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To the Graduate Council:

I am submitting herewith a thesis written by Janet D. Jolly entitled "Metabolism of selenium-75 in the erythrocytes of sheep and growing calves." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Animal Science.

M. C. Bell, Major Professor

We have read this thesis and recommend its acceptance:

Joseph Fuhr, E. W. Swanson

Accepted for the Council:
Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

To the Graduate Council:

I am submitting herewith a thesis written by Janet D. Jolly entitled "Metabolism of Selenium-75 in the Erythrocytes of Sheep and Growing Calves." I recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Animal Science.

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We have read this thesis
and recommend its acceptance:

Joseph E. Tuhy

Erwin W. Swanson

Accepted for the Council:

Walter A. Smith

Vice Chancellor
Graduate Studies and Research

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METABOLISM OF SELENIUM-75 IN THE ERYTHROCYTES OF SHEEP AND GROWING CALVES

A Thesis

Presented for the

Master of Science

Degree

The University of Tennessee

Janet D. Jolly

March 1976

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ABSTRACT

Four mature wethers and four Holstein-Hereford steers were dosed with a single intrajugular injection of ^{75}Se as selenious acid in order to determine red blood cell life spans. Blood samples collected in heparin were taken at two and eight hours, daily for 10 days, weekly for 12 weeks, and biweekly until the end of the experiment. One ml of each sample was washed in buffered saline, corrected to 100% with the packed cell volume, and used to determine the amount of ^{75}Se present in one ml of washed cells. One ml of plasma was collected in order to determine the selenium activity. The remainder of the blood sample was used in ^{75}Se chromatographic analysis and ^{75}Se incorporation studies.

The red blood cell life span of the sheep averaged 123 days, while that of the younger and older calves averaged 114 and 141 days respectively. Analysis of the selenoprotein by Dowex 50-8X ion exchange chromatography failed to reveal either selenomethionine or selenocystine. Other short-term incorporation studies suggested that ^{75}Se was bound to the sulfur of amino acids and not actually incorporated into the protein molecule.

^{75}Se proved to be adequate in determining the red cell life span of calves and sheep. Although studies on the manner of selenium incorporation into protein were not conclusive, methodologies evolved will be useful in continued studies on the metabolism of selenium in sheep.

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CHAPTER I

INTRODUCTION

Through the years selenium (Se) metabolism has become a very controversial subject. Extensive research has been conducted concerning its toxic effects, its addition to feedstuffs for farm animals, and its relation to several diseases. Numerous attempts have been made to interpret its method of incorporation into tissue proteins.

The isotope ^{75}Se has become a useful radioactive tracer in the study of red blood cell (RBC) life spans (Wright, 1965; Withrow and Bell, 1969). Several other tracers used to study RBC life spans include ^{59}Fe, ^{51}Cr, and glycine-2-^{14}C. Each isotope has a characteristic metabolic pathway that influences the data and the life spans determined previously using ^{75}Se agree rather closely with the glycine-2-^{14}C method. However, the manner in which selenium becomes attached to the protein fraction of animal tissues is still unknown. Numerous attempts are being made to solve the puzzle. The problem is complex because selenium as selenite, selenate, and selenomethionine and selenocystine all behave differently.

In the following study ^{75}Se as selenious acid (H_2SeO_3) was used in order to confirm the length of the RBC life span of the sheep and to investigate the use of selenium in the determination of the length of the bovine RBC life span. Other objectives in this study were to attempt to resolve the incorporation controversy.

In order to accomplish these goals the following areas were investigated:

(1) Determine the ^{75}Se uptake in the plasma and RBCs of the sheep and calf.

(2) Use ion exchange chromatography in order to quantitate the amount of radioactivity in the chromatographic areas of cystine and methionine.

(3) Carry out short term in vitro incorporation studies using a protein synthesis inhibitor, simple dialysis and the incubation of selenium with purified cystine and methionine.

(4) Roughly quantitate the amount of cystine and methionine in the globin fraction of sheep and calf blood.

CHAPTER II

LITERATURE REVIEW

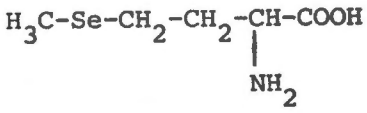
It is impossible to cover the vast topic of selenium in the biosphere. Numerous comprehensive reviews concerning the element are available (Rosenfeld and Beath, 1964; Withrow, 1967; Shrift, 1958; Stadtman, 1974; Ammerman and Miller, 1975). Therefore, the literature review will be limited to those topics pertaining directly to the thesis problem.

I. CHEMISTRY OF SELENIUM

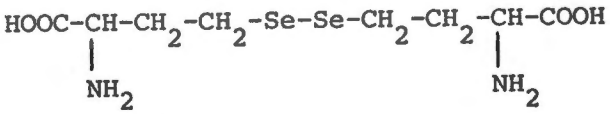
In order to comprehend the activity of selenium a review of its chemical nature is in order. Selenious acid is a weak dibasic acid which often acts as an oxidizing rather than a reducing agent. Two series of salts, the normal selenites and the acid selenites, are formed by selenious acid. Selenite readily oxidizes sulfhydryl compounds forming disulfide and unstable RS-SE-SR compounds. The following reaction between sulfhydryl compounds and selenious acid was suggested by Painter (1941): $4\ RSH + H_2SeO_3 \rightarrow RSSR + RS\text{-}Se\text{-}SR + 3\ H_2O$. The seleno-compound is unstable, with the selenium separating more rapidly under basic than under acidic conditions. This phenomenon was confirmed by Cummins and Martin (1967) who showed that dialyzed alkaline liver homogenates labeled with ^{75}Se-selenite had a considerable loss of radioactivity in the form of selenite. Under acidic conditions the selenite ion appeared to be firmly bound to the sulfur compounds.

In the literature selenium is mentioned in several other forms, such as selenium tetrachloride ($SeCl_4$), sodium selenate (Na_2SeO_4), selenomethionine, and selenocystine. A study by Jenkins and Hideroglou (1971) demonstrated that the chemical form of selenium can markedly affect its metabolism.

Two amino acids which deserve special attention in considering the chemical behavior of selenium are selenomethionine and selenocystine.



Selenomethionine



Selenocystine

These selenoamino acids are unstable as are their sulfur analogs. Selenocystine is less stable than selenomethionine. It has been established that a mixture of selenoamino acids and their sulfur analogs cannot be separated by either resin or paper chromatography. (Peterson and Butler, 1962; Rosenfeld, 1961; Tuve and Williams, 1957).

II. BIOCHEMISTRY OF SELENIUM

Selenium in Plants and Bacteria. Selenium compounds, either in an excess or deficiency may affect the biological activities of both plants and animals. Cowie and Cohen (1957) discovered that replicating Esherichia coli could completely replace methionine with selenomethionine, without

altering the growth curve. Further studies suggested that E. coli incorporated the selenium into methionine, since selenomethionine was detected by paper chromatography (Tuve and Williams, 1961).

When hydrolyzed protein from seleniferous wheat was chromatographed, selenocystine and selenomethionine were detected (Rosenfeld and Beath, 1964). An attempt was made to incorporate radioactive sulfate, selenate, and selenite into the proteins of growing wheat plants (Rosenfeld and Beath, 1964). No radioactive amino acids were detected although a peptide of undetermined nature was isolated from the leaf cytoplasm.

Selenium in Animals. As early as 1938 it was demonstrated that ingested selenium was incorporated into the body tissues, apparently as part of the tissue proteins (Smith, et al., 1938). McConnell and Cooper (1950) discovered that selenium as Na_2SeO_4, injected subcutaneously in dogs, concentrated to a greater extent in the red blood cells than in the plasma. Later studies with $^{75}SeCl_4$ suggested that selenium was apparently incorporated into amino acids (McConnell and Wabnitz, 1957).

In a study with chicks (Jenkins, 1968), it was observed that 80% of the radioactive selenium found in the serum could be liberated in the form of elemental selenium and selenite, by the action of disulfide cleaving agents. Extensive dialysis of the serum indicated the radioactivity was protein bound. It was of interest to note that if the serum were drawn after four hours post-incubation, the selenoprotein appeared resistant to the action of the cleaving agents, and this resistance increased with time.

Recently several mammalian selenoproteins have been isolated. A selenoprotein was discovered in the muscle of lambs receiving supplemental selenium (Whanger, et al., 1972). This protein was not detected in lambs on a selenium deficient diet. Selenium is also thought to be an important component of glutathione peroxidase. The addition of glucose normally prevents the hemolysis of red blood cells caused by oxidative peroxides. Enzyme preparations of the red blood cells of selenium deficient animals were found to be low in glutathione peroxidase activity, thus promoting the RBC oxidative damage. Neither the addition of glucose nor glutathione helped to prevent this oxidation (Rotruck, et al., 1973). Therefore, selenium seems to be critical to the glutathione peroxidase activity.

In vitro work involving the incorporation of selenium into ovine red blood cells (Wright and Bell, 1963), has shown that ^{75}Se as selenite can move into the red cells if oxygen is supplied, and the amount of movement is inversely proportional to the dietary intake of selenium. The incubation of human blood with ^{75}Se-selenate (Lee, et al., 1969) disclosed the presence of a selenoprotein in the plasma. This protein was not evident when the plasma alone was incubated with the isotope and it appears possible that a selenoprotein is being produced by the red cells as a product of metabolism. The identity of the compound remains obscure, although the authors feel that it may be this compound, which Wright and Bell (1963) thought to be selenite, that is slowly incorporated into the red cell in vitro.

Selenium metabolism in ruminants is rather controversial. The ruminant's ability to synthesize essential amino acids by the action of rumen microorganisms gave Rosenfeld (1962) a basis to believe that ^{75}Se as selenite could be incorporated into wool proteins. It became evident that when radioactive selenite was fed to sheep, that seleno-methionine and selenocystine were present in the wool hydrolysates, although very little of the radioactivity was actually an integral part of the protein. The administration of ^{75}Se to the rat failed to indicate the presence of selenoamino acids in the hair. When, however, the same injection was given to dogs a very small amount of activity was found in the cystine fraction of the sulfokeratine of the hair. In a previous study (Withrow, 1967) it was noticed that the ruminant incorporated a greater percentage of the total dose of ^{75}Se in the RBCs than did the nonruminant.

III. USE OF ^{75}Se IN RED BLOOD CELL LIFE SPAN STUDIES

Several isotopes have been used in order to determine the RBC life span of farm animals. ^{51}Cr has been employed to measure the life span of sheep, swine, cattle, and burro red cells (Hansard and Kincaid, 1956). Average survival time was calculated as 71 ± 10 days for mature swine and roughly 48 - 64 days for the sheep, cattle and burro. Young lambs injected with ^{59}Fe (Baker and Douglas, 1957) gave very low results of 46 days for three month old lambs and 52 days for those a year old. Estimated red cell survival time for 21 calves using ^{59}Fe averaged 47 ± 8 days (Hansard, et al., 1959), which is far below the 115 days reported

for calves by other methods (Mizuno, et al., 1958).

A more accurate estimate of 146 days was obtained by injecting sheep with glycine-2-^{14}C (Judd and Matrone, 1962) which employed a more direct method. The importance of using a direct physiological method of estimating RBC life spans was first emphasized by Shemin and Rittenberg (1946). Previous human RBC life span estimates ranged from 5 to 200 days! Glycine labeled with ^{15}N was used to label the heme fraction based on the theory that glycine is the precursor of the protoporphyrin of hemoglobin. In this way a more reliable human RBC life span was estimated at 127 days.

The isotope selenium-75 has a half life of approximately 120 days, emits a gamma ray of .4 mev. and decays to form stable arsenic (Wang, 1969). The isotope's relatively long half life and capability of being incorporated in the cell, coupled with being a gamma emitter it requires no special preparation before radioactive detection, make ^{75}Se a good candidate for RBC life span studies. Dogs were the first to be used in long term studies (McConnell and Roth, 1962), which revealed a drop in radioactivity at 100 to 120 days after injection. The results were interpreted to mean that once selenium became incorporated into the dog RBC, it remained there throughout the life span of the cell.

McConnell et al. (1953) were the first to use ^{75}Se in red cell life span studies. Red cells from ducks injected with sodium selenate were administered to other ducks, giving a life span of 11.7 days. This estimate was much lower than a later estimate of 42 days (Brace and Atland, 1956). Several years later it was discovered that selenium remained attached to

the RBC of the dog until the death of the cell (McConnell, 1963). In order to establish the usefulness of ^{75}Se in estimating the RBC life span of farm animals, Wright (1965) dosed several sheep with $H_2{}^{75}SeO_3$. He obtained an average RBC lifespan of 157 days for mature sheep. An average of 127 days was found in growing lambs dosed with radioactive selenite (Withrow and Bell, 1969).

IV. CHROMATOGRAPHIC ANALYSIS OF SELENOPROTEINS

In attempts to isolate amino acids from a selenoprotein, one generally turns to chromatographic analysis. Moore and Stein (1951) provided a very detailed procedure for separating amino acids on sulfonated polystyrene resins. Using Moore and Stein's method, ion exchange resins are generated to possess a charge which causes amino acids to selectively adhere to the surface of the resin due to their individual chemical makeup. When a buffer of weak ionic strength is washed through the column, the amino acids are eluted as shown in Figure 1. The temperature and pH of the buffer are changed at designated intervals in order to continue the elution. Figure 2 shows a chromatogram containing methionine sulfone and cysteic acid. Selenomethionine and selenocystine, when oxidized with performic acid most probably behave as methionine sulfone and cysteic acid.

Numerous attempts have been made to chromatograph animal selenoproteins. Bovine RBCs incubated <u>in vitro</u> with ^{75}Se-selenite were hydrolyzed and chromatographed with 68% of the activity appearing in the glutathione disulfide (GSSG) area (Jenkins and Hideroglou, 1971). About half of this

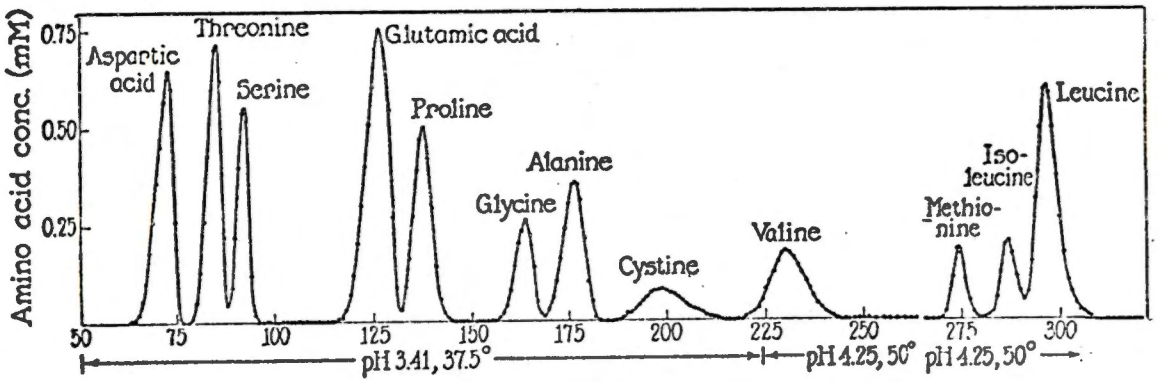


Figure 1. Separation of synthetic amino acid mixture on a column of Dowex-50 (Na^+), .9 x 100 cm. (From Moore and Stein, 1951)

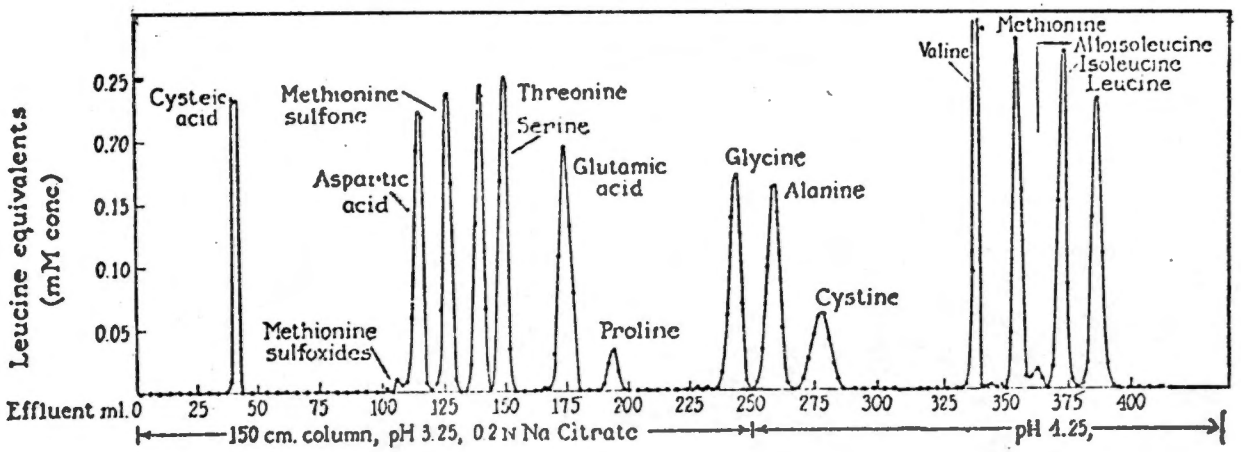


Figure 2. Separation of a synthetic amino acid mixture on a column of Amberlite CG-120 (Na^+), .9 x 150 cm. (From Moore, Spackman and Stein, 1958)

activity was found to be GSSeSG and the rest chromatographed as elemental selenium. Liver hydrolysates from dogs injected with ^{75}Se were chromatographed with peaks of activity being found in the regions of selenomethionine and leucine (McConnell and Wabnitz, 1957). It was noted that several times the prescribed volume of buffer was required to elute the radioactivity, while a small portion was never removed and was thought to be irreversibly bound.

In contrast to McConnell and Wabnitz's finding with liver hydrolysates, the selenoprotein described by Whanger et al. (1972) in lambs supplemented with selenium was chromatographed with only traces of cystine and methionine found. Furthermore, limited analysis of both sheep and pig globin revealed ^{75}Se to be in areas of isoleucine, tryptophane and phenylalanine (Withrow, 1967).

The first indication of a true bacterial selenoamino acid was found in a hydrolysate of E. coli incubated with radioselenite (Tuve and Williams, 1961). The radioactive portion of the hydrolysate chromatographed as selenomethionine. Only low yields of radioactivity were obtained using a Dowex-50 (H^+ form) column, due to the fact that much of the activity remained bound to the column. Neither paper nor column chromatography revealed any fraction corresponding to selenium analogs of cystine. In a later study (Cowie and Cohen, 1957) ^{75}Se in E. coli protein was chromatographed as selenocystine with no evidence of ^{75}Se-selenomethionine.

Studies involving ^{75}Se in plants, animals, and bacteria present

conflicting data. One should remember, though, that each system has its own particular specificities and each of these systems must be completely characterized before valid comparisons can be made.

CHAPTER III

MATERIALS AND METHODS

I. EXPERIMENTAL ANIMALS

Four mature wethers and four Holstein-Hereford steers were used in the study. Two of the calves were six months old weighing 136 and 140 kilograms, while the other two calves were ten months old, weighing 187 and 193 kilograms. The mature sheep weighed 56, 63, and 64 kilograms. All animals were placed in metabolism stalls for a two week adjustment period. While in the metabolism stalls the wethers received approximately .9 kilogram and the calves 1.8 and 2.3 kilograms of mixed feed daily, according to their weight. This mixed feed was a complete balanced ration containing 12% crude protein and 18% crude fiber. Ingredients included steam rolled yellow corn, soybean meal, alfalfa meal, cottonseed hulls, molasses, animal fat, Vitamin A and minerals. The feed mixture contained 0.093 PPM of selenium. After a period of eight weeks the calves were moved to a pasture and after twelve weeks the sheep were moved to a paddock.

II. ISOTOPE DOSING PROCEDURE

Each animal received a single dose of ^{75}Se as H_2SeO_3 in dilute HCl intravenously into the jugular vein by means of a polyethylene catheter and a 14 gauge California bleeding needle. A 10 ml volume of saline was used to flush all of the isotope into the vein. A dose of .8 mCi was

given to the sheep, 2 mCi to the large calves, and 1.6 mCi to the smaller calves. The isotope had a specific activity of 160 mCi/mg of stable selenium. Radioactivity of the feces and urine was monitored for 10 days and checked frequently thereafter until a safe level was reached.

Jugular blood samples of 10 to 20 ml were drawn into heparinized syringes at two and eight hours after dosing, each day for 10 days, once a week for 12 weeks, and every other week thereafter until the end of the experiment. One ml of whole blood and one ml of plasma were removed for radioactive counts, with the remainder being used in selenium binding studies and column chromatography work.

III. EXPERIMENTAL PROCEDURES

Erythrocyte Life Span Study: The heparinized sample from each animal was gently mixed and one ml of whole blood and a microhematocrit sample were removed. The one ml sample was washed twice with .85% buffered saline at 3,000 RPM in a refrigerated centrifuge. Hematocrits were spun in a microhematocrit centrifuge for 3-1/2 minutes. The initial sample of whole blood was then centrifuged for 10 minutes at 3,000 RPM and a one ml sample of plasma removed for counting. The remaining cells were washed three times with buffered saline and used in other studies. The samples of washed cells and plasma were assayed for ^{75}Se activity in a well-type solid crystal counter. A dilution of the $H_2{}^{75}SeO_3$ used in dosing the animals was counted with the samples and used as a reference standard of ^{75}Se activity.

In all animals the radioactive counts for the washed cells were corrected to 100% with the hematocrit. An additional correction factor to account for the increasing blood volume was used in calculating the values for the calves. Blood volume was roughly estimated by a calculation involving the total body weight.

Chromatographic Analysis: Twenty ml of sheep RBCs were washed three times with buffered saline, brought back to the original volume with distilled water, frozen for 24 hours, thawed, and the stroma removed by centrifugation at 18,000 RPM's for 20 minutes. The pooled hemolysates were frozen if not used immediately.

A cold acetone-acid mixture was used to precipitate the globin (Anson and Mirsky, 1930). A mixture of four ml of conc. HCl and 100 ml of acetone was cooled to -15°C. A 10 ml aliquot of hemolysate was added dropwise to the mixture and stirred with a magnetic stirrer for 30 minutes at -15°C. The precipitate was allowed to settle at the same temperature and washed three times in cold acetone.

Hydrolysis of the globin fraction was carried out in a small separatory funnel. Samples were either treated with 6N HCl alone for 20 hours at 110°C (Hirs, 1967), or first incubated for 4 hours with performic acid and then hyrolyzed (Schram, et al., 1954). Before hydrolyzing all samples were evacuated, flushed with nitrogen, and evacuated again. If more than one sample was to be hydrolyzed, an evacuated dessicator was used. After

treatment with performic acid and treatment with HCl, all samples were brought to a slurry stage in an evacuated rotary evaporator at 50°C. Drying of the sample took about 10 minutes.

In most of the column chromatography work the original procedure of Moore and Stein (1951) was followed closely. A .9X100 cm column equipped with a water jacket was packed with Dowex-50 cation exchange resin in the sodium form and mounted on a fraction collector. The hydrolyzed sample was brought to a desired volume with a .2M sodium citrate buffer at, pH 3.42, and carefully applied to the column which had previously been equilibrated with the same buffer. In order to elute the sulfur amino acids which are suspected to be the carriers of ^{75}Se, only the first two buffers pH 3.42 and 4.25, and the first two temperatures of 37°C and 50°C were used. The column was adjusted to run at 4 ml per hour, collected in one ml fractions. Tubes were searched for radioactivity in a well type gamma counter.

Incorporation Studies. Several studies were performed in order to get an insight into the mechanism of the attachment of ^{75}Se to proteins. Following a procedure of Fuhr and Gengozian (1973) ^{75}Se as H_2SeO_3 was incubated *in vitro* at 37°C with washed ovine red cells for one hour. Cycloheximide was added to half of the samples in order to inhibit protein synthesis. Reactions were terminated with cold physiological saline, the excess isotope washed free of the cells, the cells lysed and stroma removed by centrifuging at 18,000 RPM for 20 minutes. Radioactivity of

both the control and the samples containing cycloheximide was determined.

A simple dialysis procedure described by Cummins and Martin (1967) was performed in order to determine the binding status of ^{75}Se. A hemolyzed sample of ovine red cells with the stroma removed was brought to pH 9.0 with .5N NaOH and was dialyzed against H_2O at 0-3°C for 24 hours. Globin samples were also treated in the same way.

Encouraged by the evidence of Schwarz and Sweeney (1964) that several sulfur compounds can bind the selenite ion, a 5 mg sample of purified cystine was incubated with .4 uCi of $H_2{}^{75}SeO_3$ for 24 hours at a pH of 4.0 at room temperature. The sample was then chromatographed on the previously described column of Dowex-50 through the first buffer of pH 3.42 and temperature of 37°C. All fractions were incubated at 60°C for several minutes after the addition of 1/2 ml of ninhydrin reagent. In order to detect the amino acid, the fractions containing the amino acid were then checked for ^{75}Se activity.

In order to determine the amount of ^{75}Se present in the enzyme portion of the red cell, which includes glutathione peroxidase, a procedure of Bonnichsen and Brink (1955) was followed in order to precipitate the hemoglobin. A mixture of 90% ethanol and chloroform was added dropwise to the hemolysate and stirred rapidly at 5°C. A spongy mass of hemoglobin was separated from the soluble glutathione peroxidase and other enzymes by filtration. The fraction was checked for ^{75}Se activity.

Analysis of Sheep and Calf Globin for Cysteic Acid and Methionine. In order to compare the amount of sulfur amino acids in the globin from sheep and calves, samples from each experimental animal were either hydrolyzed with 6N HCl for 20 hours at 110°C then dried on a rotary evaporator, or were treated first with performic acid then hydrolyzed. In the group treated with performic acid, methionine would be oxidized to methionine sulfone and cystine to cysteic acid. In this way, rather than to risk the possible loss of either amino acid to oxidation, each was completely oxidized and expressed as complete individual peaks. All samples were resuspended in water and analyzed on a Beckman/Spinco Amino Acid Analyzer.

CHAPTER IV

RESULTS AND DISCUSSION

I. ERYTHROCYTE LIFE SPAN STUDY

Sheep. Figure 3 represents an average of the ^{75}Se uptake of the red blood cells and plasma of the three sheep dosed with selenious acid. The rapid decrease in plasma activity and slow increase in red cell activity at approximately day 8 agrees with previous results (Withrow, 1967). The peak of erythrocyte activity was found at 44 days. A rapid decline in red cell activity was seen at 152 days indicating that the majority of cells that had originally incorporated the label had been eliminated from the circulation.

The ovine RBC life span was estimated at 123 days with representative values of 120, 121, 127 days. The procedure of Neuberger and Niven (1951) was followed which estimated the mean life span as the time interval between the point at which 50% of the maximum activity is obtained and that point at which the activity falls again to 50% of the maximum activity. These results are slightly lower than previous ovine RBC life span determinations (Judd and Matrone, 1962; Wright, 1965), but agree favorably with those of Withrow and Bell (1969).

Calves. Figure 3 represents the ^{75}Se uptake of the erythrocytes and plasma of four calves. The general shape of the curves is similar to that of the sheep, yet more than four times the radioactivity was found in the ovine erythrocytes. As with the sheep, a rapid decrease of activity was

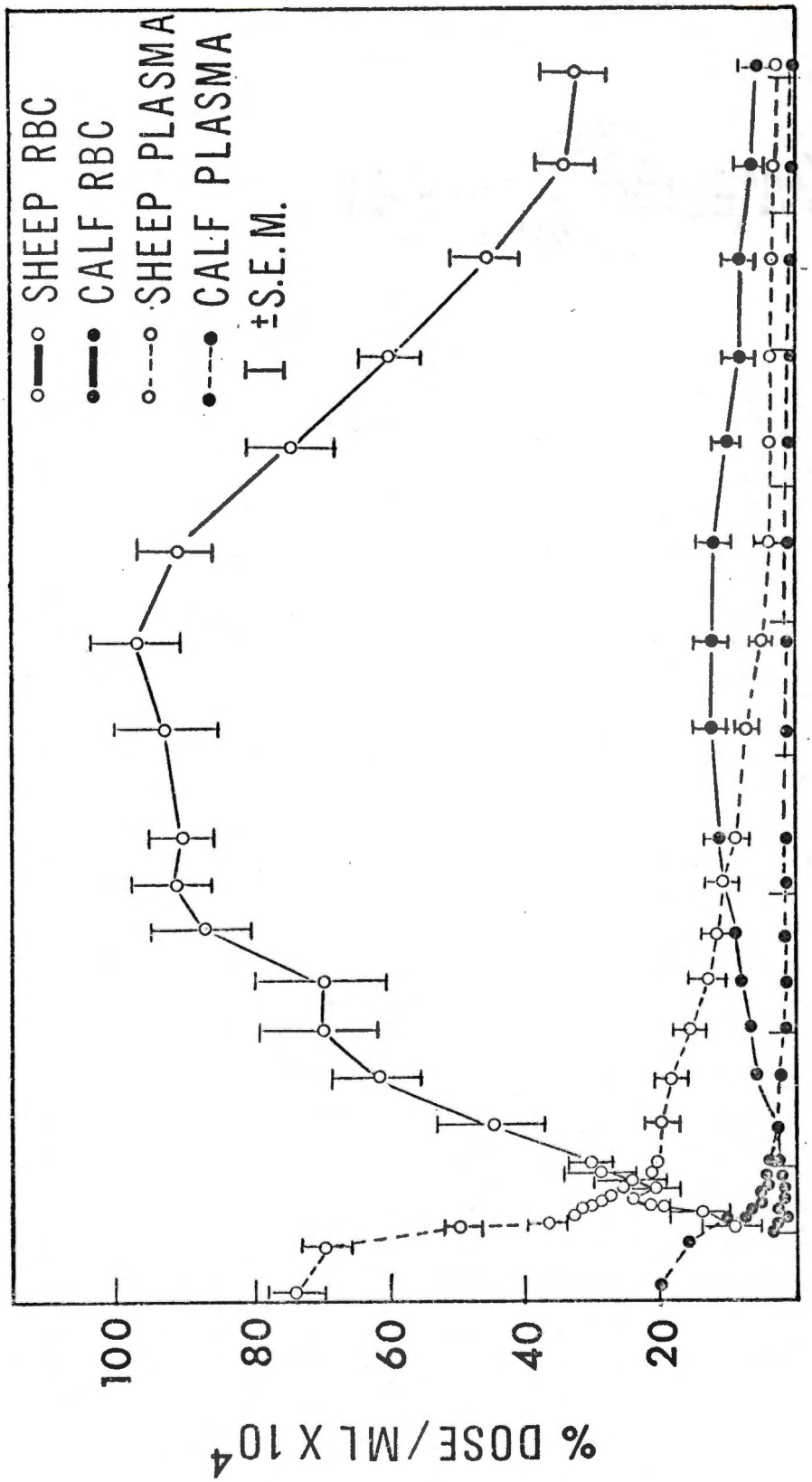


Figure 3. The ^{75}Se uptake in the RBCs and plasma of 3 sheep and 4 calves.

seen in the plasma and a slow increase in the erythrocyte activity which peaked at 73 days.

Two distinct bovine red cell life spans were determined by the method of Neuberger and Niven (1951). The older calves gave estimates of 146 and 137 days, while the younger calves RBC survival time was estimated at 109 and 119 days. The average of 141 days is slightly less than the 157-162 days estimated by Kaneko, et al., (1971) for mature cows, and the shorter life span average of 114 days is in accord with the 115 day estimate on calves by Mizuno et al., (1958). Estimates for young calves using ^{14}C as above are as low as 70 days (Johnson and Schwartz, 1970).

Erythrocyte life span is believed to be an age dependent factor (Schalm, et al., 1975). A study using ^{51}Cr to determine the red cell life span of cyclic hematopoietic dogs revealed three distinct life spans in both the CH and normal dogs (Lange, et al., 1976). The shorter life span correlated with the younger age group, while the longest life span correlated with the oldest group. If the RBC life span is, in fact, age dependent, then this may explain the difference in the two determinations. Even though the difference in age was only four months, the rate of growth of the younger calves indicates that they were not, by far, fully grown. As to why the RBC should not survive as long as those of a mature steer, it is possible that, although the red cell volume is increasing to meet the demands of growth, these demands are only fully met at the expense of the oldest red cells. Gradual attrition of erythrocytes energy-generating metabolic activities occurs with aging resulting ultimately in cell death and removal from the circulation (Schalm, et al., 1975)

In a life span the length of approximately 120 days, an increase in activity of about .8%/day should be observed. This was found to be true in the sheep with an increase of approximately 1.2%/day, but in the calves only an increase of .2%/day was demonstrated. An autoradiogram from sheep dosed with ^{75}Se as H_2SeO_3 revealed a high concentration of radioactivity in the hematopoietically active areas of the bone marrow (Wright, 1965).

The sheep have a RBC count of 9-15 million/mm^3 of whole blood, while the calves RBC count averages from 5-10 million/mm^3. This may provide a possible reason for the increased uptake in ovine RBCs. The sheep red cells are much smaller, in some cases only half the size of those of the calf, yet the normal hemoglobin content expressed as g/100 ml of whole blood is the same in both animals. Unless the incorporation is a process involving attachment of the ^{75}Se to the red cell membrane then the higher RBC count cannot be used as a basis for increased uptake.

The uptake of ^{75}Se by the bovine RBCs was similar to that of porcine RBCs in a previous study (Withrow, 1967). In this case the ^{75}Se incorporated or attached to the ruminant RBCs was comparable to that of a non-ruminant. Rather than propose that the ruminant, in this case the sheep, is able to incorporate more ^{75}Se due to the action of ruminant bacteria, it appears that the sheep has some quality peculiar to it, which enables the RBCs to incorporate greater amounts of the isotope.

II. CHROMATOGRAPHIC ANALYSIS OF SHEEP GLOBIN CONTAINING ^{75}SE

All chromatography work dealt entirely with sheep globin. The search for significant radioactive amino acids lay in the belief that selenium

in some way either replaces sulfur in sulfur amino acids, or becomes covalently bound to the sulfur molecule in them. All attempts were directed toward the determination of the presence of ^{75}Se as selenocystine and selenomethionine. It has been established that most of the radioactivity is found in the globin rather than the heme fraction of animals dosed with ^{75}Se (Wright, 1965; Withrow, 1967). This fact was also confirmed in the present study and it was the globin fraction that was used in all chromatographic work.

The first successful attempts at a chromatographic analysis of sheep globin containing ^{75}Se revealed an almost immediate release of radioactivity occurring between 50 and 60 ml of effluent. In comparing this elution point to those in Figure 1, it is evident that this peak appears even before the first amino acid. This activity in the eluant continued at a level of three times background through the first buffer. The fractions making up the peak were lyophilized and analyzed on the Beckman analyzer. Numerous amino acids were found in large quantities. Due to the large amount of protein applied to the column in order to obtain detectable radioactivity, it was thought that the column was probably loaded beyond its capacity. A manual ninhydrin determination on each tube collected through the first buffer revealed a definite overlapping of peaks, confirming the above theory.

In order to eliminate the basic amino acids and therefore minimize the amount of protein applied to the column, a sample previously treated with performic acid was run through a column of Dowex-50 in the ammonia form. All basic amino acids should have been adsorbed on the column, with the neutral and acidic ones being eluted. If our previous radioactive

peak was due to an overload, then the same peak should have been eluted using this column. Selenomethionine and selenocystine, if present, would have been eluted as would methionine sulfone and cysteic acid, in a single peak with the acid and neutral amino acids. The radioactivity, in this case, did not wash through the column, and was only released upon regeneration of the column with .2N ammonium hydroxide.

A large column of Amberlite CG-120 (Na^+) was used in order to maximize the surface area available to the sample. This resin is similar to Dowex-50 but varies in particle size and was available in larger quantities. A standard sample of ^{75}Se-selenomethionine treated with performic acid was eluted with 120 ml of sodium citrate, while a sample of hydrolyzed globin treated with performic acid was not eluted at all. The large column (4.5 x 100 cm) proved to be impractical to operate due to the much larger effluent volumes and time required to elute the amino acids.

It is possible that the radioactive peak eluted at approximately 50 ml of effluent could have been ^{75}Se as selenite. Selenious acid, when chromatographed, appeared within 8-10 ml of effluent, and in a previous study (Cummins and Martin, 1967) radioactive selenite was eluted after 35 ml. A ^{75}Se-selenite standard was not available at the time of this study.

The hydrolyzed samples of sheep globin did not appear to contain either ^{75}Se-selenomethionine or ^{75}Se-selenocystine. In numerous chromatographic analyses on three separate columns using two separate procedures, at no time did the radioactive fraction even give a hint of behaving as either

selenoamino acid. ^{75}Se-labelled animal tissues, when chromatographed on both paper and ion exchange columns, always present the problem of sample overload. The radioactivity/protein ratio is too low to properly correlate one with the other by a chromatographic analysis. There now exist several highly cross-linked resins used specifically for the retrieval of sulfur amino acids from a hydrolyzed sample, but their cost was prohibitive in the present study.

Another problem which exists in working with the sulfur amino acids is their extreme vulnerability to oxidation (Smith, 1960). It is almost impossible to completely hydrolyze the protein without destroying at least a portion of the cystine and methionine present. Enzymatic digestion in this case may be worth exploring.

The knowledge that sulfur amino acids bound by radioselenite chromatograph as do the corresponding selenoamino acids (Cummins and Martin, 1967), makes it difficult to interpret such chromatograms. It is obvious that selenium's biochemical nature makes it very difficult to obtain decisive results.

III. INCORPORATION STUDIES

In the brief attempt to inhibit protein synthesis in the reticulocytes of sheep with cycloheximide, it was found that the radioactivity in the cells treated with cycloheximide was 67% of that in the untreated cells.

Cycloheximide inhibits protein synthesis by blocking the transfer of amino acids from the transfer RNA to the polypeptide chain. If the radioactivity which appears to be incorporated into RBCs *in vitro* is due entirely to the protein synthesis in the small number of reticulocytes in peripheral ovine blood, then there should have been very little radioactivity in the RBCs treated with cycloheximide. It appears from this experiment that most of the ^{75}Se was merely attached to the protein fraction of the red cell, while only a small portion could have been incorporated into the protein. Previous studies of this kind (Fuhr and Gengozian, 1973) using marmoset red cells and radioactive leucine, revealed almost no radioactivity in the cells incubated with cycloheximide.

In the procedure in which the globin fraction was adjusted to a pH of 9.0 and dialyzed against water at 0-3°C it was found that most of the radioactivity was dialyzed out before 16 hours. A portion of the hemoglobin fraction precipitated with alcohol and chloroform (Bonnichsen and Brink, 1955) was redissolved in 1 N KOH and dialyzed with similar results. These findings correspond to those of Cummins and Martin (1967) indicating that the selenium bound to the globin was influenced by pH, a phenomenon which should not occur in incorporated proteins.

Purified cystine, when incubated with selenious acid under acid conditions, gave only a slight indication of the *in vitro* binding phenomenon described by Schwarz and Sweeney (1964). In most cases the selenious acid emerged from the column very early, while cystine, as detected by the

ninhydrin reaction, followed later with no overlapping of peaks. In one instance a broad but very low peak appeared in the area of proline and glycine. This peak at the time was considered insignificant and was not checked for the presence of cystine.

The amount of activity found in the enzyme fraction remaining after the removal of hemoglobin was very low. It did indicate, though, that some ^{75}Se was incorporated into the enzyme fraction of the sheep blood.

The exact method of selenium incorporation is not yet known. Numerous hypotheses and methods exist, but a single answer is probably not to be found. Answers most commonly arrived at in dealing with the incorporation questions usually include either: (1) an exchange reaction in which selenium displaces sulfur as $SeO_3^{2-}+H_2S \rightleftharpoons H_2Se+SO_3^{2-}$; (2) a series of exchange reactions in which selenite is converted to organic selenium, which, through enzymatic pathways, is incorporated into proteins during protein synthesis (McConnell and Roth, 1966); or (3) in which selenium as selenite is covalently bound to the sulfur compound. The reason for the alternative explanations appearing frequently in the literature is probably due to the different ways in which selenium acts in different animals, plants, or bacteria. Cowie and Cohen (1957) provide evidence which supports the incorporation theory, Wright (1967) presents data that correlates with the incorporation of selenium into proteins, and the results of Cummins and Martin (1967) lead us to believe the theory

of selenium binding to sulfur. Each investigation deals with the binding of selenium in different biological materials.

IV. AMINO ACID ANALYSIS OF SHEEP AND CALF GLOBIN

Tables 1 and 2 represent the values for cysteic acid and methionine as chromatographed on the Beckman analyzer. Although it is not possible from these data to quantitate the amino acids in the two species, it can be stated that the greater amount of ^{75}Se activity found in the ovine was not due to a difference in the amount of sulfur amino acids found in the globin. A four-fold increase in the erythrocyte uptake due to an increased amount of cystine and methionine should have been detected in the analysis by the Beckman analyzer. The possible explanation for this increased uptake can not be explained by the amount of methionine and cystine residues found in the alpha and beta chains of the hemoglobin. While the beta chain of sheep hemoglobin contains two and three residues of methionine, and the calf only one methionine, it is also true that the sheep alpha chain contains only one residue of methionine, while the calf has two (Dayhoff and Eck, 1968). The number of residues of cystine are also equally distributed between animals within the alpha and beta chains.

It appears that fractionating both sheep and calf globin on a Sephadex column, and in this way determining the ratio between the alpha and beta chains, would be of great value. If the ratios are known, then

TABLE I

AMINO ACIDS OF CALF AND SHEEP GLOBIN AS ANALYZED ON BECKMAN ANALYZER AND EXPRESSED AS RATIOS OF SEVERAL AMINO ACIDS TO METHIONINE

	Calves				Sheep		
Amino Acid	588	589	544	543	406	411	470
methionine	1.00	1.00	1.00	1.00	1.00	1.00	1.00
aspartic acid	11.70	8.18	11.21	11.32	8.31	7.89	9.77
glutamic acid	8.80	5.31	9.84	6.55	5.00	6.03	6.39
glycine	12.03	5.94	5.95	10.06	7.26	5.68	6.70
alanine	18.60	10.63	18.63	13.76	7.34	8.79	9.91
valine	18.26	9.31	10.53	14.85	12.48	9.20	9.86
phenyalanine	6.71	4.68	8.26	6.55	3.33	4.94	4.34

TABLE II

AMINO ACIDS OF CALVES AND SHEEP EXPRESSED AS A RATIO TO CYSTEIC ACID

	Calves				Sheep		
Amino Acid	588	589	544	543	406	411	470
cysteic acid	1.00	1.00	1.00	1.00	1.00	1.00	1.00
aspartic acid	24.80	21.59	22.68	26.77	20.10	15.61	14.53
glutamic acid	11.30	13.44	7.54	21.15	12.96	10.00	11.02
glycine	11.87	23.02	13.37	17.46	17.06	8.96	11.74
alanine	21.82	28.95	38.91	45.38	24.10	21.10	20.60
valine	11.20	12.64	18.06	20.69	14.15	12.66	11.26
phenylalanine	.36	.94	1.51	1.12	.417	.26	.23

the number of residues of cystine and methionine can be corrected according to the ratios, and only then will this knowledge be valid in determining the reason for the greater ^{75}Se uptake by the sheep.

CHAPTER V

SUMMARY

Information obtained from this investigation suggests that ^{75}Se is adequate in determining the red blood cell life spans of calves and sheep. The life span of three sheep averaged 123 days, while that of the younger and older calves was 114 and 141 days respectively.

The incorporation of ^{75}Se into the sheep red cells was approximately four times greater than that in the calves. Upon analyzing the globin fraction of both the sheep and calves on a Beckman amino acid analyzer, no gross quantitative difference in the amount of sulfur amino acids was found.

^{75}Se as selenious acid does not appear to exist as ^{75}Se-selenomethionine or ^{75}Se-selenocystine in the globin of sheep. The selenoamino acids, if present, did not behave as previously described upon eluting the amino acids from an ion exchange resin. The radioactive peak from oxidized globin samples did not correlate with a selenomethionine standard under the same conditions. If selenocystine had been present in the sample it should have been eluted previous to methionine sulfone.

The radioactive label of ^{75}Se was quickly removed by dialysis under alkaline conditions. A very small amount of activity was found in the enzyme fraction of the sheep blood. Glutathione peroxidase is found in this fraction. Upon incubating nonradioactive sheep red cells with selenious acid with and without cycloheximide *in vitro*, it was found

that 67% of the activity observed in the control cells was seen in those cells in which protein synthesis was inhibited.

The selenium problem has been a topic of research for many years. As more questions are answered, new and more complex ones arise. Before further knowledge involving the incorporation of selenium into the globin fraction of the red blood cells of farm animals can be obtained several things must first be accomplished:

1) All excess protein not containing the selenium label should be removed. Withrow's procedure (1967) involving separation of the cells according to their age allows one to isolate only that age fraction which incorporated the isotope in the reticulocyte stage. If this fraction were hydrolyzed and applied to the highly-crosslinked resin mentioned earlier, then the possibility of isolating the radioactive peak is greatly enhanced.

2) Several ^{75}Se-labelled standards including selenite, selenious acid, selenomethionine, and selenocystine should be applied to the resin of choice and their elution points carefully noted. All conditions must be maintained exactly as each standard is chromatographed.

3) A person knowledgeable in aspects of column chromatography should be available to initially help set up the experiment and be on hand to try to answer any questions that arise.

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TABLE III

^{75}SE UPTAKE IN RBCS OF SHEEP AS % DOSE/ml x 10^4

Day After Dosing	406	411	470	Mean	S.E.M.
1	8.6	9.5	7.8	8.63	.5
2	6.9	6.6	6.1	6.53	.2
3	10.5	19.5	8.4	12.8	3.4
4	16.7	27.3	15.8	19.9	3.7
5	20.0	36.2	13.9	23.4	4.3
6	17.6	28.6	14.2	20.1	4.3
7	22.2	34.1	16.4	24.2	5.7
8	19.9	36.3	18.6	24.9	5.1
9	28.8	37.7	20.0	28.8	5.4
10	28.1	39.6	21.0	29.6	3.5
16	43.9	51.3	39.1	44.7	7.1
23	62.7	73.0	48.3	61.3	7.7
30	74.0	81.2	55.2	70.1	8.7
37	63.3	86.4	58.1	69.2	9.8
44	93.1	99.8	67.4	86.8	7.0
51	94.5	101.7	78.0	91.4	5.2
58	90.7	98.7	80.8	90.1	4.5
74	96.8	97	83.3	92.4	8.0
87	104.2	104.4	80.2	96.2	7.5
101	95.2	100.2	75.6	90.3	5.4
116	80.1	77.4	62.8	73.4	6.1
129	60	68.4	47.4	58.6	4.8
143	49.2	51.7	36.2	45.7	5.5
157	37	40.5	22.4	33.3	5.3
171	35.3	40.4	22.2	32.6	5.2

TABLE IV

^{75}SE UPTAKE IN PLASMA OF SHEEP AS % DOSE/ml x 10^4

Day After Dosing	406	411	470	Mean	S.E.M.
2 hr.	69.2	67.7	81.7	72.9	4.4
3 hr.	68.5	63.2	75.1	68.9	3.4
1	44.8	47.1	53.7	48.5	2.7
2	35.8	34.5	35.8	35.4	.4
3	32.8	32	32	32.3	.3
4	30.8	27.8	30.8	29.8	1.0
5	28.8	27.6	28.3	28.2	.3
6	28.5	26.4	27.0	27.3	.6
7	27.6	23.7	24.7	25.3	1.2
8	24.1	21.0	21.2	22.1	1.0
9	22.0	20.6	20.4	21	.5
10	21.4	19.7	20.7	20.6	.5
16	19.7	22.6	16.3	19.5	1.8
23	21.1	18.0	17.3	18.8	1.2
30	18.1	15.7	12.2	15.3	1.7
37	16.4	13.2	10.6	13.4	1.7
44	14.3	12.6	11.8	12.9	.7
51	12.9	12.0	5.8	10.2	2.2
58	11.5	9.4	4.9	8.6	1.9
74	9.2	7.9	3.4	6.8	1.8
87	6.3	5.4	2.8	4.8	1.0
101	5.3	3.7	2.4	3.8	.8
116	4.0	3.2	2.3	3.2	.5
129	3.8	3.1	2.3	3.1	.4
143	3.6	3.1	2.2	3.0	.4
157	2.9	2.4	2.4	2.6	.2
171	3.1	3.1	2.3	2.8	.3

TABLE V

^{75}SE UPTAKE IN RBCS OF CALVES AS % DOSE/ml x 10^4

Day After Dosing	588	589	544	543	Mean	S.E.M.
1	3.4	1.8	2.4	4.8	3.1	.7
2	2.3	1.4	1.7	1.8	1.8	.2
3	2.5	1.6	2.0	2.1	2.1	.2
4	2.2	1.2	2.2	2.2	2.0	.3
5	2.1	1.4	2.3	2.1	2.0	.2
6	2.1	1.2	2.1	2.1	1.9	.2
7	2.3	1.5	2.5	2.9	2.3	.3
8	2.5	1.7	2.7	3.3	2.6	.3
9	2.5	1.7	2.8	3.4	2.6	.4
10	2.6	1.5	3.0	3.4	2.6	.4
16	3.3	2.1	4.2	4.9	3.6	.6
23	5.7	3.6	6.9	7.9	6.0	.9
30	6.6	4.7	8.7	10.5	7.6	1.3
37	7.8	5.9	10.2	12.0	9.0	1.3
44	9.1	6.8	11.4	14.2	10.3	1.6
51	10.1	7.0	11.9	15.5	11.1	1.8
58	11.9	7.5	9.7	17.7	11.7	2.2
74	11.2	8.4	19.5	16.3	13.8	2.5
87	12.1	8.8	15.2	14.4	12.6	1.4
101	12.0	8.1	12.7	15.0	12.0	1.4
116	11.1	7.3	13.0	12.8	11.0	1.3
129	9.4	5.8	10.0	8.9	8.5	.9
143	8.9	5.9	9.63	8.9	8.3	.8
157	6.7	4.5	8.8	7.1	6.8	.9
171	6.9	5.0	8.1	6.9	6.7	.9

TABLE VI

^{75}SE UPTAKE IN PLASMA OF CALVES AS % DOSE/ml x 10^4

Day After Dosing	588	589	544	543	Mean	S.E.M.
2 hr.	17.8	18.6	20.2	23.7	20.1	1.3
8 hr.	14.9	15.9	16.6	20.0	16.9	1.1
1	9.7	9.6	10.0	13	10.6	.8
2	6.9	7.1	7.2	9.4	7.7	.6
3	6.2	6.0	6.5	7.4	6.5	.3
4	5.5	5.1	5.6	6.6	5.7	.3
5	4.9	4.6	5.6	5.3	5.1	.2
6	4.7	4.7	4.6	5.3	4.8	.2
7	4.0	4.0	4.4	4.6	4.3	.2
8	3.5	3.6	4.1	4.4	3.9	.2
9	3.4	3.6	3.9	4.2	3.8	.2
10	3.0	3.2	3.5	4.0	3.4	.2
16	3.7	3.4	3.7	4.2	3.8	.3
23	3.3	2.5	3.5	3.9	3.3	.3
30	1.8	2.0	2.7	3.2	2.4	.2
37	2.0	2.0	2.5	2.7	2.3	.2
44	1.9	2.0	2.4	2.6	2.2	.2
51	1.5	1.8	1.4	2.1	1.7	.1
58	1.3	1.6	1.8	1.8	1.6	.1
74	.8	.85	1.1	1.0	.94	.0
87	.7	.82	.8	.9	.82	.0
101	.5	.6	.65	.7	.61	.0
116	.5	.5	.4	.6	.5	.0
129	.4	.4	.36	.5	.42	.0
143	.4	.3	.3	.4	.35	.0
157	.3	.3	.28	.3	.3	.0
171	.3	.35	.35	.4	.35	.0