [^{75}Se]selenite following excretion. (Part 3) Sulfur compound mixture treated with [^{75}Se]selenite. The peaks in part 3 are as follows: 1 cysteic acid, 2 taurine, 3 GSH, 4 GSSG, 5 cysteine, 6 homocysteine, 7 cystine, 8 cystathionine, 9 methionine, and 10 homocystine.

TABLE III: Per Cent Recovery of Radioselenite in the Ion-Exchange Fractions Collected from Urine Samples and from a Synthetic Sulfur Compound Mixture.

Fraction Corresponding to Fig. 4	Cor % Recov without Carrier Selenite[a]	Cor % Recov with Carrier Selenite[a]
1-A	46.4	56.1
1-B	32.3	51.0
2-A	78.2	92.9
2-B	61.7	81.2
3-A	65.6	83.9
3-B	51.3	87.7

[a] The reported values are the average of three analyses and these values are corrected to 100% recovery.

(sp act. 63 μc/μg of selenium in sterile isotonic saline at a concentration of 415 μc/ml) was injected into the marginal ear vein and 65 μc of ^{75}Se was given orally. Since the rabbit did not urinate in the 24-hr period following the injection, the urine sample (referred to as the *in vivo* labeled urine sample) was taken directly from the bladder. These urine samples were kept frozen until analyzed.

Analysis of Urine Samples and the Sulfur Compound Mixture. The *in vivo* labeled urine sample was filtered and the pH of the filtrate was adjusted to 2.0 with 7.0 N HCl. This sample (2 ml) (containing 2,153,000 cpm) was placed on the ion-exchange column and fractions 1-A and 1-B (Figure 3) were collected. The unlabeled control urine sample (2 ml) was incubated with an amount of ^{75}Se as $Na_2{}^{75}SeO_3$ equivalent to that in the *in vivo* labeled urine sample for 14 hr at 38° at a pH of 2.0. (This sample is referred to as the *in vitro* labeled urine sample.) Fractions 2-A and 2-B (Figure 3) were collected from this analysis.

The sulfur compound mixture (2 ml) was analyzed in the same manner as the urine samples. Fractions 3-A and 3-B were collected when this mixture was chromatographed. The six fractions (1-A, 1-B, 2-A, 2-B, 3-A, and 3-B) were then analyzed both with and without carrier selenite to determine the amount of selenium that exists as selenite in each fraction (Table III).

Experimental Results

Evaluation of the Analytical Recovery of Radioselenite. In the analytical method the initial count of activity represented the total selenium content of the sample, whereas the final count represented only that selenium which existed as the selenite ion because the DAB used in this analytical procedure complexes selenium only as the selenite ion. Therefore, the final count of the toluene extract represented only the amount of free selenite in the sample.

The efficiency of the method both with and without carrier for the recovery of radioselenite in nanogram quantities was determined by analyzing spiked aqueous samples. In both cases the average actual recovery was 81% of the added radioselenite. All subsequent determinations were, therefore, adjusted to yield 100% recovery by the following formula.

$$\text{corrected \% recovery} = \frac{\text{actual \% recovery} \times 100\%}{81.0\%}$$

Table I shows there was an almost threefold increase in the recovery of radioselenite when aliquots of the sulfur compound mixture were analyzed after adding carrier selenite. Since essentially nothing is known about sulfur compound–selenite binding, the possibility existed that when carrier selenite was mixed with the sulfur compounds, the selenium was actually incorporated into the compound to produce a selenide. If this were the case, then possibly the increased recovery of radioselenite with carrier was due to some type of a destructive oxidation of selenides. To test for such destruction, 1.0 mg of each of the following organic selenium compounds was analyzed by the "carrier technique": selenocystine,[3] selenomethionine,[3] and selenohomocysteine,[4] and selenotetrahomocysteine.[4] None of the compounds tested was destroyed under the conditions employed. Since these compounds did not contain radioactive selenium, the criterion used for selenite determination was colorimetry as described by Cummins *et al.* (1965). To further evaluate the specificity of the carrier technique elemental selenium and Na_2SeO_4 were also analyzed. Again, based on

[4] Courtesy of Donald F. Petersen of the Los Alamos Scientific Laboratory, Los Alamos, New Mexico.

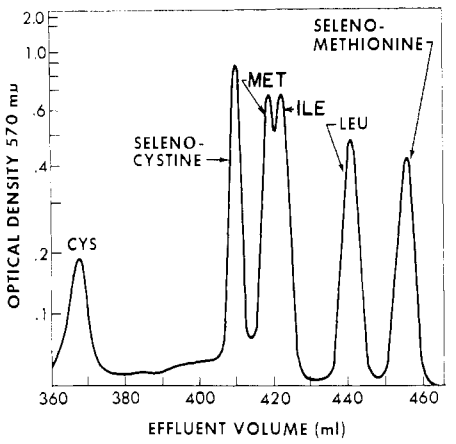

FIGURE 4: A portion of an ion-exchange chromatogram of a standard amino acid calibration mixture. The mixture contained 1.0 μmole of each amino acid.

colorimetry as described by Cummins *et al.* (1964), no selenite was detected. (The method published in 1964 can detect selenate whereas the 1965 method cannot.) It was, therefore, concluded that the analytical method was capable of determining only free selenite.

Dialysis of Hepatic Protein and Radioselenite Recovery from the Dialysates. From Figure 1 it can be seen that the major portion of the radioselenium associated with the liver proteins can be removed by dialysis when the protein solution is held under alkaline conditions. To ascertain whether this release of selenite was the result of protein denaturation, additional proteins were dialyzed while the pH was maintained at values ranging from 1 to 3. A protein solution was also made alkaline (pH 12) then neutralized and dialyzed. In both cases the amount of radioactivity removed from the proteins was far less than that released by constant maintenance at an alkaline pH. The protein conformation does appear to play an important role in selenium binding, because after the removal of activity was almost complete, urea denaturation brought about the release of more radioselenium.

From Table II it can be seen that 91.2% of the selenium in the dialysates was present as selenite. These data strongly suggest that probably all the selenium was present as selenite. It should be pointed out that a value of 91.2% represents excellent recovery since the analytical determination is based on the ability of DAB to complex selenite, and the DAB must also compete with sulfur compounds present in the dialysate in order to bind selenite.

Enzymic Hydrolysis and Analysis of Hepatic Proteins. Figure 4 illustrates a portion of an ion-exchange chromatogram obtained when a standard amino acid calibration mixture plus selenocystine and selenomethionine was analyzed according to the method of Martin and Cummins (1966). Under these conditions selenocystine comes off at an elution volume of 410 ml and selenomethionine at 455 ml.

Figure 2 represents a chromatogram obtained when the enzymic hydrolysate of the purified liver protein was analyzed. The absence of any radioactive peak in the vicinity where the selenium amino acids are known to appear infers that neither amino acid was present in the protein, or, if present, in concentrations so low that their presence could not be detected by our methods of analysis. The single radioactive peak at an effluent volume of 35 ml suggested that all the selenium was present in the purified liver protein as selenite which appears at an effluent volume of 35 ml.

To rule out the possibility that selenocystine or selenomethionine was destroyed during the hydrolysis, these two amino acids were subjected to the same hydrolytic treatment and there was no evidence of destruction.

Analysis of Radioactive Fractions Collected from Urine Samples and from the Sulfur Compound Mixture. When the *in vivo* labeled urine sample was chromatographed on the amino acid analyzer the fractions labeled 1-A and 1-B in Figure 3 were of special interest. Fraction 1-A appears at an elution volume of approximately 390 ml and 1-B at about 425 ml. Figure 4 shows that selenocystine occurs at an elution volume of 410 ml, and selenomethionine at 455 ml. Therefore, fractions 1-A and 1-B were originally suspected to be selenocystine and selenomethionine, respectively. Although neither radioactive fraction had the exact elution volume that selenocystine and selenomethionine should have, the possibility of their presence could not be dismissed. Differences in elution positions could be attributed to variations that might occur when a complex solution such as urine is chromatographed. The results were surprising when the *in vitro* labeled urine sample was analyzed (part B, Figure 3). Instead of obtaining the single selenite peak at an elution volume of 35 ml, eight radioactive fractions were detected.

The last two peaks at 390 and 440 ml were of particular interest since, again, they appeared at elution volumes very nearly the same as those of selenocystine and selenomethionine, respectively. Part 3 of Figure 3 represents the chromatogram obtained when the sulfur compound mixture was analyzed. Here, again, the last two radioactive peaks (3-A and 3-B) suggested the presence of selenocystine and selenomethionine.

From Table III it can be seen that more than 80% of the radioselenium in fractions 2-A, 2-B, 3-A, and 3-B was recovered as selenite when analyzed by the carrier technique, and more than 50% of the radioselenium in fractions 1-A and 1-B existed as selenite.

Discussion

Binding of Selenite by Sulfur Compounds. Although Schwarz and Sweeney (1964) have shown that certain sulfur compounds bind selenite, the exact nature of the binding is not known. This study does show that the binding is pH dependent since under acidic condi-

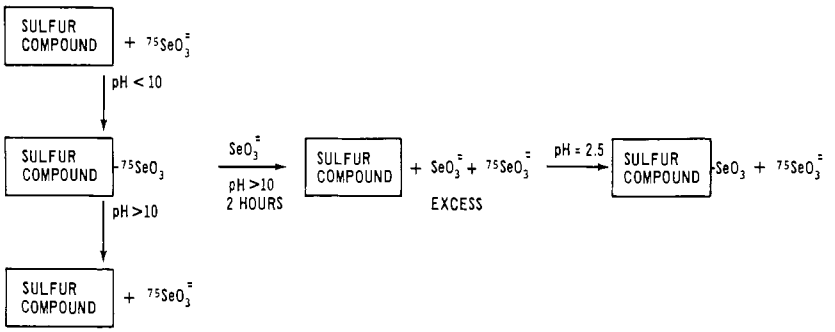

FIGURE 5: Schematic diagram of the proposed binding of selenite by sulfur compounds.

tions, the selenite ion appears to be firmly bound to sulfur compounds, whereas it is released when the solutions are made alkaline. This phenomenon was first observed when the isolated liver proteins were purified by dialysis. Under alkaline conditions ^{75}Se is easily removed from proteins by dialysis. However, upon acidification the removal of ^{75}Se greatly diminished.

With this knowledge that the sulfur compound–selenite binding is pH dependent, it was possible to develop a method which was capable of quantitatively determining radioselenite in the presence of sulfur compounds. Figure 5 represents a schematic diagram of the chemistry involved. Initially, the pH of the solution was raised to between 10 and 11, thus freeing the bound selenite; then an excess amount of nonradioactive selenite was added. At this point there are three entities consisting of the sulfur compound and two types of selenite. Upon lowering the pH, the selenite is again bound, but since there is such a large excess of nonradioactive selenite, the major portion of the selenite that is rebound would be nonradioactive. As illustrated in the final step of the diagram shown in Figure 5, it was necessary to lower the pH to 2.5 because it is only under these conditions that DAB will complex selenite.

Additional support for this theory that sulfur compounds bind selenite is provided by the data in Table I showing an almost threefold increase in the recovery of radioselenite from the sulfur compound mixture after the addition of carrier selenite.

The explanation for the greater selenite recovery when individual fractions from the sulfur compound mixture were analyzed (Table III) than when the entire mixture was analyzed (Table I) can only be explained by the fact that the recovery appears to be inversely proportional to the sulfur content of the sample or to the number of different sulfur compounds present in the sample. This is the reason that the thiodiglycol (an antioxidant) was omitted from the eluting buffers. Its presence not only seriously hindered the recovery of radioselenite from the isolated fractions, but it also gave an extra radioactive peak when samples were chromatographed.

An attempt was made to determine which sulfur compounds in the synthetic mixture were responsible for the last two peaks in part 3 of Figure 3. After several analyses of various combinations of sulfur compounds, it was concluded that the last two peaks occur only when the following compounds were present: methionine, cystathionine, cysteine, cystine, homocysteine, and both GSH and GSSG. If GSH was omitted, only the initial selenite peak at an elution volume of 37 ml and peak 3-A were produced. This, therefore, suggests that more than one sulfur compound is involved in the selenite binding and additional work is being conducted to determine exactly which sulfur compounds are necessary for the production of peaks 3-A and 3-B in Figure 3.

Protein Binding of Selenite. The first observation suggesting that selenium is bound to proteins and is not present as the incorporated selenium amino acids was that, on dialysis, radioactivity was continually removed. This information, coupled with the fact that 91.2% of the total selenium in the concentrated dialysates was present as selenite (Table II) suggested that the major portion of the selenium was present as bound selenite.

Secondly, almost unequivocal proof that selenium is not present as the selenium amino acids in the protein was obtained from chromatographic analysis of the enzymic hydrolysate of the purified protein. Such chromatograms revealed no radioactive fractions in the vicinity where the selenium amino acids are known to appear.

Pronase was chosen for the enzymic hydrolysis for two reasons. One, it appears to be a good proteolytic enzyme in that it cleaves 87% of the total peptide bonds in egg albumin and 75% of the total peptide bonds in casein (Boyer *et al.*, 1960); secondly, and more important, it was necessary to hydrolyze the proteins under very mild conditions to ensure that no degradation of the selenium amino acids occurred. In a preliminary experiment, it was determined that there was considerable degradation of both selenocystine and selenomethionine when these acids were subjected to acid hydrolytic conditions. These observations are in support of the findings of Painter (1941) and Peterson and Butler (1962) who demonstrated that selenocystine was decomposed during acid hydrolysis. Blau (1961)

also observed that selenomethionine is destroyed by acid hydrolytic conditions.

Urinary Excretion of Selenium. Since there was no evidence for the existence of either selenocystine or selenomethionine in the liver protein hydrolysate or in liver and kidney extracts (J. L. Martin and L. M. Cummins, unpublished data), the urine was investigated.

When the *in vivo* labeled urine sample was first analyzed on the amino acid analyzer, peaks 1-A and 1-B (Figure 3) were suspected of being selenocystine and selenomethionine, respectively. This same conclusion could also be drawn concerning peaks 2-A, 2-B, 3-A, and 3-B if the similarities in elution volume were the only criterion of identification considered. It was not until the six peaks were isolated and analyzed for selenite that any reliable conclusions could be drawn in regard to the possible presence of the selenium amino acids in the sample. When fractions 1-A and 1-B were analyzed, 56.1 and 51.0%, respectively, of the activity was determined to be selenite. This observation was still not conclusive enough to completely rule out the possibility that the remaining activity was not due to the authentic selenium amino acids. However, the fact that more than 80% of the radioactivity in the four fractions 2-A, 2-B, 3-A, and 3-B was selenite strongly suggested that they were not the selenium amino acids. The fact that carrier selenite increased the recovery of radioselenite adds additional evidence that probably all the radioselenium was present as selenite. These data suggested that the radioselenite was tightly bound, presumably by sulfur compounds.

The possibility that the analytical methods employed could cause oxidative destruction of selenides has been eliminated because the authentic compounds (serving as controls) were carried through the entire procedure and no selenite was detected.

If the selenite recovery values of the fractions from the *in vivo* and *in vitro* labeled urine samples were reexamined, it appears that the selenite was not bound identically in both samples because there was more than 80% recovery from the *in vitro* fractions and only about 50% from the *in vivo*. However, by analyzing the residues of the *in vivo* fractions once again with the carrier technique, approximately 40% of the remaining radioselenium was recovered as selenite. An explanation for the additional recovery of selenite by multiple analysis of the same fraction is that some sort of equilibrium exists in the binding of selenite, and under alkaline conditions a certain amount of selenite is released each time. It was hoped that all the radioselenium could be recovered in this manner, but after the second analysis the residue was so concentrated with reagents that it was not feasible to analyze the fraction again. The variations in the recoveries between the urine samples must be due to their differences in composition. In other words, it was assumed that the control urine was of the exact composition as the *in vivo* labeled urine with the exception that radioactive selenite was not initially present. Comparison of the ninhydrin-positive peaks on the two chromatograms (Figure 3) points out considerable variation from one sample to the other, and the ninhydrin-positive peaks represent only a fraction of the compounds found in urine. Therefore, perhaps the use of this sample as a control was not fully justified, but for lack of a better control there was no other alternative.

Conclusion

Information obtained from this investigation strongly suggests that in the rabbit there is no biosynthetic pathway by which selenium as selenite can replace sulfur in cystine and methionine. Although previous investigators have reported that these selenium amino acids are present in biological material, their criterion of identification was based on the observation that radioselenium was detected on chromatograms in the vicinity where their corresponding sulfur analogs are known to appear. However, now that it is known that sulfur compounds complex with selenite under acidic conditions, such proof is no longer sufficient.

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