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Title: SELENOMETHIONINE AND METHIONINE IN IN VITRO RABBIT RETICULOCYTE HEMOGLOBIN SYNTHESIS AND IN ESCHERICHIA COLI tRNA AMINOACYLATION

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Paul H. Weswig

Selenomethionine and methionine have been postulated to be similarly involved in protein synthesis. This thesis has evaluated this hypothesis from a molecular and genetic approach. Selenomethionine and methionine were incorporated into rabbit reticulocyte hemoglobin at the same rates and in equal quantities. Each methionine demonstrated competitive inhibition for its analog incorporation into the globin. There were no significant differences in α or β chains for the methionine or selenomethionine rates of incorporation. Ion-exchange peptide analysis from a tryptic hydrolysate from this globin showed that selenomethionine and methionine were probably incorporated into the same peptides. However the peptides containing selenomethionine were somewhat displaced from the peptides containing methionine. When methyl labeled methionine and selenium labeled selenomethionine were incorporated into the globin, both their activities could be reduced 56-76% with dialysis against sodium sulfite. It was concluded that this sulfitolysis procedure was removing activity from the

methionine and selenomethionine residues in the globin.

There was no difference in selenomethionine or methionine aminoacylation of tRNA using materials from normal or selenium adapted Escherichia coli B. Both methionine and selenomethionine were rapidly bound in this system. Assay by plating on membrane filters demonstrated a higher binding of selenomethionine than methionine. However, assays by reverse phase chromatography showed that methionine and selenomethionine were bound in equal amounts and to the same two tRNA species. The two methionines were competitive inhibitors of each other for the aminoacylation of tRNA.

Aminoacyl synthetase and tRNA were then prepared free of ribonuclease. Both selenomethionine and methionine had about the same optimum aminoacylation conditions: enzyme and tRNA concentration, ATP/Mg ratios, and pH range. Both aminoacylations were completely dependent upon added ATP. Sodium ion inhibited both aminoacylations, but could be overcome by potassium ion. Selenomethionine was aminoacylated faster and in preference to methionine, but showed no saturation of tRNA when selenomethionine concentration was increased. Aminoacylated selenomethionine- and methionine-tRNA could be deaminoacylated at pH 8.8, and then reaminoacylated to the same degree. Reverse phase column chromatography indicated that from this ribonuclease free material, there was only one methionine accepting tRNA species, which was identical in position and stoichiometry to the selenomethionine accepting tRNA species.

Selenomethionine and Methionine in *In Vitro* Rabbit Reticulocyte Hemoglobin Synthesis and in *Escherichia coli* tRNA Aminoacylation

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This thesis is dedicated to the author's wife, Betty, and to his mother, Martha, for their love and understanding.

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INTRODUCTION

Much of the biochemistry of selenium has resulted from the report of its apparent essentiality in animal nutrition. Schwarz and Foltz in 1957 first reported that selenium would protect torula yeast-fed rats against death from liver necrosis (93). Later selenium was shown to give protective effects to a wide range of animals under both field and laboratory conditions (89). In these early nutritional experiments vitamin E and selenium were shown to have a synergistic relationship, whereas sulfur and selenium showed an antagonism. Nutritionally, the most effective form of selenium for the rat has been factor 3, which has been postulated to be an aliphatic selenic or selenoic acid, which has not been related to protein synthesis (93, 94).

CHEMISTRY OF SELENIUM

Chemical and physical properties of sulfur and selenium are remarkably similar due to their vertical family relationship. Biochemical similarities have, therefore, also been expected. An understanding of the similarities and disparities between sulfur and selenium has been vital to understanding their metabolism in the biological system. Table 1 lists some important properties of these two elements.

Table 1. Physical and chemical properties of sulfur and selenium.

properties	sulfur S	selenium Se
atomic weight	32.064	78.98
covalent radius, A^o	1.02	1.16
atomic radius, A^o	1.27	1.40
ionic radius, A^o (valence)(89)	1.84 (-2)	1.98 (-2)
bond angle, H-X-H, calculated	92.1^o	91.5^o
bond angle, H-X-H, observed	92.2^o	91.5^o
bond distance, H-X, A^o (78)	1.34	1.47
electronegativity	2.60	2.55
electron structure	(Ne) $3s^2p^4$	(Ar) $3d^{10}4s^24p^4$
oxidation potential, $H_2XO_3 \rightarrow XO_4^{--}$	-0.17 volts	-1.15 volts
oxidation states(89)	6,3,4,-2	6,4,-2

The physical and chemical analogies between sulfur and selenium have led investigators to postulate similar biochemical roles for these two elements, eventhough the optimum concentration of selenium in the animal diet is about 0.1 ppm while the level of sulfur is about 1000 ppm. The need for selenium in microbial systems has not been generally demonstrated. Conversely the fact that selenium at only slightly higher concentrations (5 ppm) is toxic to many forms of life has also stimulated research.

The reaction of selenium with sulfhydryl groups was documented early by Painter (76). He demonstrated the reaction of selenite with sulfhydryl compounds cysteine and reduced glutathione:

$$4\ RSH + H_2SeO_3 \longrightarrow RSSR + RSSeSR + 3\ H_2O$$
$$\downarrow$$
$$RSSR + Se^o$$

The selenite readily oxidized the sulfhydryl compounds to disulfide

and selenium dicysteine. The organic selenium compound decomposed more rapidly in basic than acidic solution. Ganther has expanded this work and has shown that 2-mercaptoethanol and coenzyme A react similarily to form moderately stable derivatives, which can be separated on thin layer chromatograms from the disulfides. The reaction was fastest at low pH's and no decomposition to red elemental selenium occurred unless the RSH/selenite ratio was greater than five. The compound also decomposed in 15 minutes at 70^{o} (28). Stekol demonstrated that the overall yield for this type of reaction was high, 80-85% (106).

Tsen and Tappel have manometrically demonstrated the molecular oxygen oxidation of cysteine, reduced glutathione (GSH), dihydrolipoic acid and reduced coenzyme A catalyzed by Fe^{+++}, Cu^{++}, Co^{++}, Mn^{++} or SeO_3^{--} (113). Selenite was the best overall catalyst, and remarkably so for GSH. Based on the isolated intermediate, selenium diglutathione, they proposed the mechanism:

$$4\ GSH + SeO_3^{--} \longrightarrow GSSG + GSSeSG + 2\ OH^- + H_2O$$

$$2\ OH^- + GSSeSG + O_2 \longrightarrow GSSG + SeO_3^{--} + H_2O.$$

Another chemical phenomenon of biological interest has been the sulfhydryl-disulfide exchange reaction:

$$R^1SSR^2 + R^3SH \rightleftharpoons R^1SSR^3 + R^2SH.$$

A series of these reactions has been studied by Dickson and Tappel using selenocystine, selenocysteine and GSH (19). Generally when selenocystine and cysteine or GSH exist in the same solution, the selenium species remains in the oxidized form. Results at neutral pH indicated that cysteine or GSH can completely reduce selenocystine only if in an excess of one thousand fold. Therefore in biochemical

systems where GSH is normally 10^{-3}M and selenium is 10^{-6}M, any selenocystine present will be in the reduced or selenol state.

Papain, a sulfhydryl type of enzyme, was activated by selenomethionine or selenocystine in the presence of cysteine and molecular oxygen. In the absence of selenocystine a high proportion of active papain was irreversibly oxidized. Selenocystine increased the rate and extent of loss of ribonuclease activity on treatment with cystine since ribonuclease is a disulfide type of enzyme. Dickson and Tappel postulated that selenomethionine and selenocystine were binding to the sulfhydryl groups of active papain to form substrate displaceable complexes which protect the enzyme from oxidative inactivation. The disulfide bonds of ribonuclease must have been reduced conversely (18).

It has been shown that selenols readily reduce disulfides:

$$RSeH + RSSR \rightleftharpoons RSeSR + RSH.$$

Gunther (32) also demonstrated that GSH was not a good reducing agent for the diselenide or selenide-sulfide bond, but that only a slight excess of dithiothreitol (oxidation potential, -SH = -0.322 volts) would instantaneously reduce the diselenide bond at neutral pH. Gunther reduced aliphatic and aromatic diselenides with hypophosphorous acid, whereas the corresponding disulfides were not reduced under these conditions unless catalyzed with a small amount of the diselenide (31).

The disulfide-sulfhydryl exchange reaction takes place as a function of the nucleophilic attack of the sulfhydryl or selenol group of the disulfide bond. A measure of this nucleophilic ability is the character of the -SH or -SeH groups. Generally RSeH is a much stronger acid than RSH. Huber and Criddle have compared pH and spectrophoto-

metric titration curves of cysteine and selenocysteine, Table 2 (37).

Table 2. Selenocysteine and cysteine pK values (37).

compound	group			
	-COOH	-SeH	-SH	$-\overset{+}{N}H_3$
selenocysteine	2.01	5.24	--	9.96
cysteine	2.30	--	(-- 8-10 --)	

Nygard has reported a pK value of 5.70 for the selenol group in selenocysteine (74). Beyond showing that the -SeH group is more acidic than the sulfhydryl group and that the selenol group is ionized at neutral pH's, this work indicates that the ionic configuration of the other functional groups in selenocysteine may be different from cysteine at physiological pH's. Huber and Criddle also reported that selenocysteine is more than twice as reactive as cysteine with iodoacetate even at pH values below the selenol pK values. Selenocysteine has an apparent half wave potential of -0.212 volts compared to +0.021 volts for cysteine (37). This confirmed the work by previous investigators in that selenocysteine is more readily oxidized than cysteine.

Ionized selenol groups have a high absorption in the ultraviolet region of the spectra with the main absorption peak at 248 mu which facilitate their study, especially since RSH groups do not absorb strongly in this region (19, 32, 37).

Dielectric constants for alkyl and aromatic sulfides, selenides, disulfides, diselenides are remarkably similar, although the sulfur compounds usually are a little higher (55). Krakov *et al.* studied the $-CH_2-S-S-CH_2-$, $-CH_2-Se-Se-CH_2-$ and $-CH_2-Se-S-CH_2-$ bonds and found no

significant polarization differences (47, 48). Mono- or diseleno-oxytocin could be separated from its sulfur analog by counter current distribution (126). Differences in hydrogen bonding or dipole moments were too insignificant to account for separation or biological activity differences. There were no polarization differences of the disulfide bonds when selenium replaced one or both of the sulfur atoms. Dielectric constants are related to charge separations and their vector angles. If these constants are not dissimilar then one would not expect differences in hydrophobic or hydrophilic bonding.

Theodoropoulos, Schwartz and Walter reported that tosylated serine residues in peptides or proteins can be reacted with sodium benzyl selenolate to convert the residues into selenocysteines (111). Chiu et al. (14) reported that the isotopic replacement of Se for S could serve as a heavy atom marker in x-ray crystallographic analysis. Chiu used the selenium marker in oxytocin, a small hormonal peptide with one disulfide bridge, and compared it to the normal peptide. Comparison of the diffraction patterns showed such similarity that they concluded that the overall conformation of both peptide molecules were identical. Cystine residues in protein lend a large amount of rigidity to the molecule not only through the disulfide bridge, but also in its hydrogen bonding ability which leads to a large specific rotation (114).

Separation of selenoamino acids has been demonstrated by McConnell and Wabnitz (63), Walter et al. (120) and others. Using the usual ion exchange columns and buffers of amino acid analyzers, selenoamino acids are displaced to a larger elution volume than their sulfur analogs. Walter found that selenocystine gave nonhomogeneous profiles when

chromatographed alone. He found that 2-mercaptoethanol, an impurity in thiodiglycol which is normally used in amino acid analyzer buffers, was undergoing diselenide-sulfhydryl exchange with selenocystine to give the extraneous peaks (120). Usual paper chromatography has not been able to differentiate R_f values of these amino acid analogs (83, 84).

Quantitative results for yields in acid hydrolysis of selenocystine and cystine were compared by Huber and Criddle (38). After 6 hours of standard acid hydrolysis, 95% of selenocystine and 5% of cystine were destroyed. Pyruvate and ammonia represented a third of the decomposition products with the other products not characterized. They concluded that reports of selenocystine in acid hydrolysates must therefore be carefully considered. Walter (120) in agreement, found that selenocystine was completely destroyed under acid hydrolysis in 22 hours when hydrolyzed alone, but that when selenooxytocin was hydrolyzed only 60% of the selenocystine was destroyed.

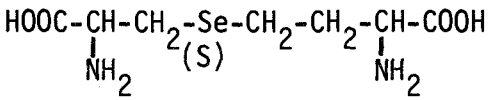
BIOCHEMISTRY OF SELENIUM

This section will discuss the metabolism of selenium compounds related to proteins as compared to similar sulfur compounds. Many possibilities exist in such a comparison. Selenium compounds may be metabolized in the same manner as their sulfur analogs using the same pathways and enzymes to yield isologous sulfur and selenium compounds which are biologically similar. Any variation in pathways, enzymes, biological identity of products, or kinetics will lead to a disparity of these compounds in the biological system. Many problems face the investigator working with selenium since the normal S/Se ratio approaches 800. Most selenium compounds are toxic and systems usually cannot yield direct selenium-compound identification. Due to their similarities, selenium and sulfur compounds are difficult to resolve. Lability of both selenium and sulfur compounds is a problem. Perhaps the largest problem has been that selenium may not always be incorporated into protein in the same manner as sulfur.

Selenium Compounds in Plant Extracts

Certain plants grown in regions of large amounts of selenium in the soil accumulate up to 5,000 ppm selenium (80, p. 61-140). This genus, Astragalus, has species in which growth is either inhibited or stimulated by the presence of selenium. Homogenates from Astragalus grown on high selenium soil are not biologically active, probably due to selenium inhibition of their enzymes.

There must be a division between plant extracted selenium compounds and those obtained from acid hydrolysis of plants due to the lability of selenium compounds and the possible production of selenium artifacts. Historically Horm in 1941 isolated a crystalline amino acid complex containing S and Se which was extracted from Astragalus pectinatus. He tentatively identified it as a mixture of selenocystathionine and cystathionine:



(36).

Trelease, Disomma and Jacob have isolated S-methyl cysteine and Se-methylselenocysteine from an extract of Astragalus bisulcatus (112). Shrift and Virupsksha also found this compound in several other selenium accumulators using TCA soluble extracts from excised leaves that were supplied with radioactive selenate, selenite, and sulfate. All identification was by paper chromatography or electrophoresis. R_f's for selenium and sulfur isologs were found to be the same. Selenate was less efficiently used than selenite. The major selenium compound was Se-methylselenocysteine, followed by selenocystathionine (104, 118) and trace amounts of Se-methylselenomethionine and a selenium containing peptide, but no selenoglutathionine. Plants supplied with radio sulfate made equal amounts of S-methylcysteine and glutathionine (104).

Jenkins and Hidiroglou extracted Brome grass supplied with radio selenite with an 80% ethanolic solution. They found the radioactivity in selenocystine (46%), selenite (12%), selenomethionine (13%), an unknown (28%) and a trace amount of Se-methylselenocysteine. An

ethanolic solution of barium chloride selectively precipitated the selenite and selenate from the selenoamino acids. The selenite and selenate barium salts could then be separately dissolved (43).

Selenium Compounds from Plant Hydrolysates

Using paper chromatography Peterson and Butler found 21 radioactive spots in shoots and roots of five plants supplied with radio selenite. They found elemental selenium (soluble in bromine water), selenocystine, selenoglutathione, Se-methylselenomethionine, selenocysteic acid, selenocysteine, selenic acid, selenite, selenomethionine selenoxide, selenomethionine and other unknown spots or streaks. They found no known amino acids in Neptunia or wheat where 90% of the radioactivity remained as selenite. Red clover, white clover and rye grass showed the selenoamino acids with highest activity in the roots. Recoveries of ^{75}Se in acid hydrolysates (6 N HCL, 110^{o}) were unsatisfactory and decreased with time. However 75% of the radioactivity could be extracted from the roots with a chymotrypsin and trypsin treatment (84). Jenkins and Hidiroglou also reported that $^{75}SeO_3^{--}$ treated pasture grass could be digested with trypsin and pepsin to release 60% of the radioactivity as selenocystine, 29% as selenomethionine and 5% as elemental selenium; but no selenite was found (42).

A great disparity exists between plant species in the metabolism of selenium. One may only speculate on some of the selenium metabolites. They may have a function in amino acid metabolism, for example cystathionine is an intermediate in methionine biosynthesis,or they may represent a method for the detoxification of selenium (103).

Sulfur and Selenium Antagonism in Microbial Growth

Due to the chemical similarities of sulfur and selenium, work has been directed towards their competitive or antagonistic nature in microbial systems. Fels and Cheldelin demonstrated that growth inhibition of Saccharomyces cerevisiae was partially reversed by the addition of methionine (26) and sulfate (27). Cysteine and to a lesser extent glutathione reversed selenate toxicity in Escherichia coli, but methionine was ineffective.

It has been generally found that microorganisms cannot grow in media without sulfur irrespective of selenium. A technique sometimes used with selenium experiments has been to grow microorganisms in a low sulfur (sulfate) media with varying amounts of selenium compounds. After the sulfur depletion point, which is indicated by a decreased growth rate, selenium has been more rapidly absorbed by the cells (16, 100).

Shrift has reported a series of experiments with bacterial growth with selenium compounds. He found that the selenium concentration in Chlorella vulgaris remained constant through a 16 fold increase of selenate in the media (97). In these experiments sulfate improved growth and inhibited selenate from entering the cells. Next he showed that these cells incorporated selenomethionine to a level which depended only upon the selenomethionine/methionine ratio and that sulfate did not affect the selenomethionine incorporation (99).

Shrift et al. showed that C. vulgaris could be mass adapted to selenomethionine in the absence of sulfate. Half of the cells were enlarged into giant forms, but this condition was temporary. Cells

adapted to selenomethionine maintained the property of utilizing selenomethionine for at least 200 generations in the absence of selenomethionine. However when methionine was introduced in the absence of selenomethionine, the adapted cells lost their ability to grow normally with selenomethionine. Adapted cells did not incorporate radioactive methionine, but did incorporate radio sulfate. Shrift postulated that selenomethionine did not function in the proteins concerned with cell division, but that selenomethionine could substitute for proteins concerned with protein synthesis and growth. He noted that adaptation towards or against selenomethionine involved a transformation of all cells rather than a selection of mutants. He did not find this type of adaptation in E. coli or Torulopsis utilis. Although no selenium analysis or radioactive selenomethionine were used, Shrift concluded that the cell permease system in adapted cells was adapted to exclude both methionine and selenomethionine. Usually, but not in this case, repression is reversible after removal of metabolite (seleomethionine), but that cells in these experiments could only be derepressed if sulfate was omitted for a period of time (97, 102).

Escherichia coli showed a characteristic lag phase before exponential growth when grown on a high selenate media (100). At a constant sulfate concentration of 0.2 mM the growth of E. coli was inversely proportional to the concentration of selenate (20-80 uM). Above 160 uM there was a lag phase of 24-48 hours and no growth if the selenate concentration was greater than 800 uM. Adapted cells grown for 12 days without selenate remained adapted with no lag phase when reintroduced to selenate (100). Huber and Criddle found that growth with a selenium-

tolerant substrain K-12 of E. coli in 10 mM selenate was linear instead of exponential and that cell growth efficiency (g cells/g glucose utilized) was less. They concluded that cells could only utilize selenium if some sulfur was also present (38).

Perhaps the classic work in this field was done by Cowie and Cohen when they demonstrated that selenomethionine could completely replace methionine in the growth of a methionine requiring mutant of E. coli. Sulfate was supplied but was not incorporated into methionine. The growth using selenomethionine was exponential and only slightly slower than the methionine control (15). Mautner and Gunther showed that selenopantethine could replace the sulfur analog in Lactobacillus helveticus which requires preformed pantethine. Here again the organism had only a slight preference for the sulfur over the selenium compound (54).

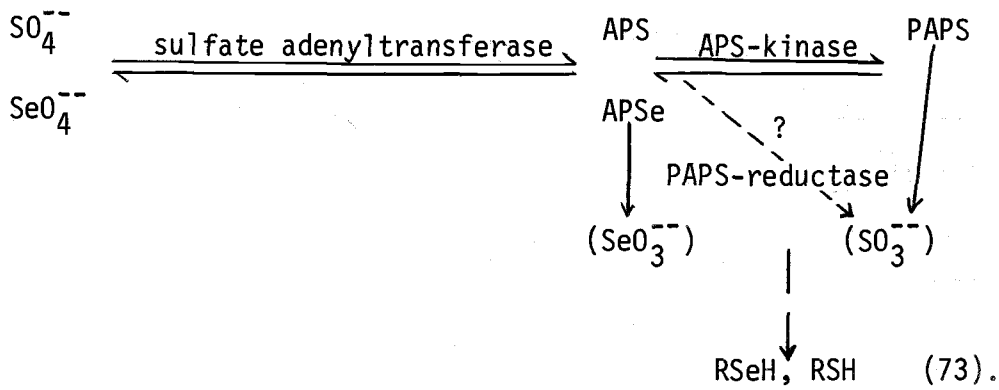
Only one reference suggests an essentiality for selenium in microorganisms (46). Russian workers isolated microbes from seleniferous soils and compared their growth to normal soil bacteria. Optimum growth for the seleniferous bacteria included 0.01-0.1 ppm selenium whereas normal soil bacteria needed less than 0.005 ppm selenium in their media. The high selenium microbes could resist a selenium concentration 50 fold over the normal microbes, and they could reduce soluble selenium compounds to elemental and volatile products.

Microbial Selenoamino Acids and Proteins

Reduction of sulfate is the important mechanism for making sulfur amino acids. To man this is important because he cannot utilize sulfate

so he must depend upon dietary methionine. Microbial reduction of selenium salts to colloidal red elemental selenium was one of the first observations made by investigators of selenium metabolism (51). Falcone and Nickerson demonstrated that cell free preparations of Candida albicans needed glucose-6-phosphate, NADP, GSSG, menadione, and an 18,000 x g supernatant to reduce selenite to elemental selenium (25, 70). Ahluwalia demonstrated that selenite reducing activity in E. coli was not induced, but was present all the time (1).

Wilson and Bandurski found evidence for the occurrence of "active selenate," adenosine 5'-phosphoselenate, APSe. This compound was produced less rapidly than APS and it decomposed into AMP and selenate. No PAPSe, 3'-phosphoadenosine 5'-phosphoselenate, was found (125). Nissen and Benson proposed the following mechanism for reduction of selenate and sulfate:



(73).

The final step has been suggested to be a reversal of the cysteine desulfhydrase reaction combining ammonia, pyruvate and H_2S or H_2Se (124).

Classical methods of amino acid chemistry using electrophoresis, paper chromatography and acid hydrolysis have failed to properly resolve selenoamino acids qualitatively as well as quantitatively.

Tuve and Williams identified selenomethionine in E. coli grown on radio selenite in sulfur deficient media. In hydrolysis of the protein with pepsin, pancreatin and erepsin followed by paper chromatographic fingerprinting, the radioactivity was shown to be associated with synthetic selenomethionine. They also demonstrated that sulfate, chloramphenicol and 2,4-dinitrophenol inhibited selenite uptake. The selenite uptake was not due to adaptation, adsorption of selenite or deposition of elemental selenium. They also demonstrated the biosynthesis of selenomethionine, but not selenocystine. They postulated that selenocystine was produced since cysteine is the precursor of methionine, but that selenocystine was not found because of the lability of diselenides. Although no selenocystine was detected with enzyme hydrolysates, acid hydrolysis followed by paper chromatography produced a spot corresponding to selenocystine. This spot did not co-chromatograph with synthetic selenocystine, nor did its H_2O_2 oxidation products. This compound was partially decomposed to selenite upon oxidation with dilute peroxide as was authentic selenocystine (116, 117).

Huber et al. showed that their selenium tolerant strain of E. coli K-12 could replace only 30-40% of the sulfur with selenium. They could detect only selenomethionine (39). Blau demonstrated the biosynthesis of labeled selenocystine and selenomethionine. His technique was to use yeast in low sulfur media to get 50-75% incorporation of radio selenite, then to add 100 mg of methionine and cystine before acid hydrolysis for 5 hours. A Dowex-50-X8 column yielded 20-40% of the original radioactivity as selenomethionine and selenocystine. The ratios of selenomethionine to selenocystine varied from 1:3 to 3:1 (8).

In contrast Tuve and Schwarz reported that in similar experiments with Baker's yeast only 7% of the radioactivity was TCA soluble. With either acid or enzyme hydrolysis followed by two dimensional paper chromatography, they identified only selenium tetracysteine (probably selenium dicysteine, 28). They found no selenium analogs of methionine, cystine, glutathione or selenium tetraglutathione, although they had a large unidentified spot (115).

Falcone and Giambanco succeeded in preparing a cell-free extract of Candida albicans (a selenium resistant strain) which was capable of reducing radio selenite into proteins and elemental selenium. By paper chromatography they identified radioactivity as being in selenite, selenocystine and selenomethionine (24). Weiss et al. compared selenite metabolism in E. coli, Proteus vulgaris and Salmonella thompson. Apparently the E. coli were more susceptible to selenium toxicity because they incorporated it twice as fast as the others in early stages of growth. E. coli was completely inhibited with 77 mM selenite whereas it took up to 500 mM to completely inhibit the other strains. Radioautographs showed selenocystine in all three, but selenomethionine only in E. coli, along with many other spots (121).

Rumen microorganisms, a less well defined system, have been important in selenium research due to their symbiotic relationship with sheep and other ruminants which show a selenium requirement. Inorganic selenium may be metabolized to unusable elemental selenium, or to selenoamino acids or other active forms of selenium. Paulson et al. compared the metabolism of radioactive selenite, selenate, selenomethionine and sulfate. They found only 9% of the sulfur, but 90% of the

selenium to be TCA insoluble in three hours. Excess sulfate inhibited radiosulfur incorporation into cystine and methionine, but had no effect on inorganic selenium-75 incorporation as determined by pronase hydrolysis followed by amino acid analysis. Methionine strongly inhibited selenomethionine incorporation. The selenomethionine remained unaltered upon analysis. Dialysis of labeled protein against GSH released 37% of the selenomethionine, 56% of the inorganic selenium and 25% of the sulfate activities. Radioactive selenite or selenate incorporated less than 0.1% of the activity by analysis on paper chromatograms or by analysis on the amino acid analyzer. N-methylmaleimide did not prevent selenite binding in these microbes probably because the reagent did not penetrate the cells (80). Another comparative study using paper chromatography, radio selenite and radio sulfite showed activity in methionine, selenomethionine selenoxide and a trace in selenocystine, whereas the sulfur went equally into cystine and methionine (34).

Microheterogeneity of Microbial Proteins

The microheterogenity of proteins, or the analog replacement of one amino acid for another, is an important concept in biology. Do these altered proteins have any specialized functions, are they active or inactive, are they synthesized, and what are their concentrations?

p-Fluorophenylalanine can replace 47% of the tyrosine and 23% of the phenylalanine residues in wild *E. coli* (15). A phenylalanine requiring mutant of *E. coli* can use this analog. In a methionine requiring mutant of *E. coli*, selenomethionine gave exponential growth but p-fluorophenylalanine, beta-2-thienylalanine, and norleucine were

incorporated into protein but gave only linear growth and cessation of synthesis of one or more essential enzymes. Biosynthesis of β-galactosidase and growth rates with 6-methyl-tryptophan and p-chloro-phenylalanine in the media were not affected. These last two analogs were found not to be incorporated into proteins based on hydrolysis and two dimensional paper chromatography (68). Gross and Tarver showed incorporation of ethionine into labeled peptides and that hydrolysis yielded labeled ethionine, aspartate and glutamate (30). Cowie et al. found that labeled methionine and norleucine were incorporated into the same broad spectrum of E. coli proteins as seen by an ion exchange column. Formation of incomplete or uncoupled protein synthesis would have drastically altered this elution profile. Norleucine at 0.02 M decreased the incorporation of 10^{-4} M sulfur labeled methionine by 35%. Similarily a 50 fold excess of p-fluorophenylalanine decreased incorporation of phenylalanine by 50% (16).

In Cowie and Cohen's experiments growth was reduced if the selenomethionine/methionine ratio reached 10. However they could also reduce radioactive methionine incorporation with selenomethionine. Increasing the selenomethionine/methionine ratio resulted in decreased activity of β-galactosidase in a crude homogenate assay. Growth on selenomethionine led to a 35% decrease of control activity. They postulated that this could be due to synthesis of an enzyme with modified affinity for the substrate, reduction of total enzyme synthesized, or synthesis of an enzyme of a lower specific molecular activity. This mutant did incorporate sulfate into cystine, but not methionine. Sulfate and selenite reduced the incorporation of each other, and upon hydrolysis

and paper chromatography there was selenium radioactivity in the area of cystine (15).

A further refinement of this work by Huber and Criddle used galactosidase purified from E. coli grown on selenate or sulfate. During purification the specific activity was always lower for the selenate grown enzyme, which meant that about twenty times less enzyme was produced. However after the last purification the specific activities were the same. By analysis both enzymes had the same number of cystine residues, but in the selenate enzyme 80 of the 150 methionine residues were replaced. There was no selenium in the sulfate protein. The two enzymes had nearly identical K_m, V_{max} and pH optimas. The selenium enzyme was less stable to urea and temperature denaturation. It also had a better renaturing capability than the sulfur enzyme (38). One possibility that the authors failed to note was that the selenocyst(e)ine residues could have possibly been present, but leading to an inactive enzyme which was later separated from the active enzyme. This possibility arises because no analysis for selenocystine was performed on the crude enzyme preparation.

Scala et al. concluded that selenite inhibited cell growth if the microbes had to induce an enzyme first (β-galactosidase)(90). Ahluwalia and Williams concluded that E. coli grown on increasing concentrations of selenite gave a lower specific activity of alkaline phosphatase and that selenium incorporation was only 8-10% of sulfur (2).

Very little has been done on the actual mode of the incorporation of selenoamino acids into protein. Generally the method used has been to assay for TCA insoluble activity, presumably protein bound. Nisman

and Hirsch have shown that the soluble fraction of E. coli which contains aminoacyl synthetase incorporated ^{32}P from pyrophosphate into ATP when selenomethionine was added to the media. Methionine was curiously one of the best substrates for this reaction; the preferences are as follows: try> met> val>>others. The synthetase activity in mu moles ^{32}P incorporated into ATP/15 minutes-mg protein, was L-methionine, 1155; D-methionine, 0; DL-selenomethionine, 1211; DL-ethionine, 384; and DL-norleucine, 221 (72). The primary step in protein synthesis has been postulated to be catalyzed by a single enzyme (124, p. 599):

amino acid + ATP + enzyme $\rightleftharpoons$ aminoacyl-AMP-enzyme + PPi

sRNA + aminoacyl-AMP-enzyme $\rightleftharpoons$ aminoacyl-sRNA + AMP + enzyme.

Evidence has been presented that each amino acid has its own set of aminoacyl synthetases and transfer or soluble RNA's; some amino acids have more than one enzyme or tRNA species (120, 129).

Inorganic Selenium Incorporation into Higher Animal Proteins

Whereas most higher animals except ruminants cannot utilize inorganic sulfate for the biosynthesis of sulfur amino acids, they must rely on intake of these amino acids from plants or bacteria. The reduction of sulfate has not been generally known in animals, although it has been reported for selenium compounds. In vivo and in vitro studies with mammalian tissues have demonstrated the reduction of selenite or selenate to elemental selenium (88). Reduction and methylation of selenite to dimethylselenide has been shown to be a method of selenium detoxification in animals (89, p. 175). S-adenosylmethionine has been shown to be a methyl contributor to the formation of dimethylselenide.

Dimethylselenide was formed from selenite, but not from selenomethionine or selenocystine (108).

It has long been known that inorganic selenium was incorporated into animal proteins, but the mode of incorporation has been the center of controversy. Radioactive selenite rapidly associates with serum proteins of the dog and the rat. Albumin is the initial acceptor (64) but alpha-2- and beta-1-globulins have the highest activity after 24 hours (40, 61). Dialysis of the serum against water showed less incorporation of radioactivity than TCA precipitation.

Lipoproteins, the main carriers of vitamin E, were shown to bind selenium from selenite (59), but was later questioned by Roffler et al. who showed that the selenium-75 activity in lipoproteins was due to contamination by serum proteins (87). Jenkins working with chicken serum labeled with radio selenite showed that dialysis against 2-mercaptoethanol, cysteine, GSH, dithiothreitol, sulfite or dilute base in the presence of 8 M urea released 75-90% of the activity. The forms of the released selenium was selenite and elemental selenium. No radioactive spots for selenocystine or selenomethionine or other ninhydrin positive spots were noted (41).

Selenite uptake by red blood cells has been shown to be inversely proportional to the dietary levels of selenium and therefore has been useful in diagnosing selenium status in sheep (52, 128). This uptake was increased under oxygen, iodoacetate, and arsenate, whereas the radioactivity may be displaced or inhibited by cyanide (127). McConnell and Cooper found that selenium was present in both globin and hemin fractions (58). Whereas Siew et al. postulated that red blood cells

consistent with selenite, but not selenate, binding with diaminobenzi-

converted selenite into selenomethionine and selenocystine which entered conventional protein synthesis (105). Cavalieri et al. compared leucine and selenite incorporation into human leucocytes and found that puromycin inhibited leucine, but not selenite incorporation. Iodoacetamide, cyanide and p-chloromercuricbenzoate inhibited $SeO_3^=$ uptake. A time course showed immediate selenite binding followed by a more gradual uptake (13).

Cummins and Martin postulated that selenocystine and selenomethionine are not synthesized in mammals. A rabbit was fed radio selenite for 35 days. Liver, dialyzed at pH 10, lost 90% of its activity and negligible radioactivity was detected under any of the amino acids after hydrolysis with pronase. They showed that ^{75}Se activity could be found in the regions of selenite, cystathione, cystine and homocystine on the amino acid analyzer if selenite was incubated with methionine, cystathione, cysteine, cystine, homocysteine, GSH and GSSG. In vivo and in vitro labeled urine had about eight radioactive peaks, two of which were in the regions of selenocystine and selenomethionine. When this urine was treated with cold selenite and adjusted to a high pH, the radioactivity was released as selenite (17). Liver homogenates bound 75% of added selenite in 30-60 seconds. Mixing selenite with methionine, cysteine, GSH, homocysteine, cysteic acid or methionine sulfoxide and immediately spotting on a paper chromatogram resulted in no radioactivity associated with the amino acids. However experiments with cystine, GSSG and homocystine resulted in ^{75}Se association with these amino acids with no free selenite occurring. These results were consistent with selenite, but not selenate, binding with diaminobenzi-

dine, another diamino compound which has been used in selenium (selenite) analysis (95).

Paulson et al. found no effect of sulfate on the distribution of ^{75}Se from selenate after injection into the rumen which suggested no antagonism between these two elements. Most of the radioactivity (70%) was eliminated in the feces presumably as elemental selenium (79). The ability of rumen bacteria to reduce selenium to the elemental state may be the reason that sheep have been very susceptible to selenium deficiency disease (white muscle disease, so called because of a calcification of skeletal and cardiac muscles). A comparison of selenite and selenomethionine in selenium deficient lambs revealed no drastic differences in the general ^{75}Se distribution in the feces or urine. After six days there was only slightly more radioactivity in the blood from selenomethionine, and after 13 days, significantly more activity in the tissues from selenomethionine. Vitamin E had no effect on these selenium distributions (22).

Selenoamino Acid Incorporation into Higher Animal Proteins

The attempt to use ^{35}S labeled cystine as a tool in the study of protein synthesis has resulted in many of the same type of artifacts found when selenium has been similarily used. Melchior and Tarver working with rat liver homogenates and slices could remove two-thirds of labeled cystine with excess thioglycolic acid. Boiled homogenates showed a lesser but still large incorporation, most of which could be removed by reduction. Upon analysis they found 10% of the label as sulfate, 26% was not reducible and 44% was reducible (65). Labeled

methionine was converted to cystine, 5-20%, and 1-9% was converted to sulfate. In protein they found equal amounts of label as methionine and cystine. Added cysteine increased, whereas homocysteine decreased methionine incorporation in liver slices (66).

Early work with unlabeled selenocystine showed that the compound did not parallel cystine in liver proteins (123). Due to the lability and difficulty of working with selenocystine, most of the work in this field has been done with selenomethionine. Awwad et al. showed that some of the selenomethionine was reducible upon sulfitolysis with $NaHSO_3$ and that the amount reducible decreased with time. No binding of selenomethionine to plasma proteins was observed in vitro. Because some binding was reducible they concluded that some selenomethionine must have been converted to selenocystine (selenohomocysteine would probably have given the same results). They found that the uptake of selenomethionine was faster than that of methionine. Free amino acids in the liver showed that the ^{75}Se label was equally distributed between selenomethionine and selenocystine (3, 4). In protein hydrolysates 80% of the radioactivity was found in methionine and 16% in cystine. The interconversion of selenomethionine to selenocysteine was faster than for methionine to cysteine.

Selenomethionine has been well studied in blood proteins. Sternberg and Imbach found most activity in the α- and γ-globulins. Rate of incorporation remained unchanged in rats with total hepatectomies and only a small amount would bind in vitro. On this basis they suggested transpeptidation (107). Awwad et al. demonstrated selenomethionine incorporation into human albumin, fibrinogen and hemoglobin after

stimulating protein synthesis with triiodothyronine (5). Red blood cell maximum incorporation of selenomethionine was in young cells as determined by density centrifugation and lifetime of the ^{75}Se-selenomethionine tagged cells (5, 82). In vitro experiments with plasma showed no protein bound selenium-75 after incubation for 24 hours with ^{75}Se-selenomethionine. In vivo experiments showed that radioactivity associated with TCA insoluble plasma proteins could be reduced by sulfitolysis, and that the amount reducible decreased with time: 20%, 10 minutes; 10% 4 hours; and none in 24 hours. They again suggested conversion of selenomethionine into selenocysteine. Halflife of selenomethionine labeled plasma proteins agreed well with values obtained with ^{35}S-methionine (109) and other methods (5).

Hansson showed the same degree of incorporation of selenomethionine and methionine into pancreatic juice proteins. Pretreatment with methionine reduced the selenomethionine incorporation. Chromatographic analyses showed that selenomethionine was incorporated into these proteins without prior change (33). Ochoa-Solano and Gitler also proposed that no metabolic changes occur for selenomethionine and methionine. Injection of the two methionines into the hen wing vein prior to ovulation resulted in their incorporation into egg white protein. After carboxymethylation with iodoacetate the proteins were hydrolyzed along with standards of carboxymethylated selenocysteine and cysteine. An amino acid analyzer showed ^{35}S in CM-cysteine and methionine peaks, but ^{75}Se was only under the selenomethionine peak. No loss of activity occurred when ovalbumin was carboxymethylated or when there was no protection. Oxidation with performic acid then acid hydrolysis resulted in 40%

loss of the ^{75}Se activity. The $^{35}S/^{75}Se$ ratio was 1.61 in ovalbumin (which was misleading since the specific activity of the selenomethionine was 4 times higher than the methionine). They concluded that the ratio in favor of the methionine over the selenomethionine was because selenomethionine was not converted into selenocystine or that selenocystine was not incorporated into proteins (75).

A comparison of selenite, selenomethionine and selenocystine incorporation into rat liver ribosomes revealed drastic differences. In zero time experiments there was instantaneous incorporation of the selenoamino acids, but not for the inorganic selenium (61). Previously it was reported that the microsomal fraction had the greatest ^{75}Se activity per unit of nitrogen of all cell fractions (62). Selenite binding to the ribosomes was increased by cell sap, ATP, phosphocreatine and phosphoenol pyruvate, but not by Mg^{++} or guanosine triphosphate. Selenite binding was inhibited by cyanide but not by 2,4 dinitrophenol or puromycin. Selenomethionine incorporation was increased only 50% by cell sap, wheras GTP, Mg^{++}, 2,4-dinitrophenol and puromycin were without effect. Cyanide did not inhibit selenomethionine binding as much as it inhibited selenite. Cyanide, puromycin and phosphocreatine had no effect and ATP only slightly affected the incorporation of selenocystine, but cell sap doubled its binding. The conclusions were that selenium was incorporated into the ribosomes in an exchange reaction or by nonspecific binding (61).

Perhaps the only pure selenoprotein (peptide) that has been shown to have biological activity has been synthetic selenooxytocin. This peptide hormone was shown to have 85-95% of the activity of oxytocin

in the milk ejection response. There was a biological nonequivalence of the two selenium atoms in the selenide-sulfide or diselenide bridge (119).

Selenium compounds in higher animals have activity in other enzyme systems not related to protein synthesis: transmethylation with selenomethionine (10, 67, 81), methionine adenosyl transferase with selenomethionine (77) and active transport with selenomethionine but not with selenite or selenocystine (56, 57).

Selenomethionine and Methionine in *In Vitro* Rabbit Reticulocyte Hemoglobin Synthesis and in *Escherichia coli* tRNA Aminoacylation

STATEMENT OF THE OBJECTIVES

The literature review has demonstrated that there is a need for the actual comparison of selenomethionine and methionine in protein synthesis, qualitatively and quantitatively. This comparison should be made using the latest techniques and methods for the avoidance of artifacts. The objectives of this study were: (1) to determine if selenomethionine and methionine are incorporated into hemoglobin of rabbit reticulocytes at the same rates and positions; and (2) to determine whether the tRNA species for the two methionines are the same, to compare their aminoacylation kinetics with a cell free system, and to investigate if selenium adaptation of *Escherichia coli* is manifested in the tRNA or aminoacyl synthetase.

SELENOMETHIONINE AND METHIONINE IN *IN VITRO* RABBIT RETICULOCYTE HEMOGLOBIN SYNTHESIS

EXPERIMENTAL

Production and Treatment of Reticulocytes

Reticulocytosis was produced in adult male New Zealand White rabbits by daily subcutaneous injection of one ml of neutralized 2.5% aqueous solution of phenylhydrazine hydrochloride each day for one week (9, 50). Blood samples taken from the ear vein on the seventh day and treated with brilliant cresyl blue stain, microscopically confirmed the preponderance of reticulocytes. To decrease mortality it was found necessary to omit the phenylhydrazine injection on the sixth day (85). The rabbit was anesthetized with ethyl ether on the eighth day and blood was taken via heart puncture with a heparinized 20 ml syringe fitted with an 18 gauge, 2.5 inch needle. Blood was washed and centrifuged at 2,000 x g three times with ten volumes of Ringer-phosphate bicarbonate solution (49). All centrifugations were at 4^{o} unless otherwise specified. The hematocrit varied from 10-36%.

Cells were diluted according to need (approximately 1:8) with isotonic solution. All amino acids except methionine were added from a frozen (-20^{o}) stock solution to the same molar ratio as found in the combined α and β chains of hemoglobin x 10^{-5}. Iron, 0.5 ug/ml incubate, as $FeSO_4 \cdot (NH_4)_2 \cdot SO_4 \cdot 6H_2O$ was also added from a frozen stock solution.

Glassware and solutions were sterilized by autoclaving. Incubations were performed at 37° in a shaking water bath in 25 ml flasks stoppered with cotton plugs. Radioactive amino acids were added last.

L-Selenomethionine-^{75}Se was received from The Radiochemical Centre in Amersham, England with an initial specific activity of 100 uCi/u mole. Another lot of this radioactive amino acid was obtained with an initial specific activity of 1000 uCi/u mole. Halflife of ^{75}Se of 121 days was used for decay corrections throughout the experiment (86). L-Methionine(methyl-^{14}C) had a specific activity of 56.8 uCi/u mole. All solutions were diluted with water and kept frozen in capped bottles to insure sterility and to prevent evaporation. The molar amounts of the two methionines were adjusted by adding cold carrier to the respective reaction flasks along with the radioactivity.

At the end of the incubation the cells were washed three times as previously mentioned. Where timing was critical, reaction vessels and contents or aliquots were quickly frozen in a dry ice, ethanol, water mixture at -25° without prior washing. Cells were then frozen in sealed test tubes.

Hemoglobin Purification and αβ Chain Separation

Frozen cells were thawed and hemolyzed with a minimum amount of water (1:1) and centrifuged at 12,000 x g. The hemolysate was placed on a Sephadex G-100, 1.4 x 50 cm column and eluted with 0.1 M, pH 7.0 phosphate buffer. Homogeneity for more than one hemoglobin species was checked on the first sample of an experiment according to Jones

and Schroeder on a 1 x 35 cm column of Bio-Rex 70 (equivalent to Amberlite IRC-50-XE-64 resin) 200-325 mesh obtained from Bio-Rad laboratories in Richmond, California. Developer number one was used, which was 0.025 M $NaH_2PO_4 \cdot H_2O$, 0.025 M $Na_2HPO_4 \cdot 7H_2O$, 0.01 M KCN, pH 7.22 at 25^o. The hemoglobin solution was dialyzed against 0.01 M $K_3Fe(CN)_6$ previous to column application which was performed in the cold room at 4^o. Hemoglobin color was read at 540 mu in a 1 cm cuvette using a Beckman Model DU spectrophotometer fitted with a Gilford Model 222 attachment. This optical density multiplied by 1.44 gave hemoglobin concentration in mg/ml (45).

Where further Bio-Rex 70 columns were found to be impractical, the globin was prepared from the hemoglobin after the Sephadex column. It was found necessary to deheme the hemoglobin slowly at -20^o with 10 volumes of 0.15 N HCl in acetone according to Rabinowitz and Fisher to prevent a deep yellow color in the final globin preparation (85). Precipitated globin was washed twice with -20^o acetone, centrifuged each time at the same temperature and finally dissolved in the starting buffer used to separate the α and β chains.

Separation of the α and β chains of rabbit hemoglobin required a different set of conditions from that reported in the literature (20,85). Optimum separation occurred with a 2.5 x 40 cm carboxymethylcellulose (CMC) column using a 500 ml total volume linear concentration gradient of 1X-10X. The 10X buffer was 2 M formic acid, 0.2 M pyridine adjusted to pH 2.00 from pH 2.40 with HCl; 1X buffer was diluted from the 10X buffer. Smaller columns of 1.5 x 15 cm had optimum resolution with a linear concentration gradient of 250 mls. The CMC was obtained from

Bio-Rad laboratories under the trade name of Cellex CM having a capacity of 0.66 milliequivalents/g. The CMC was washed with tenth normal HCl and NaOH before pouring the columns, then equilibrated at room temperature with 1X buffer. The large column was used continuously for a large number of separations. Between separations the columns were washed with 250 mls of 10X buffer, then enough 1X buffer to bring the eluant to 1X concentration as checked by titration with 0.01 N NaOH and phenolphthalein indicator. This titration was also used to check the gradient throughout a run. Protein profiles of the column were determined according to Lowry (53): 0.2 ml sample, 0.8 ml water, 5.0 ml alkaline copper reagent (0.5% $CuSO_4$ in 1% sodium potassium tartrate), after 10 minutes 0.5 mls of phenol reagent (diluted 1:1 with water; Van Waters & Rogers, Inc., Portland, Oregon). The resulting blue color was then read at 660 mu with a Coleman Universal spectrophotometer in 1.7 cm cuvettes after 30 minutes. Only 0.2 mls of the pyridine buffer eluant could be used since more would cause precipitation of the phenol reagent. The sensitivity of the method was better than protein assay at 280 mu because the 280 mu baseline increases with the pyridine gradient.

For samples not to be enzymatically digested, α and β samples were pooled according to their protein profiles and precipitated by adding 50% trichloroacetic acid, TCA, to make a final concentration of 7% TCA. The precipitate was collected by centrifugation at 8,000 x g and washed with 7% TCA, collected, dissolved in water with the addition of a small amount of dilute 0.1 N NaOH and frozen until the radioactivity was counted. Samples to be enzymatically digested were

freeze-dried, dissolved in water, dialyzed against water, then frozen. TCA precipitation, unlike freeze-drying, resulted in most of the α and β chains being insoluble in water unless the pH was raised to 10 or lowered to 3.

Radioactivity Determinations

Selenium-75 samples of 2.0 mls were counted with a 49% efficiency in a Packard Autogamma solid scintillation counter, Model 500 D, and corrected for decay. Carbon-14 samples were counted in a Packard Tri-carb liquid scintillation counter with an unquenched efficiency of 62%. Liquid scintillation fluid was two parts toluene to one part methyl-cellosolve with 5.5 g PPO/liter. This solution held up to 0.2 mls of aqueous solution/10 mls. Another scintillation fluid was used for counting up to 1 ml of aqueous sample per 15 mls of fluor solution: 28% Triton X-100, 72% toluene, 5 g Omnifluor/liter (6). Efficiency and quenching was determined by spiking 20 ul of ^{14}C-toluene containing 9680 dpm into each sample scintillation vial after initial counting. Selenium-75 crossover (19.7% efficiency) in the liquid scintillation counting was subtraced before the carbon-14 activity was calculated when dual labeled experiments were performed. Samples were counted twice for 10 minutes. Activity was generally above 5,000 cpm for samples, and of course less for fractions from columns.

To determine the selenomethionine-^{75}Se and methionine-^{14}C distribution in tryptic peptides, the carbon-14 and selenium-75 samples were combined before $\alpha\beta$ separations.

Hemoglobin Hydrolysis with Trypsin

Water dialyzed samples of globin were adjusted to pH 9.5 using 0.1 N NaOH. Dense precipitation occurred and persisted if the pH was below 9.5. A 1% water solution of trypsin (Worthington Biochemical Corporation, Freehold, N. J., Bovine pancreas trypsin, TRL) was added to give a ratio of 1 to 100, trypsin to globin. The pH was maintained at 9.5 by adding 0.1 N NaOH using a 50 microliter syringe and a single probe electrode pH meter. The digestion, as evidenced by base addition, was fastest during the first half hour, but was allowed to proceed for 1-1.5 hours. After digestion the solution was clear at neutral pH. The reaction was stopped by the addition of two drops of glacial acetic acid.

Hemoglobin Peptide Separation

The double labeled peptides were separated by a modification of Jones (44). A Technicon amino acid analyzer column of 130 x 0.43 cm with Technicon chromobeads type A resin and pumping system were used. Column temperature was 50^{o} and the flow rate was maintained at 0.5 mls per minute. The general gradient used was pH 3.1, 0.2 M pyridine to pH 5.0, 2.0 M pyridine with the pH's adjusted with acetic acid. (See RESULTS AND DISCUSSION for specific quantities used.) This buffer seemed to have a deteriorative effect on the valves and packing of the pump, and on the resin itself since subsequent amino acid separations performed on this column were not as sharp. Therefore use of these buffers with this amino acid analyzer cannot be recommended.

All of the eluant from the column was collected by a Buchler fraction collector. Fractions of 2.5 mls were collected and counted in Autogamma tubes. Then aliquots of 0.2-0.5 mls were counted in the liquid scintillation counter. Ninhydrin profiles were determined manually: 0.5 ml sample, 1.0 ml ninhydrin reagent (7), 15 minutes at 100^{o}, cooled with tap water and read in a 1 cm cuvette at 570 mu using the Beckman-Gilford instrument.

Amino Acid Analysis

Acid hydrolysis of freeze-dried or rotary vacuum evaporated (40^{o}) globin or peptides were performed according to Blackburn (7). Hydrochloric acid was triply distilled and then made to 6 N. Two mls of the 6 N HCl were added to five mg of dried protein in a five ml heavy walled Kontes round bottom drying ampoule. Nitrogen was bubbled through the sample for several minutes. The ampoule was fitted to a T-joint so that it could be repeatedly flushed with nitrogen and evacuated by aspiration. The ampoule was then sealed under vacuum and hydrolyzed at 110^{o} for 24 hours. The sample was quantitatively transferred to a 25 ml standard tapered volumetric flask and evaporated twice to dryness on a vacuum rotary evaporator at 40^{o} with caution against bubbling. The sample was dissolved in a small amount of starting buffer from the amino acid analyzer.

Amino acid analyses were performed with a Technicon amino acid analyzer, Model no. 1 NC-1, equipped with the same column used for peptide separations. The buffers and general technique were essentially the same as that of Efron (21). Buffer No. 1, 0.05 M sodium citrate

(0.15 M sodium), pH 2.875, was made by adding 29.42 g of sodium citrate, 50 mls of 2.0 N NaOH, 10 mls thiodiglycol, 2 g phenol, 20 mls Brij-35 (added last) and made to a total volume of 2.0 liters with double distilled water. The solution was titrated to pH 2.875 with 6 N HCl while close to final volume using a Beckman expanded range pH meter. Buffer No. 2, 0.267 M citrate (0.8 M sodium), pH 4.740, was prepared by adding 157.1 g sodium citrate and 10 mls Brij-35 (no thiodiglycol) to 2.0 liters of double distilled water and the pH adjusted as previously mentioned. The buffers were made in 10 liter volumes in containers previously rinsed with 3% formaldehyde and stored at 4^{o}. A Technicon Autograd nine chamber gradient device was filled according to Table 3.

Table 3. Autograd buffer gradient for amino acid analyses.

chamber	buffer no. 1 pH 2.875	buffer no. 2 pH 4.740
1-5	75 mls	0 mls
6	15 mls	60 mls
7-9	0 mls	75 mls

An air-stable ninhydrin reagent was used for the amino acid analyses (110). Reagent No. 1 was prepared by dissolving 20 g ninhydrin in 1500 mls methyl cellosolve (low peroxide grade), then adding 300 mls of 4 M sodium acetate buffer (pH adjusted to 5.50 with glacial acetic acid), 85 mls glacial acetic acid diluted with water, and water to 3 liters. The final pH should be between 5.45-5.50. Reagent

No. 2 was made by dissolving 0.780 g hydrazine sulfate in 3 liters of water plus six drops of sulfuric acid. The hydrazine and ninhydrin solutions were kept separated until mixed (1:2) with the sample and nitrogen. Samples were prepared to contain 12.5% sucrose from a 62.5% solution. The internal standard in each chromatogram was 0.25 u moles of norleucine. The sample was prepared in a one ml volume and then injected through the sample application device on the column with a one ml tuberculin syringe. A small amount of 12.5% sucrose was then injected with another syringe to clear the sample from the application tube. The flow rate for the chromatogram was 0.50 ml/minute. Initial column temperature was 37^{o} and was changed 2.0 hours after starting the chromatogram to 60^{o}. After the 21 hour chromatogram was finished, the column was washed with 0.2 N NaOH for 20 minutes and then washed with starting buffer No. 1 for 2 hours.

Areas for the amino acid peaks were calculated by multiplying peak height in optical density units times the peak width dots at half height.

RESULTS AND DISCUSSION

Several rabbits were prepared by the phenylhydrazine treatment. Some died from poor general condition prior to heart puncture. Only two rabbits produced blood which was satisfactorily incubated and analyzed.

Incubation of Reticulocytes from Rabbit #1

Twenty-five mls of blood drawn from the first rabbit with a recovery of 5 mls of reticulocytes was washed and diluted to 30 mls with the Ringer solution. Some hemolysis was noted during washing and incubation procedures. The radioactive solutions were: 14.6 uCi L-methionine(methyl-^{14}C)/1.41 u moles L-methionine; and 14.6 uCi L-selenomethionine-^{75}Se/1.41 u moles L-selenomethionine. The amino acid mixture, ferrous iron and radioactive solutions were added to the incubation flasks before the washed reticulocytes were added.

Table 4. Incubation of reticulocytes from rabbit #1.

flask no.	reticulocyte solution,mls	u moles		uCi		description
		met	Semet	^{14}C-met	^{75}Semet	
1	8	0.4		4.12		2 mls at 5,10,15, 30 min. for time course
2	8		0.4		4.12	
3	2	0.1		1.03		no other amino acids or iron added, 15 min.
4	2		0.1		1.03	
5	2	0.1	0.2	1.03		test for analog inhibition, 15 min.
6	2	0.2	0.1		1.03	
7	2	0.2		1.03		half specific activities, 15 min.

Preparative elution of the reticulocyte lysate after centrifugation at 10,000 x g showed a uniform specific activity as evidenced by Figure 1. The three peaks in Figure 1 are the dextran blue void volume marker, hemoglobin, and unbound selenomethionine. The void volume was usually a little turbid which was probably due to ribosomal material or other large particles. Analysis at 280 mu also showed adsorption in the void volume which was probably due to nucleic acids since there was not much Lowry protein in this region. Elution of this Sephadex column using smaller amounts of material and smaller fractions yielded a much greater resolving power of the column, but the main objective was to clear the unbound radioactive methionines from the hemoglobin to prevent a possible nonspecific binding of label in subsequent treatments.

After centrifuging at 100,000 x g and eluting on the Sephadex G-100 column, the lysate showed only one hemoglobin species on the Bio-Rex 70 column, and all of the radioactivity was associated with the one peak, Figure 2. Subsequent hemoglobin preparations from rabbit #1 used only the G-100 column prior to deheming and $\alpha\beta$ separations.

The α and β chains of globin were separated only with much difficulty after the deheming process. The carboxymethylcellulose (CMC) from the same company, but different lots gave optimum separation conditions at pH 2.00 and 2.36, whereas their capacity was not significantly different, 0.68 and 0.66 milliequivalents/g respectfully. Only the separations with the first lot of CMC will be reported.

Figures 3 and 4 represent some of the abortive attempts to clearly separate the α and β chains of rabbit globin. Some of the experiments

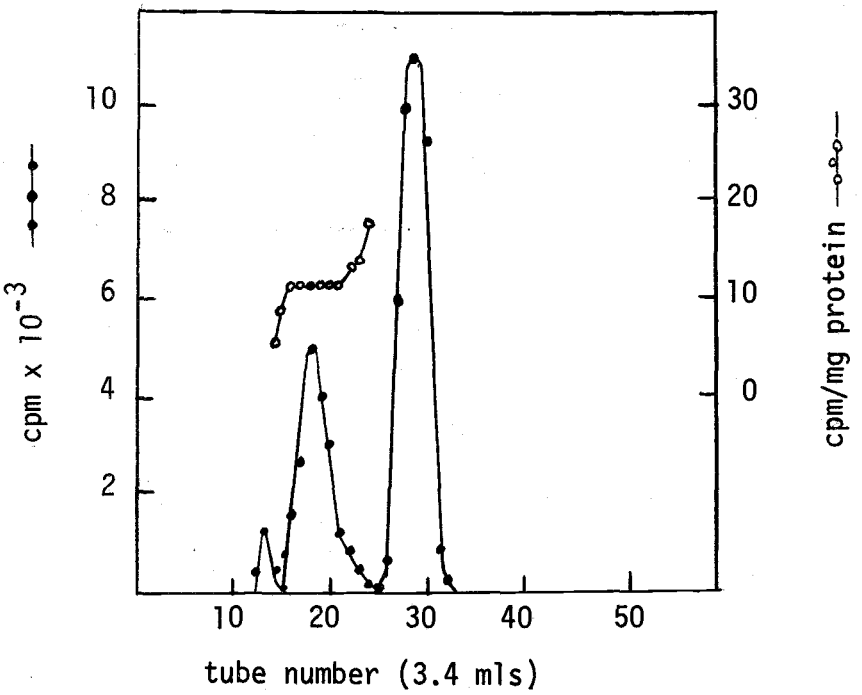


Figure 1. Hemoglobin elution from a Sephadex G-100 column.

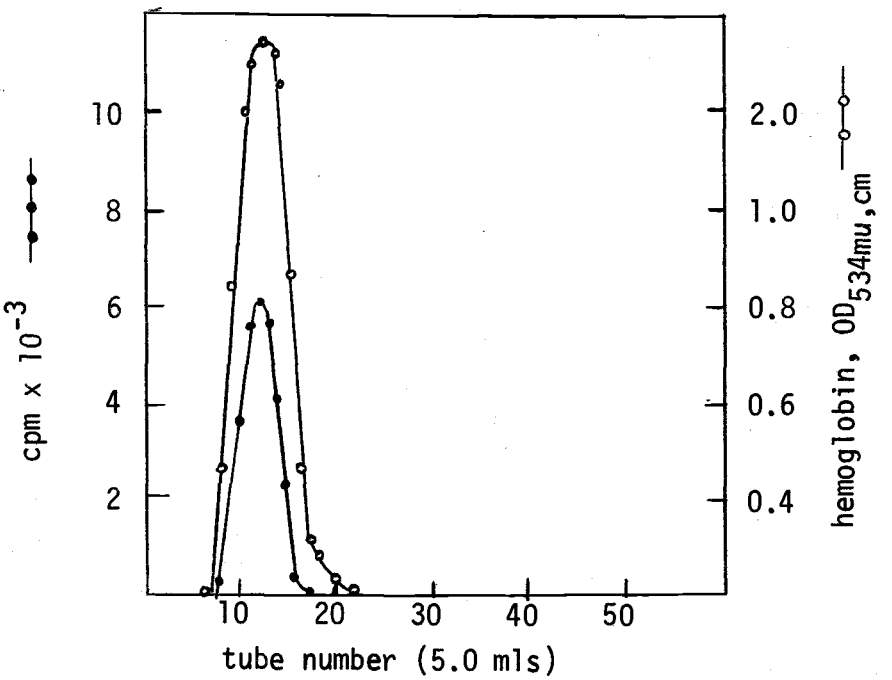


Figure 2. Hemoglobin elution from a Biorex 70 column, rabbit #1.

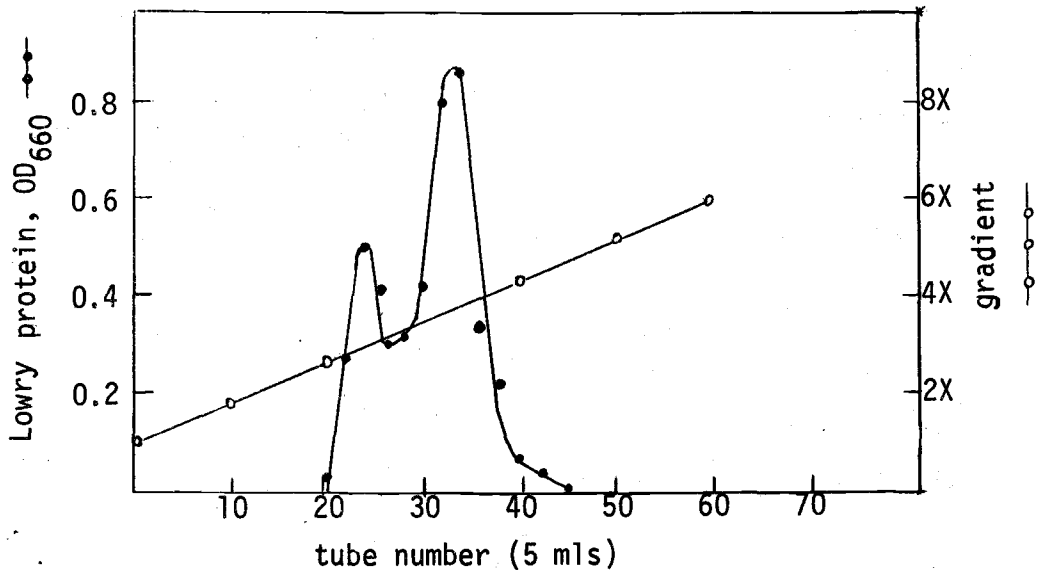


Figure 3. Carboxymethyl cellulose (CMC) column separation of globin at pH 2.40.

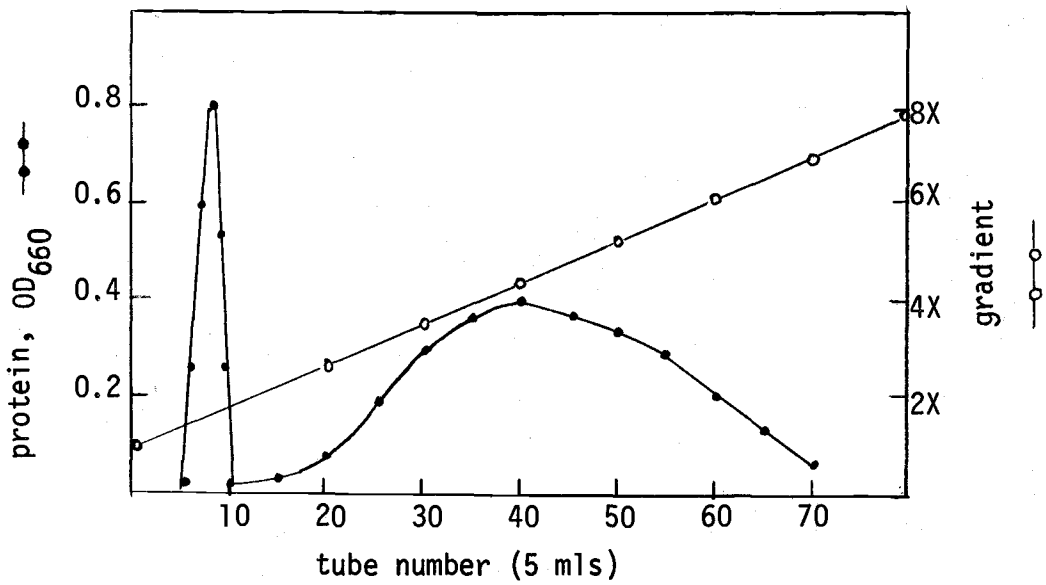


Figure 4. CMC globin separation at pH 2.40.

at pH 2.40 gave only one peak, wheras many of the chromatograms gave a preponderance of the α over the β peak. Good separations were finally achieved with a pH 2.00 and a 500 ml total volume linear gradient of 1-5X or 1-10X pyridine-formic acid buffer. Figure 5 compares separations at pH 2.00 and pH 1.90 buffers. The pH 2.00 buffer moved the peaks to a higher elution volume and gave better separation than the pH 1.90 buffer. Figure 5 represents a pair of superimposed separations using the 1-5X gradient.

The specific activities of the α and β chains during separation remained constant throughout the chromatogram and were essentially equal as demonstrated in Figure 6. Figure 7 is a chromatogram where the column was not brought down to the proper starting ionic strength before beginning the next separation. The sum of the areas under the first two peaks was equal to that of the third peak, which implies that the α peak was divided under this particular condition. The fourth peak in this chromatogram probably represents a non-hemoglobin impurity. This peak was sometimes clearly defined after the β peak, but usually it appeared to trail the β peak as a shoulder. Usually several fractions between the α and β peaks and the shoulder areas after the peaks were discarded before fractions were pooled for further analysis.

Even though the column was washed at times with dilute acid and base, its capacity seemed to decrease. Early in the experiment 80-100 mg of globin could be separated easily after conditions had been established. After repeated separations had been accomplished, loads of 100 or 50 mg of globin gave only single peaks, but 25 mg of the same globin preparation gave excellent separation. At other times globin

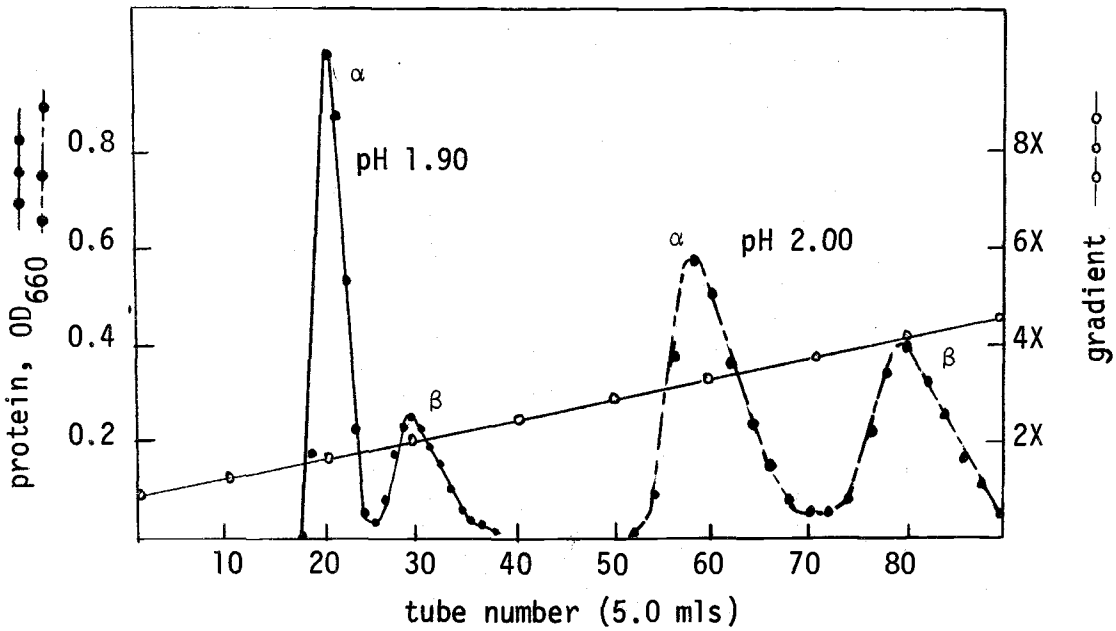


Figure 5. Two superimposed CMC globin separations at pH 1.90 and 2.00.

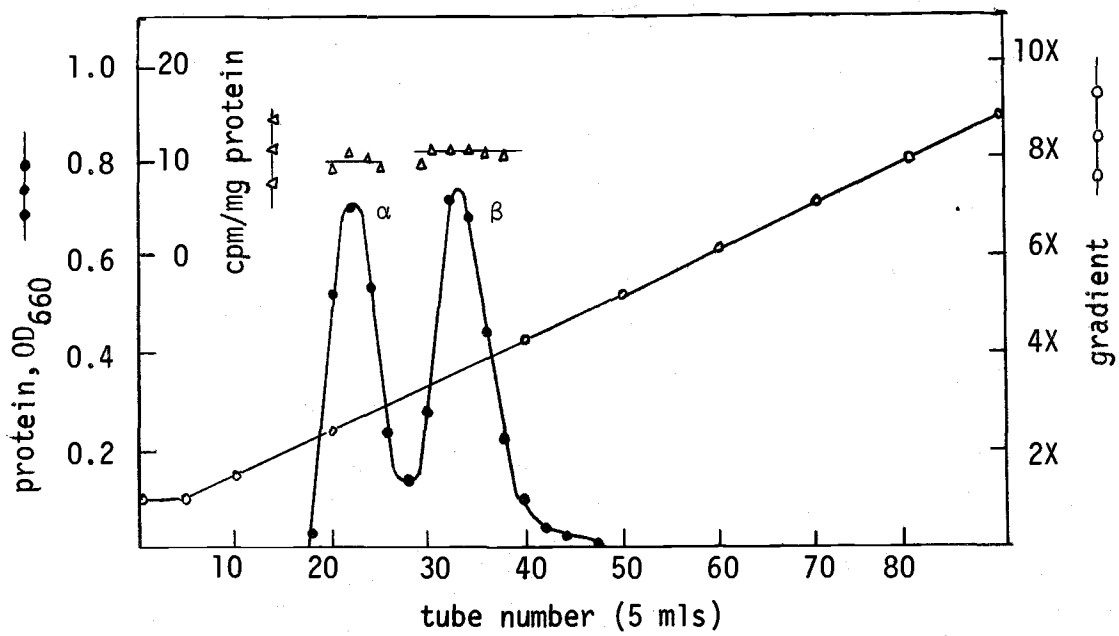


Figure 6. Successful CMC globin separation at pH 2.00.

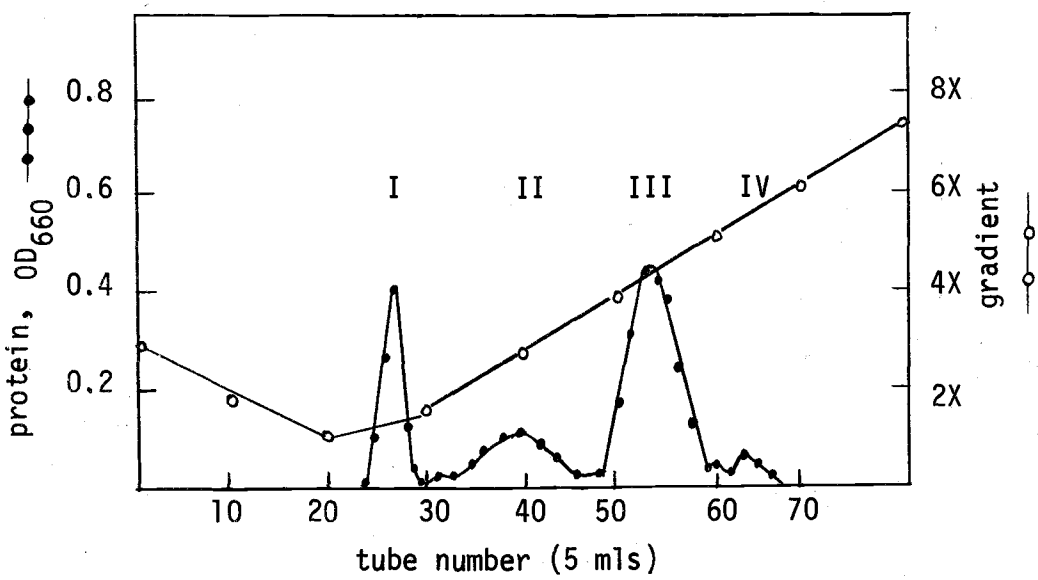


Figure 7. CMC globin separation at pH 2.00. The sum of peak I and II is equal to the third peak, III. This gradient,caused by incomplete washing of the previous column,apparently split the α peak. Peak IV may be a nonhemoglobin impurity which was sometimes noted in other columns.

could not be separated on certain preparations for one of the following reasons: when one sample was thawed at too high a temperature, some coagulation occurred and separation failed; if the acid-acetone for deheming was not cold enough (-20^{o}) separation was usually difficult or incomplete; TCA precipitated globin was generally more difficult to separate than the normally prepared globin, even after dialysis for several days against starting buffer.

Since activities were to be based on u moles of methionine per mg of protein, portions of α, β and $\alpha\beta$ globin were exhaustively dialyzed against double distilled water and dried at 50^{o} in a vacuum oven, desiccated, weighed and analyzed for protein by the Lowry method. Results indicated that α, β, $\alpha\beta$ and bovine serum albumin all had the same reading of about 4.0 OD units at 660 mu using 1.7 cm cuvettes per mg of protein. Equality of protein estimation by this method was probably due to the equal number of tyrosines in the α and β chains, Tables 9 and 10. This value varied downward with the age of the reagents, so appropriate standards were performed with each determination. All of an experiment was always analyzed at one time so as to prevent error within an experiment due to reagent or counting variations. Hemoglobin dialyzed against $K_3Fe(CN)_6$ and cyanide and read at 540 mu ($OD_{540mu,cm} \times 1.44 = mg/ml$, (45)) also confirmed the Lowry method results of 4.0 OD/mg globin. The Lowry method was linear to 0.6 OD, then dropped.

Purity of the α and β chains was checked by their amino acid analysis. Dried α or β globin from peak tubes were analyzed and the results are shown in Table 5.

Table 5. Amino acid analyses of 0.12 mg of α and β chains of rabbit globin.

amino acid	area[b]	area	moles amino acid/mole protein					
			rabbit #1			Naughton & Dintzis[a]		
			α_1	α_2	β	α_1	α_2	β
asp	2.36	2.36	5.7	6.6	6.9	6	9	11
thr	2.36	0.87	5.7	6.6	2.5	7	7	3
ser	2.12	1.88	5.1	5.9	5.5	5	6	18
glu	1.83	2.97	4.4	5.2	8.9	7	6	13
pro	1.00	0.36	2.4	2.8	1.1	5	6	3
gly	2.16	2.32	5.0	5.8	6.9	10	12	9
ala	2.55	2.67	6.1	7.2	8.0	5	8	16
val	1.88	2.88	4.5	5.2	8.6	5	5	14
cys/2	1.30	0.14	3.1	3.6	0.4	1	1	0
met	0.10	0.20	0.3	0.3	0.6	1	1	2
ileu	0.56	0.04	1.3	1.6	0.1	4	4	0
nleu[c]	4.17	4.64	-	-	-	-	-	-
tyr	0.66	0.61	1.6	1.9	1.8	3	3	3
phe	1.50	1.63	3.6	4.2	4.9	7	7	6
NH_3	2.78	2.84	6.7	7.8	8.5	-	-	-
lys	2.61	2.70	6.3	7.3	8.0	9	9	10
his	2.52	2.19	6.1	7.0	6.5	5	7	6
arg	0.59	lost	1.4	1.7	-	3	3	2

[a]Naughton and Dintzis found two types of rabbit α globin. Molecular weights were as follows: α_1 = 12,000; α_2 = 14,000; and β = 16,000 (69).

[b]Areas were corrected for color yields with ninhydrin, Moore and Stein revised values (7).

[c]Norleucine, internal standard, 0.1 u mole.

Rabinowitz and Fischer (85) reported a leucine/isoleu. ratio of 5.6 for the α chain, and 24.5 for the β chain; a serine/threonine ratio of 0.88 for the α, and 2.3 for the β. Results from rabbit #1 indicate an α_2 leu/ileu ratio of 6.2, and 111 for the β chain; and an α_2 ser/thr ratio of 0.91, and 2.15 for the β chain; thus indicating good separation of the two chains. The α chain hemoglobin appeared to be most like that of Naughton and Dintzis' second type of α chain (α_2). Results in Table 5 were calculated according to the molecular weight values of Naughton and Dintzis' amino acid analyses (69).

Table 6 lists flask number, chain and time of incubation for the incubation of rabbit #1 reticulocytes. Analysis of variance for the rates at the four time points revealed no significant differences, and therefore it was concluded that selenomethionine and methionine were incorporated into the α and β chains of rabbit hemoglobin at the same rates. The averaged rates of incorporation in uu moles/mg globin-minute were as follows: selenomethionine, α chain, 3.00; selenomethionine, β, 3.12; methionine, α, 2.66; and methionine, β, 2.22. Since the methionine and selenomethionine rates of incorporation were about the same in both chains it appeared that there were an equal number of methionine residues in both chains. (This conclusion was not substantiated in later experiments of peptide analysis.)

Table 6 indicates that ferrous iron and/or other amino acids were required for the incorporation of both selenomethionine and methionine: selenomethionine showed a 61% reduction, and methionine showed an 80% reduction when compared to the controls for the averaged α,β values. When experiments 3 and 4, Table 6, were checked before deheming or

Table 6. Results of first reticulocyte incubation, rabbit #1.

experiment no.	uu moles Semet / mg globin	average	experiment no.	uu moles met / mg globin	average	experiment description
2-α-5 min.	11.2	15.7	1-α-5 min.	9.4	10.6	time course
2-β-5 min.	20.3		1-β-5 min.	11.9		" "
2-α-10 min.	14.2	19.9	1-α-10 min.	32.1	27.6	" "
2-β-10 min.	25.5		1-β-10 min.	23.2		" "
2-α-15 min.	72.0	61.0	1-α-15 min.	38.8	39.8	" "
2-β-15 min.	50.0		1-β-15 min.	40.7		" "
2-α-30 min.	103.0	89.5	1-α-30 min.	100.2	80.1	" "
2-β-30 min.	75.9		1-β-30 min.	60.0		" "
4-α-15 min.	18.6	23.8	3-α-15 min.	8.3	8.2	no Fe^{++}, amino acids
4-β-15 min.	29.0		3-β-15 min.	8.2		" " "
4-α,β-15 min. pre-CMC [b]	26.8		3-α,β-15 min. pre-CMC	8.2		specific activity check
6-α-15 min.	54.7	76.3	5-α-15 min.	32.3	27.1	analog competition
6-β-15 min.	90.0		5-β-15 min.	22.0		" "
8-α-15 min.	51.6	99.3	7-α-15 min.	71.0	62.6	2X substrate
8-β-15 min.	147.0		7-β-15 min.	54.2		" "

[a] 2-α-5 min. means: flask no. 2-alpha globin-5 minute incubation

[b] pre-CMC is an aliquot of protein checked for specific activity after deheming and before separation.

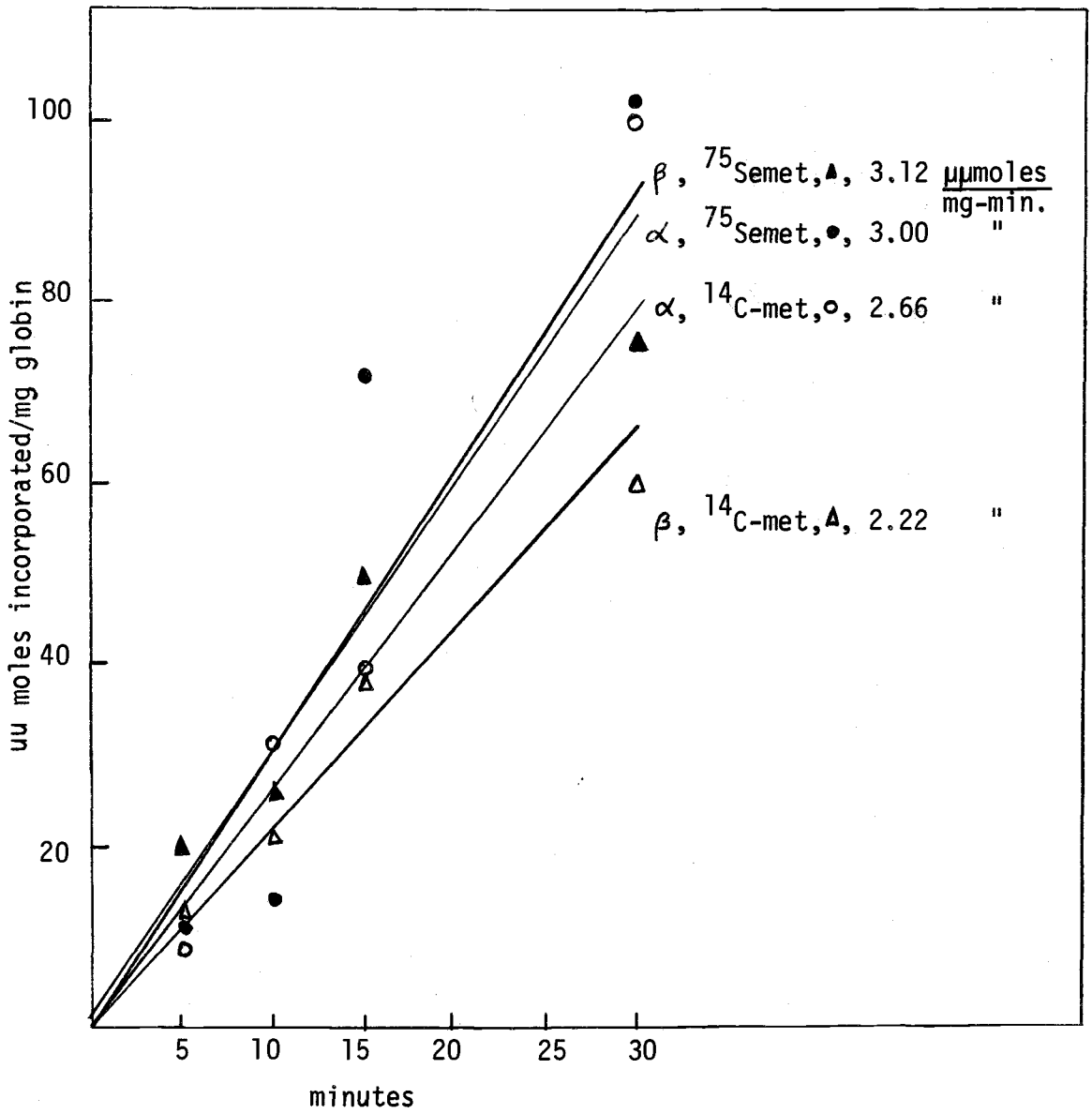


Figure 8. Time incorporation of L-selenomethionine-^{75}Se and L-methionine (methyl-^{14}C) into the α and β chains of rabbit hemoglobin. Lines and data were obtained from linear regression analysis data from the points.

separation of the α and β chains, no alterations of specific activities were found. Checking the recovery of activity was important because of the reported lability of selenium compounds and attachment in proteins.

Competitive inhibition of methionine was illustrated by a 33% decrease of methionine when twice as much selenomethionine as methionine was added. However, selenomethionine incorporation increased 18% when cold methionine was added. When twice as much selenomethionine was incubated as in the control, twice as much selenomethionine was incorporated; but only 57% more methionine was incorporated when its substrate concentration was doubled; experiments 7 and 8, Table 6.

There was considerable variation in the results reported in Table 6, especially for the selenomethionine experiments. These experiments were all completely counted and analyzed for protein twice to try to resolve the variation. Particular attention was given to correcting the quenching in the ^{14}C samples as spiking the samples after initial counting showed wide variation in efficiencies. The selenium-75 specific activities varied widely, perhaps because of handling techniques during purification. However no decomposition of labeled protein was detected. The Lowry protein determinations were reproducible.

The selenomethionine might have appeared to give a higher degree of incorporation because of the uncertainity of its initial specific activity. The selenomethionine was received with the specific activity listed as 0.51 millicuries/0.9 mg selenomethionine. The weight was probably accurate to only one place, whereas the ^{14}C-methionine had its specific activity listed to three significant figures. At the

level of specific activity used, cold methionine had to be added to the radioactive solutions in order to equalize the two methionines for incubations at equal molar quantities.

Incubation of reticulocytes from rabbit #2

The second incubation experiment was designed to overcome the shortcomings of the first experiment. In this experiment the specific activity of the selenomethionine-^{75}Se was also known to only one significant figure, however it was sufficiently high (twenty times higher than the ^{14}C-methionine) so that carrier L-selenomethionine could be added to make the two methionines the same specific activity, and to make the L-selenomethionine-^{75}Se specific activity accurate to at least three significant figures. The second experiment was designed to give significant kinetic data, and a better insight into analog competition using a higher concentration of the cold analogs.

The second rabbit yielded only 8 mls of reticulocytes when 60 mls of blood was withdrawn by heart puncture. The cells were washed and diluted to 60 mls with the Ringer solution. In this experiment both radioactive solutions were: 20 uCi L-methionine(methyl-^{14}C) or 20 uCi L-selenomethionine-^{75}Se(1-15-69)/1.41 u moles of the respective methionine.

The second rabbit was also checked for homogeneity of hemoglobin species using the Bio-Rex 70 column. Surprisingly this rabbit showed two hemoglobin species, Figure 9. The first eluting hemoglobin comprised 72% of both the radioactivity and hemoglobin, whereas the other 28% was recovered in the second hemoglobin. Therefore it was

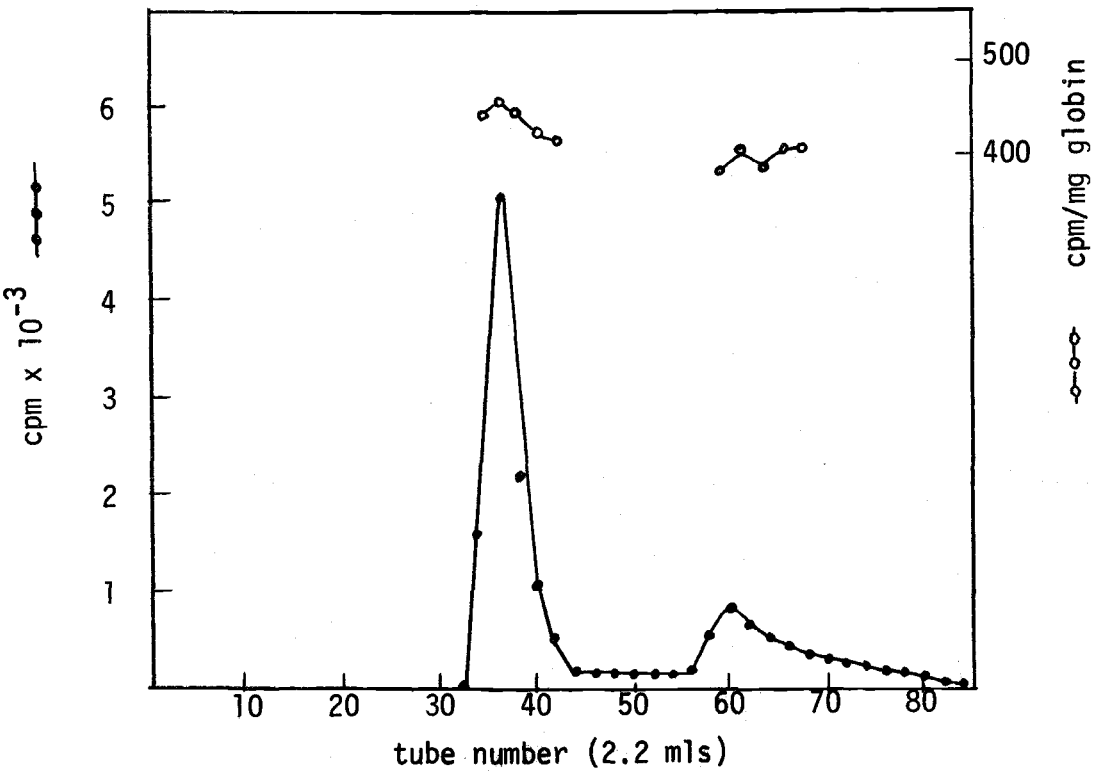


Figure 9. Hemoglobin elution from a Biorex 70 column, rabbit #2.

Table 7. Incubation of reticulocytes from rabbit #2 for one hour.

flask	reticulocyte solution,mls	mu moles met	mu moles Semet	uCi ^{14}C-met	uCi ^{75}Semet	description
1	1.5	17	170	0.95		10X analog
2	1.5	170	17		0.95	" "
3a	1.5	170		0.95		10X substrate
3b	1.5	340		0.95		20X "
3c	1.5	510		0.95		30X "
4a	1.5		170		0.95	10X substrate
4b	1.5		340		0.95	20X "
4c	1.5		510		0.95	30X "
5	24.0	268		15.2		1X control,
6	24.0		268		15.2	peptide analysis

Table 8. Results from rabbit #2 reticulocyte incubations.

flask	uu moles/mg globin ^{14}C-met α	^{14}C-met β	^{75}Semet α	^{75}Semet β	description
1	12.3	11.6			10X analog
2			-5.89-[a]		" "
3a	109	100			10X substrate
3b	105	91.0			20X "
3c	150	129			30X "
4a			-120-[a]		10X substrate
4b			-170-[a]		20X "
4c			118	126	30X "
5	47.2	39.5			1X substrate for control
6			41.8	34.2	and for peptide analysis

[a]Globin α and β chains could not be separated; flasks 2, 4a, 4b.

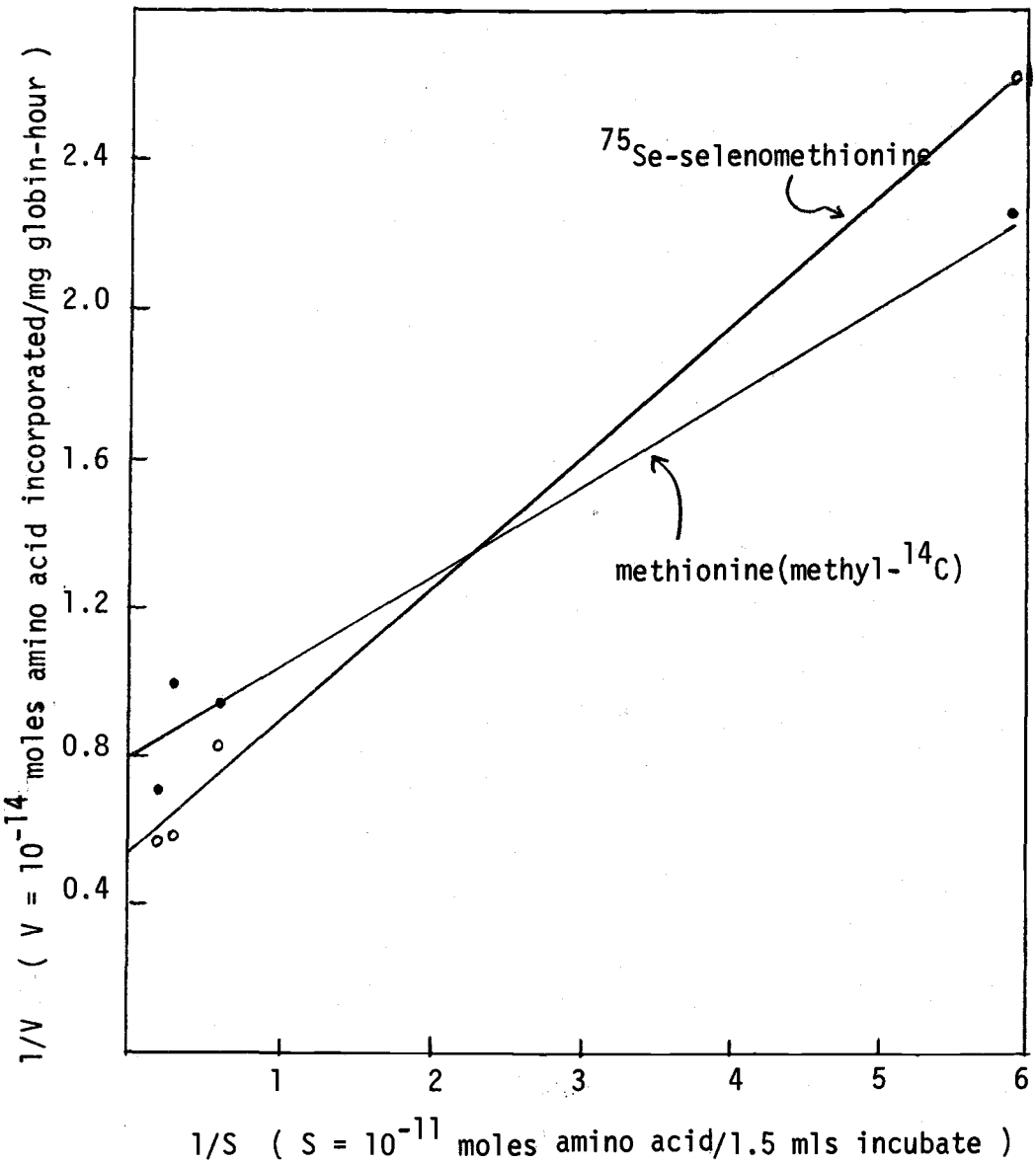


Figure 10. Selenomethionine and methionine incorporation into unseparated chains of hemoglobin. For selenomethionine V_{max} = 1.86 x 10^{-14} moles/hour compared to 1.26 x 10^{-14} moles/hour for methionine. K_m was 0.657 x 10^{-11} moles/incubate and 0.316 x 10^{-11} moles/incubate for selenomethionine and methionine respectively. Slopes and intercepts obtained from linear regression analysis of the data were plotted.

deemed necessary to prepare all of this hemoglobin by the Bio-Rex 70 column after the Sephadex G-100 column. Only the main component hemoglobin was used because the specific activities were somewhat different: 544 and 520 cpm/mg globin respectfully for the ^{75}Se tagged hemoglobins shown in Figure 9.

Due to unknown factors some of the α and β chains were not able to be separated, but the ones that were separated showed no differences in specific activities of the different chains.

Results from flasks 3-6 were plotted from the slope and intercept data from linear regression analysis, Figure 10. The linear regression analysis showed a correlation coefficient of 0.988 for the ^{14}C-methionine and 0.998 for the ^{75}Se-selenomethionine in the standard double reciprocal plot of velocity and substrate concentration, Figure 10. V_{max} for the selenomethionine was 1.86×10^{-14} versus 1.26×10^{-14} moles/mg globin-hour for the methionine. The K_m values were 4.38 mu molar for selenomethionine and 2.11 mu M for methionine. The V_{max} values were not significantly different, but the K_m values were significant at the 5% level. The values of V_{max} and K_m are useful for comparison within an experiment such as this, but may vary between experiments due to the degree of reticulocytosis, age of reticulocytes, and other factors. Most reported K_m values of selenomethionine in enzyme reactions have been higher than for methionine (56, 67).

The increased ratio of selenomethionine/methionine and methionine/selenomethionine in flasks 1 and 2, Table 8 proved that these methionines are truly competitive analog inhibitors of each other at a ten-fold concentration difference. Selenomethionine decreased methionine

72% and methionine decreased the incorporation of selenomethionine by 85% into the rabbit globin.

The striking parallelism in this experiment of selenomethionine and methionine incorporation into hemoglobin leads to the conclusion that they are genetically identical. However this identity has not been proven conclusively until the identity of the sites on the amino acid chains has been established. The experiment designed to give an insight into this proof was to show that both methionines were incorporated into the same peptide or peptides.

Hemoglobin peptide analysis

Tryptic peptides were analyzed from globin prepared from flasks 5 and 6 from the second reticulocyte incubation. The first peptide separation on the amino analyzer column used a 500 ml linear gradient of 250 mls, pH 3.1, 0.2 M pyridine to 250 mls, pH 5.0, 2.0 M pyridine with the pH's being adjusted with glacial acetic acid. Figure 11 shows the separation of β chain peptides with ^{14}C and ^{75}Se activity both occurring in two peaks. The ^{75}Se activity was displaced to a higher elution volume in both cases. The first peaks contained 203 uu moles of methionine and 220 uu moles of selenomethionine. Since 10 mg of each globin from flasks 5 and 6 were combined for tryptic digestion, the specific activities for the β chain calculated to be 20.3 uu moles methionine and 22.0 uu moles selenomethionine per mg of globin, which was only about half of the specific activities of the labeled globin reported in Table 8. This reduction in activity may be partially explained by an incomplete hydrolysis or a decompos-

ition of the methionines during the 50° chromatogram (Tables 12, 13). There was also a low but significant radioactive background throughout the column which was not included in the peak summations of the methionine or selenomethionine recoveries.

To achieve better separation the α chain tryptic hydrolysate was washed for the first 40 tubes with the pH 3.1, 0.2 M pyridine starting buffer before the 500 ml linear concentration gradient was applied, Figure 12. This chromatogram had two ^{14}C peaks and one ^{75}Se peak, with separation of the ^{75}Se and ^{14}C peaks. There were 167 uu moles of selenomethionine and 150 uu moles of methionine recovered under the two corresponding second peaks with 82 uu moles of methionine and 20 uu moles of selenomethionine under the first peak. When unseparated α,β chains were analyzed for their labeled tryptic peptides, only two peaks were found for each radioactivity. Again some separation of the ^{75}Se from the ^{14}C activity in the last or presumably the α peaks, Figure 13. The gradient of 150 mls of pH 3.1, 0.2 M pyridine to 150 mls of pH 4.3, 1.1 M pyridine buffer was started after tube no. 80. The 1.1 M pyridine buffer was made by adding equal quantities of the 0.2 and 2.0 M pyridine buffers. There was an unusual distribution of the ^{14}C activity with 63% in the first, or β peak and 37% in the second, or α peak. However there was an equal distribution of the ^{75}Se in both of its peaks, 47% and 53% respectively. This particular chromatogram was analyzed for Lowry protein in the regions of radioactivity, but there was very little reacting substance in this region, confirming that these peaks were not due to non-tryptic digested chains. The other confirmation of tryptic hydrolysis was the rapid base uptake

during hydrolysis at pH 9.5 and the many ninhydrin positive peaks during the peptide chromatograms.

Due to the apparent cross contamination of the α with the β chain and vice versa, ^{14}C and ^{75}Se labeled globin were combined prior to α,β separation on the CMC column. Excellent homogeneity of the α chain was demonstrated when only one peak for both ^{14}C and ^{75}Se was observed in Figure 14. Good separation of the peptides was also observed using an 800 ml concentration gradient of pH 3.1, 0.2 M to pH 4.3, 1.1 M pyridine buffer after washing for the first eighty tubes with the 0.2 M pyridine buffer. When the β chain was analyzed, a small amount of α impurity was noted. However two ^{14}C peaks were clearly resolved for the β peptides in Figure 15. Two ^{14}C peaks were clearly resolved for the β peptides in previous chromatograms not listed here. Some small separation of the ^{75}Se β peaks had also been noted. The ^{14}C activity appeared to lie under two distinct ninhydrin peaks in Figure 15. The appearance of two ^{14}C and possibly two ^{75}Se β peaks may be attributable to the greater resolving power of a slower concentration gradient. Chemical alteration of the peptide or incomplete tryptic hydrolysis could have led to differences in the chromatograms, however two radioactive peaks for the β peptides were not observed for either the ^{14}C or ^{75}Se. The other possibility was that there were two methionine positions on the β chain and only one in the α chain, which would agree with Naughton and Dintzis' observations, Table 10. The two β peptides containing methionine in Table 10 have very similar amino acid compositions, therefore making them difficult to resolve. Two β ^{14}C peaks could have been resolved, but the two β ^{75}Se peaks

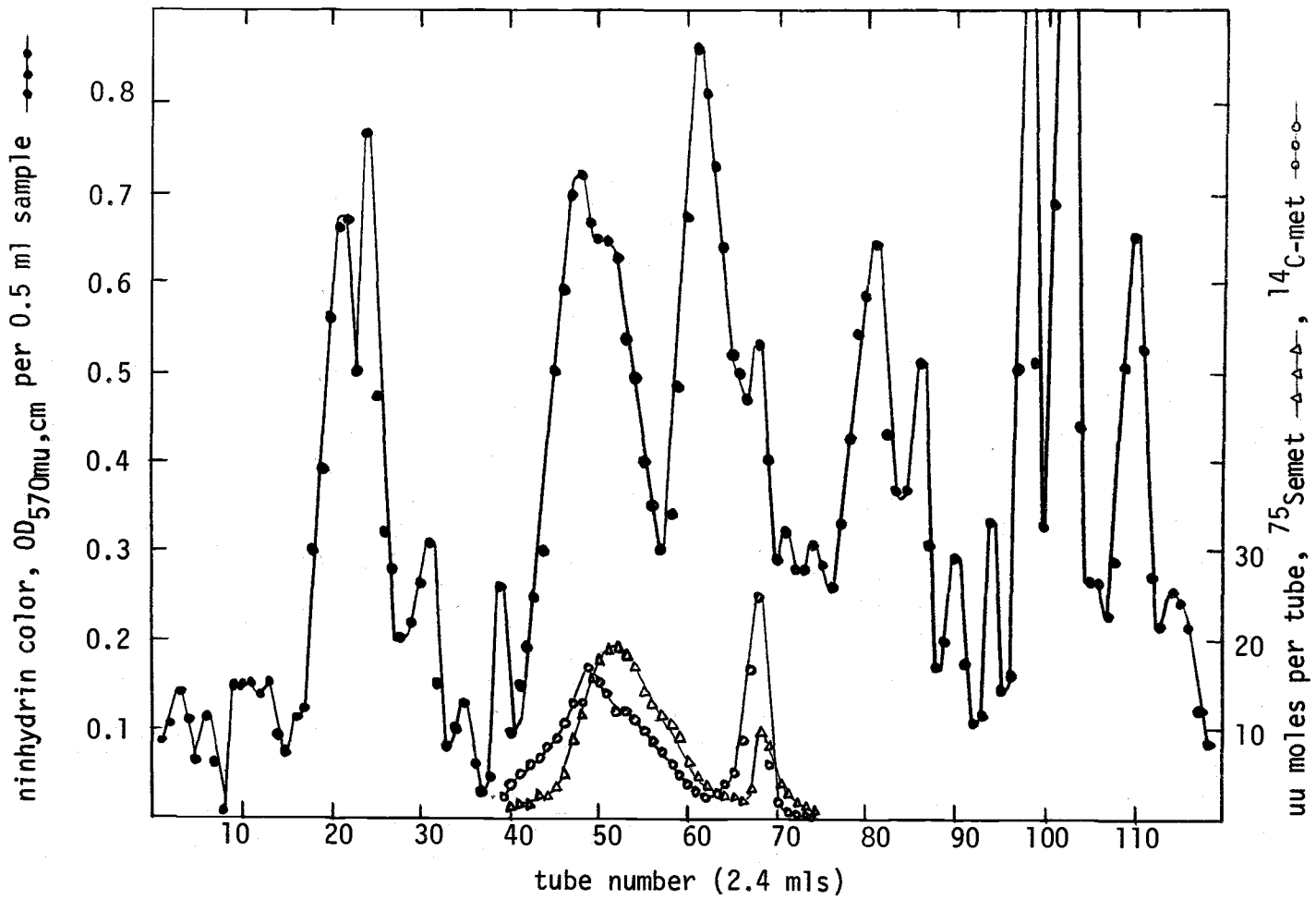


Figure 11. Peptide separation of trypsin digested rabbit hemoglobin β chain. This separation was performed on a Technicon amino acid column and resin using a 500 ml linear gradient of 250 mls of pH 3.1, 0.2 M pyridine to 250 mls of pH 5.0, 2.0 M pyridine buffer. Not all of the chromatogram is shown. 10 mg of globin from incubations 5 and 6 were each combined and analyzed together. The second radioactive peaks are apparently α chain impurities.

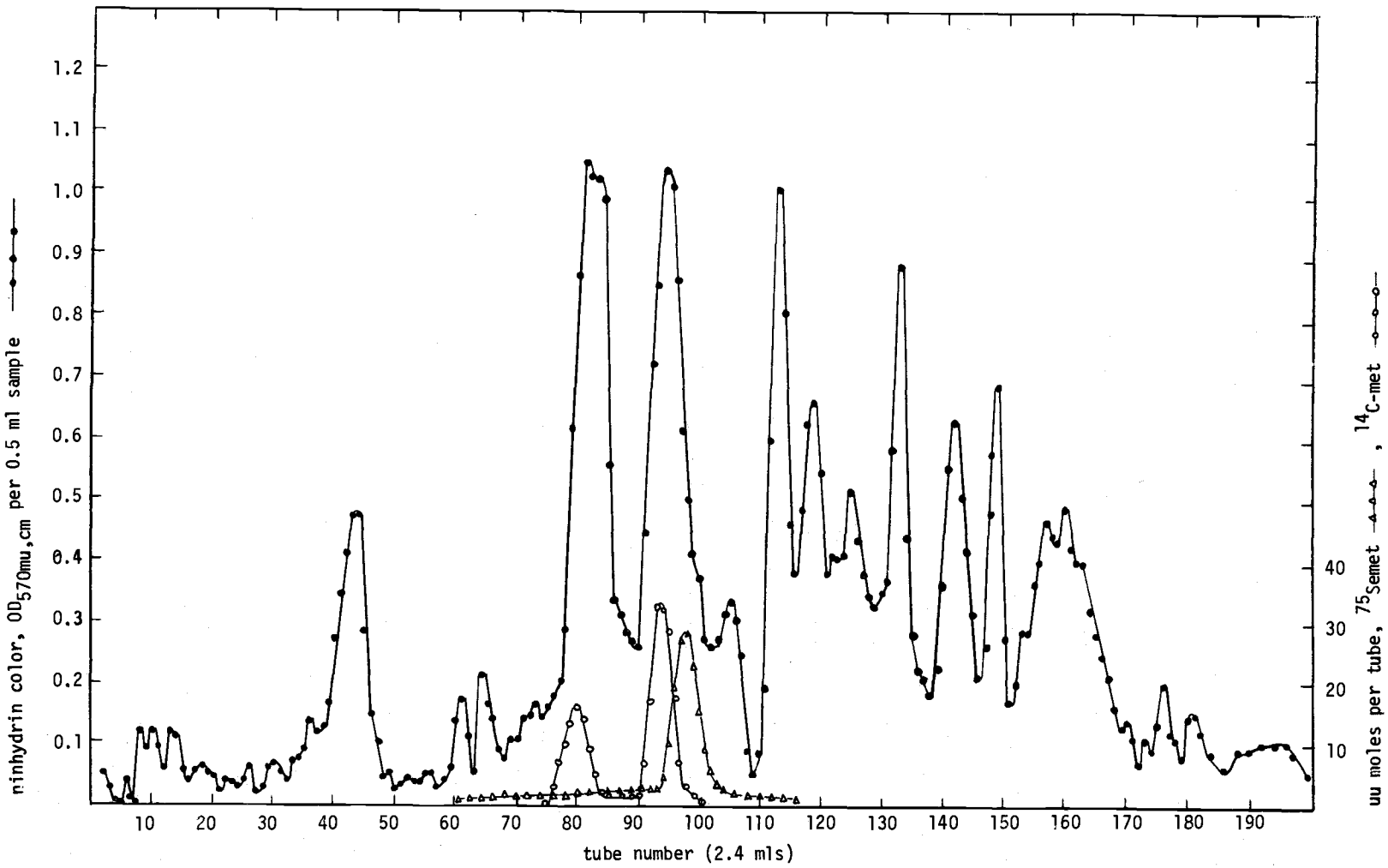


Figure 12. Peptide separation of α chain trypsin digested rabbit hemoglobin. Tubes 1-40 were washed with the 0.2 M pyridine buffer, then a 500 ml linear gradient as in Fig.11 was employed for the rest of the chromatogram. The first ^{14}C peak was probably a β chain impurity.

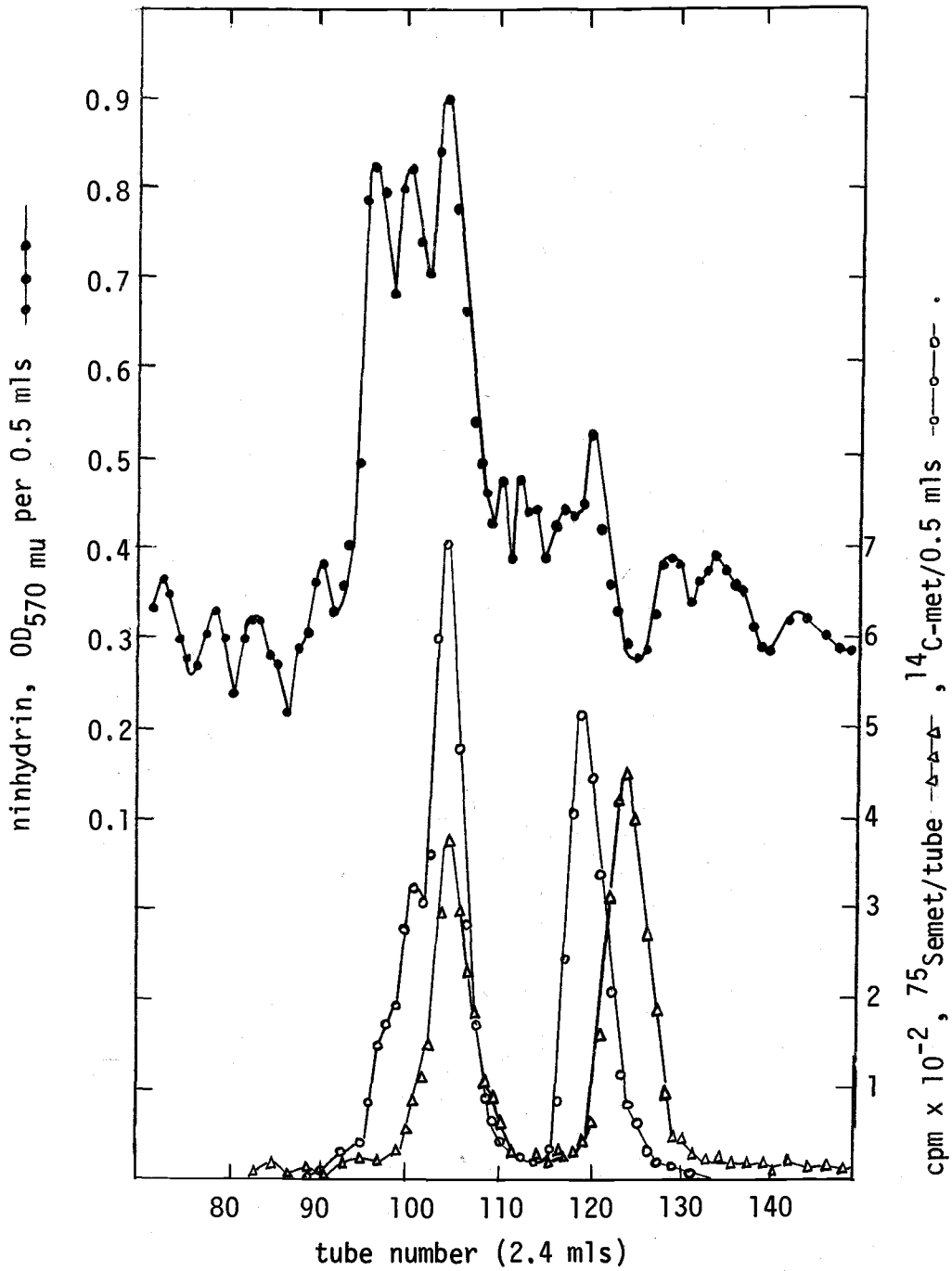


Figure 13. Peptide separation of trypsin digested, unseparated $\alpha\beta$ chains. The gradient was as follows: tubes to no. 80, 0.2 M pyridine; 81-170, linear gradient of 0.2 to 1.1 M pyridine; tubes 171-250, 1.1 M pyridine.

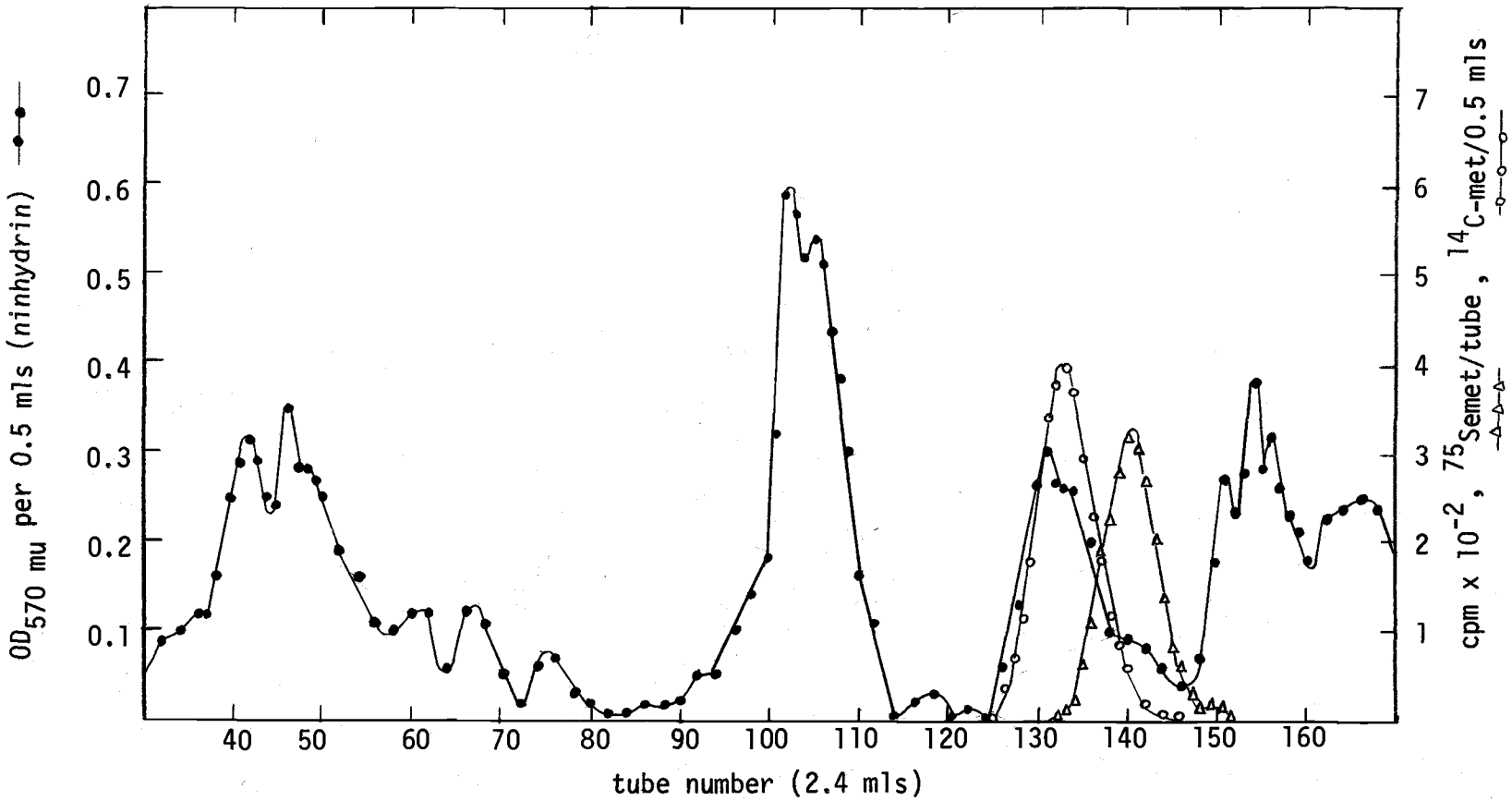


Figure 14. Peptide separation of α chain globin. Gradient was as in figure 13.

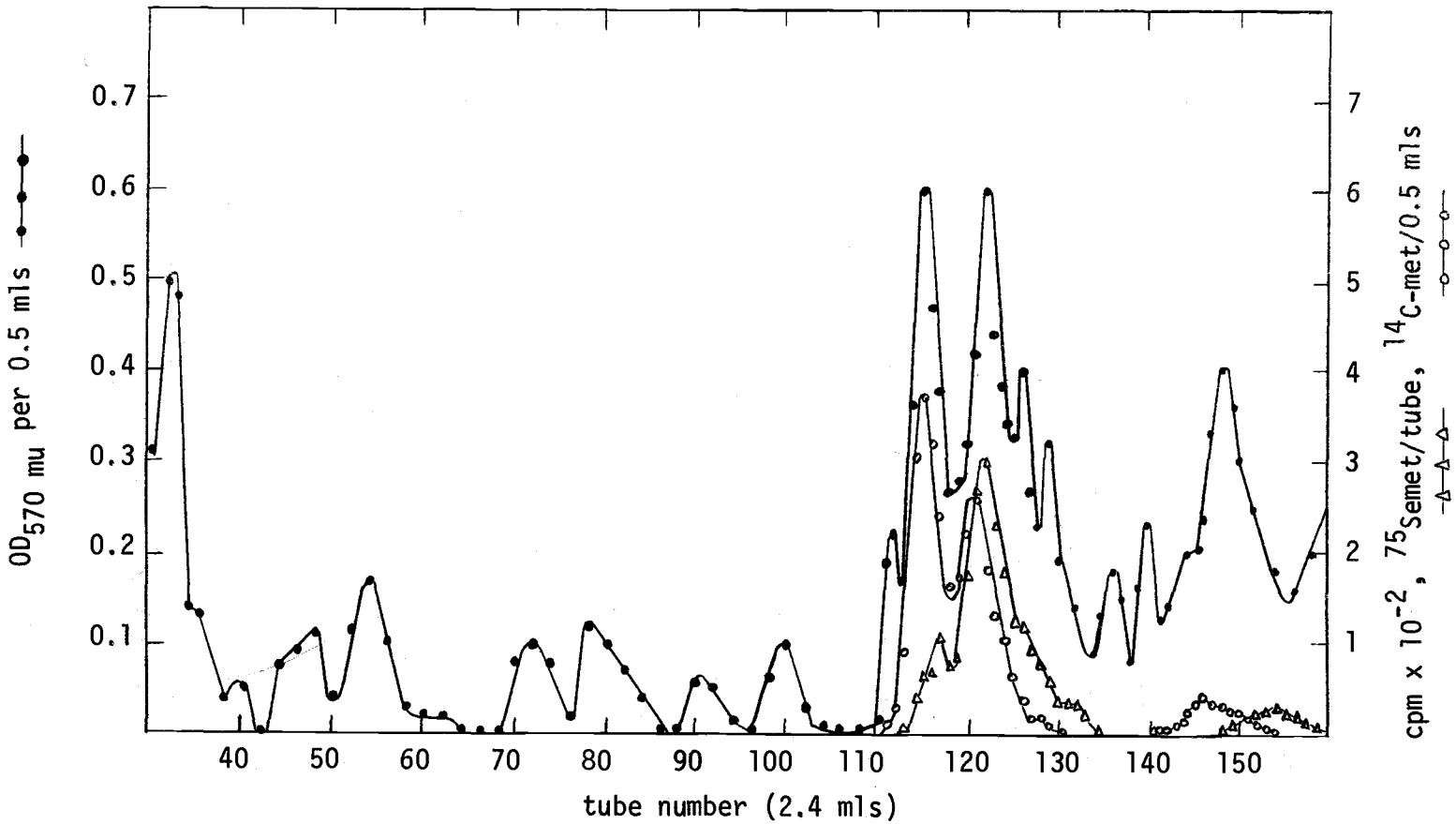


Figure 15. Peptide separation of β chain globin trypsin hydrolysate. Gradient was the same as in Figure 13.

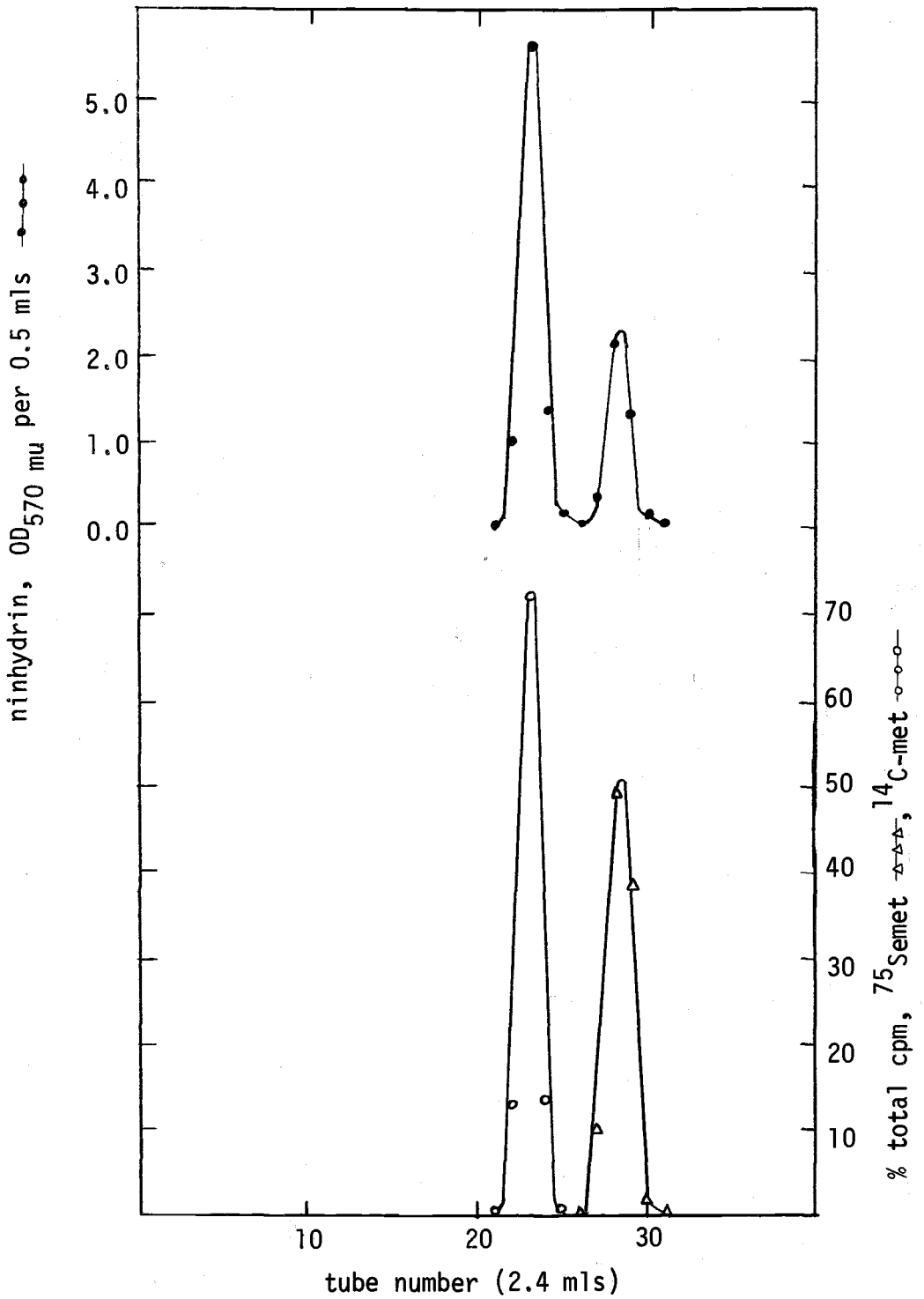


Figure 16. Separation of ^{75}Se-selenomethionine and methionine(methyl-^{14}C) on the amino acid analyzer using the peptide gradient that was used in figure 13. About 25 mu Ci of both ^{14}C-methionine and ^{75}Se-selenomethionine were placed on the column with 20 u moles cold methionine and 10 u moles cold selenomethionine.

possibly could not have been resolved due to the apparent S:Se chromatographic differences. That is, the two β selenomethionine containing peptides chromatographed similarly, but the two β methionine containing peptides were resolved.

Selenomethionine was separated from methionine on this column in Figure 16. Elution occurred early in the chromatogram with the pH 3.1, 0.2 M pyridine buffer before the gradient was applied. About 25 muCi of both methionine(methyl-^{14}C) and ^{75}Se-selenomethionine were placed on the column with 20 u moles cold methionine and 10 u moles cold selenomethionine. Half ml aliquots were removed for ^{14}C counting and half ml aliquots were used for the ninhydrin reaction after ^{75}Se counting was completed. The ability of the column to separate selenomethionine from methionine may be related to its ability to separate selenopeptides from sulfur peptides. Selenomethionine was probably incorporated into the same peptide as methionine because the selenopeptide occurred over a ninhydrin "valley" and not directly over a ninhydrin peak as illustrated in Figure 14.

Amino acid composition of rabbit hemoglobin peptides was used as a reference to check the purity of the labeled peptides, Table 11. Not all of the amino acid analyses performed are listed in this table since some were performed on incompletely separated peptides. Tables 9 and 10 list the amino acid composition of the α and β chain peptides as performed by Naughton and Dintzis (69).

Table 9. Amino acid composition of α chain tryptic peptides from Dintzis and Naughton (69).

amino acid	peptide number[a]															total[a,b]	
	I	II	III	IV	V	VI	VII	VIII	IX_1	IX_2	X	XI	XII	XIII	XIV	α_1	α_2
asp	1	1								3		2	2			6	9
thr		1			2				1	1			3			7	7
ser	1			1					1	2			2			5	6
glu				3					1				3			7	6
pro	1				1					1		1	2			5	6
gly				4	1		1			2			4			10	12
ala	1			2			1		1	4						5	8
val	1			1					1	1		2				5	5
cys/2													1			1	1
met					1											1	1
ileu		1		1									2			4	4
leu	1				1				1	5	1		1			5	9
tyr				1									1		1	3	3
phe				2								1	4			7	7
lys	1	1			1		1	1	1	1		1	2			9	9
his				1			1			2			3			5	7
arg				1							1				1	3	3
try										1						0	1

[a]Peptides are numbered according to chemical order beginning with the NH_2-terminal peptide. Numbers are listed according to human hemoglobin; there are no rabbit peptides corresponding to human peptides III, VI or XIII (69).

[b]Molecular weight calculated from this data, α_1 = 12,100 and α_2 = 14,300.

Table 10. Amino acid composition of β chain tryptic peptides from Dintzis and Naughton (69).

amino acid	peptide number[a]															total[b]
	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	XIII	XIV	XV	β
asp			1		4				3	2				1		11
thr		1		1									1			3
ser	2	1			2				1	2						8
glu	2		3	1	1				1	1			4			13
pro				1	1								1			3
gly		1	3		1		1		1	1				1		9
ala		2	1		2		1		2	2			2	4		16
val	1	1	3	2	1	1			1	1				3		14
cys/2																0
met					(+)					(+)						(2)
ileu																0
leu	1	1	1	2	2				3	2				1		13
tyr				1									1		1	3
phe					3				1	1			1			6
lys	1	1			1	1	1	1	1	1			1	1		10
his	1						1		1	1				1	1	6
arg			1	1												2
try		1		1												2

[a]Refer to Table 9, footnote a.

[b]Molecular weight calculated from this data, β = 16,100.

Table 11. Amino acid ratios of ^{14}C or ^{75}Se tryptic peptides resulting from the incubation of methionine(methyl-^{14}C) and ^{75}Se-selenomethionine.

amino acid	description of labeled peptide			
reference:	Fig. 12	Fig. 12	Fig. 14	Fig. 11
fraction:	91-95	96-102	125-135	45-55
chain:	α	α	α	β
asp	3.0	3.1	0.8	1.0
thr	3.2	1.8	2.1	0.1
ser	2.0	2.0	0.5	0.8
glu	0.4	1.2	0.2	0.8
pro	1.2	0.8	0.8	0.4
gly	2.9	3.3	1.4	1.3
ala	3.0	3.2	0.5	2.5
val	1.3	1.6	0.4	1.6
cys/2	0.0	0.0	0.0	0.0
met	0.4	0.0	0.8	0.9
ileu	0.1	0.0	0.2	2.0
leu	4.3	4.6	1.5	2.9
tyr	0.1	5.6	0.0	0.1
phe	1.3	0.6	2.0	2.0
NH_3	0.9	1.8	1.0	2.2
lys	1.6	1.6	1.6	2.0
his	2.0	3.1	0.2	1.8
arg	0.0	0.3	0.8	0.4

Although these labeled peptides were sometimes overlapping with other peptides, their amino acid composition in all cases showed amino acids that were expected and sometimes showed an absence of the amino acids which were not expected. Tubes 91-95, Fig. 12, Table 11 had an amino acid composition closely corresponding to a mixture of α peptides V and IX, Table 9. The selenium-75 peak, tubes 96-102, Fig. 12, Table 11 had no methionine or methionine sulfoxide. This was significant in that it again confirmed that the methionine and selenomethionine peptide peaks were separated. Tubes 125-135, Fig. 14, Table 11 was a hydrolysate of the leading half of a well separated α ^{14}C peak. This analysis gave an amino acid composition of the peptide whose integral values corresponded very closely to the expected α peptide V. Low yields of methionine were obtained perhaps because in standard chromatograms, methionine sulfoxide occurred only slightly before aspartic acid, but methionine sulfone occurred directly under threonine. All analyses reported were the combination of the methionine and the smaller methionine sulfoxide peak if it occurred. Amino acids are listed in order of their elution from the column.

Since methionine and selenomethionine have not been resolved by paper chromatography or electrophoresis, these peptides probably could also not have been resolved in this manner. Paper electrophoresis followed by one or two ascending paper chromatography steps was used and well characterized by Naughton and Dintzis (20, 69) to resolve the tryptic peptides of rabbit hemoglobin. They reported that the separation and identification was not uniformly good. Their techniques were tried several times on this material, but they failed to give

proper resolution, besides lending to a large amount of smearing. One dimensional ascending paper chromatography using the buffer system of Dintzis (20) (42.5% n-butanol, 27.5% pyridine and 30% water, v/v) was sufficient to demonstrate that the ^{14}C and ^{75}Se α peptides chromatographed with the same R_f value of about 0.8, although there was still considerable smearing of radioactivity. Two radioactive peptides were not resolved for the α peak in this manner or by the column chromatography which contained methionine and the methionine oxides as sometimes demonstrated by Naughton and Dintzis (20, 69).

Other Enzymatic and Chemical Treatments of Labeled Hemoglobin.

To insure that the labels remained in the methionines, the doubly labeled αβ sample was digested with one part per hundred of pronase (Calbiochem, Los Angeles, California). A small amount of material was precipitated with 7% TCA after the 24 hour digestion at pH 7.0 and room temperature. The precipitate was centrifuged out at 8,000 x g and the supernatant was concentrated and applied to an ascending paper chromatogram. Whatman No. 1MM paper was used and the solvent was 40% n-butanol, 40% ethanol and 20% water, v/v (83). Although there was some smearing the peak ^{14}C and ^{75}Se activities were associated with the methionine R_f value of 0.42. Another indication that the methionine label remained in methionine was that effluent from the amino acid analysis of Figure 14, Table 11 contained 40% of the initial radioactivity in the area of methionine. The single peak of radioactivity in only this one α peptide, combined with the destruction of methionine during acid hydrolysis, tend to confirm that the label

remained in the methionine.

Due to previous reports of non-peptide binding of selenomethionine in proteins, a study was undertaken with this hemoglobin which was pooled from ^{75}Se-selenomethionine and methionine(methyl-^{14}C) labeled samples. Since the previous work in this thesis has shown that equal molar amounts of selenomethionine and methionine were incorporated into this hemoglobin, this labeled protein would provide an excellent material for stability studies.

Duplicate two ml quantities of the dual labeled αβ globin were dialyzed against repeated changes of 0.05 M Na_2SO_3, 0.01 M Tris at 4^{o} for one week. Results indicated that both ^{14}C and ^{75}Se activities were reduced upon this sulfitolysis procedure and that the ^{75}Se activity was reduced to a greater extent at neutral and acid pH, whereas the reverse was true for the ^{14}C activity, Table 12. Certainly the labels in the methionines were not equal, but the comparison showed a striking similarity. L-methionine-^{35}S was not used because of its short half life and difficulty in resolving the ^{35}S from the ^{75}Se counting rates. A carboxyl-^{14}C labeled methionine would probably not have shown the same sulfitolysis decomposition because of the greater stability of the peptide bond.

Table 12. Dialysis of αβ globin labeled with L-selenomethionine-^{75}Se and L-methionine(methyl-^{14}C) against 0.05 M Na_2SO_3.

treatment:	SO_3^{--} pH 2.0	SO_3^{--} pH 7.0	SO_3^{--} pH 11.0	H_2O pH 7.0
^{75}Se cpm/mg globin	52.5 57.0	53.0 51.0	151.0 151.0	218.1 220.0
^{14}C cpm/mg globin	1770 1770	1710 1590	1300 1120	1994 2096
average % lost				
^{75}Se	75.0	76.2	33.2	0
^{14}C	11.0	34.0	56.0	0

Table 13. Effects of 0.05 M dithiothreitol and flash evaporation on the ^{75}Se and ^{14}C labeled globin.

treatment	cpm/mg globin		average % lost	
	^{75}Se	^{14}C	^{75}Se	^{14}C
control, H_2O dialysis	335	2260	0	0
" "				
dithiothreitol dialysis	281	1800	16	15
" "	300	1960		
evap. 40°, 48 hours	345	2131	0	3
evap. 70°, 4 hours	294	2130	14	0
evap. 70°, 4 hours with pyridine buffer	290	2160	16	2
evap. 50°, 4 hours under nitrogen	302	2150	12	2

Dithiothreitol, DTT, 0.05 M was dialyzed against this labeled globin for one week to determine whether it could also reduce any of the ^{75}Se or ^{14}C activity. Samples were also flash evaporated under vacuum at 40^{o}, 70^{o} and 70^{o} with 2.0 M, pH 5.0 pyridine buffer, then dialyzed against water. One sample was evaporated at 50^{o} under a stream of nitrogen. The evaporation experiments were performed because some reduction of the ^{75}Se activity was noticed when some of the samples were flash evaporated prior to peptide column application.

These experiments showed that there was some loss of ^{75}Se activity both upon heating and DTT treatments, but ^{14}C activity was lost only with DTT and not the heat treatments, Table 13. This might help explain some of the loss of activity in the peptide column since they were run at 50^{o} and in the presence of thiodiglycol, however no extraneous peaks were observed for ^{14}C or ^{75}Se activities. Peaks for selenomethionine or methionine activities in the peptide columns would certainly have been noticed.

Based on observations that α peptides showed only one ^{14}C and ^{75}Se peak; that both α and β chains of globin contained an equal amount of radioactivity; and that ^{14}C and ^{75}Se radioactivity was associated with the methionine after hydrolysis; it was concluded that this sulfitolysis procedure was removing radioactivity from the methionine and selenomethionine residues and not by reducing disulfide bonds or sulfide-selenide bonds which may have been formed if selenomethionine was changed to selenocysteine or demethylated to homoselenocysteine. There apparently was no selenomethionine converted and incorporated as selenocysteine. Of course with the methyl labeled methionine no observations

could be made for interconversion of methionine into cysteine.

The most important observation was that selenomethionine and methionine were incorporated into the hemoglobin in the same peptides and in the same molar quantities.

TRANSFER RNA AMINOACYLATION WITH SELENOMETHIONINE AND METHIONINE USING MATERIALS FROM NORMAL AND SELENIUM ADAPTED *ESCHERICHIA COLI*

It was previously stated that the objectives for this phase of study were to determine whether the tRNA species for selenomethionine and methionine were the same, to compare their aminoacylation kinetics with a cell free system, and to investigate if selenium adaptation of *Escherichia coli* B is manifested in the tRNA or aminoacyl synthetase for selenomethionine or methionine. This study was selected because amino acid recognition is involved during aminoacylation and not during attachment of the amino acid charged tRNA to the ribosome-mRNA complex, or in subsequent peptide bond formation.

EXPERIMENTAL

Growth and Selenium Adaptation of *Escherichia coli* B

Growth and selenium adaptation of *Escherichia coli* B were done according to Shrift and Kelly (100). The composition of the low sulfate media was: NH_4Cl, 0.0187 M; Na_2HPO_4, 0.0422 M; KH_2PO_4, 0.0220 M; $MgCl_2 \cdot 6H_2O$, 0.0017 M; $Na_2SO_4 \cdot 10\ H_2O$, 0.00031 M; and glucose, 0.0220 M. Glucose and magnesium chloride were each autoclaved separately and added to the main solution after autoclaving and cooling. Potassium selenate was added to the main salt solution in the flasks before sterilization. Growth was followed at 650 mu in a Coleman Universal Spectrophotometer in cuvettes having a 1.7 cm light path length. The microorganisms were grown on the low sulfate media in sterile one liter flasks stoppered with cotton plugs covered with aluminum foil. Sterile

pipettes and loops were employed to transfer cultures. The cultures were grown at 37^{o} with vigorous shaking in one liter flasks with 500 mls of media. Initial growth was started in the low sulfate minimal media with the addition of 2 g of Bacto-tryptone (Difco). After growth had accelerated, a one ml aliquot was transferred to the low sulfate media. One ml aliquots of the minimal media culture were then consecutively transferred to flasks containing higher and higher concentrations of selenate (4×10^{-5} M to 4×10^{-3} M K_2SeO_4) after growth had successfully taken place in the previous flask. Growth of unadapted cells was in 0.00124 M sulfate for harvesting normal cells. Growth of unadapted cells and final selenium adapted cells in 4×10^{-3} M selenate was carried out in a sterilized 15 liter carboy using a 500 ml inoculum from the smaller flask. Pressurized air was filtered through cotton and bubbled through sintered glass in order to mix and aerate the large media. Cells were harvested in log phase growth with a Sharples Super Centrifuge and washed several times with physiological saline before freezing at -20^{o}.

Isolation of tRNA

Isolation of tRNA from intact Escherichia coli cells was a combination of methods from von Ehrenstein (23) and Carnegie (11). The procedure was based on the fact that phenol denatures and precipitates unwanted protein, but the RNA remains in solution. All operations were performed at 4^{o} with heat treated glassware in order to prevent bacterial and nuclease activity.

Bacterial paste was shaken and evenly dispersed in polyethylene

bottles with an equal quantity of standard TM (Tris-magnesium) buffer: 0.01 M Tris, 0.005 M $MgCl_2$, pH 7.0. An equal volume of phenol and SDS (sodium dodecyl sulfate) to 1% were added and the mixture was vigorously shaken for one hour, then centrifuged at 20,000 x g in the refrigerated Servall centrifuge for 45 minutes. The phenol was redistilled and saturated with standard TM buffer at 4^{o}, and the SDS was purified by repeated ethanol precipitation before a 20% solution was made. Of the three phases that result from the centrifugation, the upper aqueous phase containing the nucleic acids was carefully pipetted off. The middle phase was the phenol and the bottom phase contained the precipitated proteins and other debris. The same volume of phenol and buffer were added again, shaken, centrifuged, and then the aqueous layer was combined with the first aqueous layer. The other layers were then discarded. The nucleic acids were precipitated by alcohol precipitation: 0.1 volume of 20% potassium acetate, pH 5.0 and 2 volumes of -20^{o}, 95% ethanol. The preparation was allowed to remain at this stage overnight or as long as 2-3 days. The supernatant was decanted and the precipitate collected by centrifugation for 10 minutes at 10,000 x g. The supernatant was discarded and the tubes inverted to drain the ethanol in order to facilitate the sodium chloride extraction. The precipitate was dispersed in cold 1.0 M NaCl (of about the same volume as the volume of cells used) using a spatula and a magnetic stirrer with a small spin bar for about one hour. The suspension was centrifuged at 20,000 x g for 30 minutes. The supernatant containing the soluble RNA was saved, and the precipitate was reextracted with a half volume (as above) of 1.0 M NaCl, centrifuged, and the

supernatants pooled. The crude soluble (transfer) RNA was alcohol precipitated (as above) from the sodium chloride extracts and collected by centrifugation as before.

The RNA precipitate was dissolved in 0.5 M Tris, pH 8.8 and incubated for one hour at 37^{o} to hydrolyze attached amino acids. The RNA was again alcohol precipitated and collected by centrifugation. The precipitate at this point is reported to contain about 60% tRNA, polysaccharides, nucleoside triphosphates, DNA and some RNA that is inactive as an amino acid acceptor (23).

The final purification of the tRNA was by DEAE-cellulose column chromatography. The 100 g of DEAE-cellulose (diethylaminoethyl ether cellulose, Matheson, Coleman and Bell, Norwood, Ohio) was washed in a liter of N NaOH, a liter of N HCl, titrated to pH 7.5 with NaOH then washed with 0.1 M Tris, 0.005 M $MgCl_2$, pH 7.5. A 2.5 x 20 cm column was poured, tightly packed, and eluted with about a liter of the 0.1 M TM buffer at 4^{o}. This size column was used for the RNA purification from about 40 mls of cells. Columns were only used once.

The last RNA ethanol precipitate dissolved in a small amount of the 0.1 M TM buffer was applied to the column with a flow rate of 0.5-1.0 mls per minute. Four ml fractions were collected throughout the elution. The RNA on the column was washed with about 100 mls of 0.2 M NaCl, 0.1 M TM buffer then eluted with 200-300 mls of 2.0 M NaCl, 0.1 M TM. The optical densities at 260 and 280 mu checked with the Beckman-Gilford instrument, and the peak tubes were pooled and alcohol precipitated. The tRNA was dissolved in and dialyzed against 0.01 M TM, and frozen in convenient aliquots of 1-2 mls with an RNA concentration

of 2 mg/ml. Some working figures for RNA from von Ehrenstein (23) were: for a 1% solution, $E_{260\ mu,\ 1\ cm} = 240$, or 24 OD's/mg RNA; good RNA should have a 260/280 mu ratio close to 2.0. Protein contamination checked by the 260/280 mu ratio and by the Lowry method was always found to be less than 1%.

Aminoacyl Synthetase Preparation

Aminoacyl synthetase was a crude preparation containing many enzymes, but it was essentially free of tRNA. Twenty mls of freshly harvested cells were frozen in an Evans (French) press using solid carbon dioxide. The cells were broken under 12,000 pounds of pressure using a hydraulic Carver laboratory press on the Evans cells. All steps were again conducted at 4^{o}. The lysed cells were extracted with an equal volume of the 0.1 M TM, pH 7.5 buffer containing 0.006 M 2-mercaptoethanol or reduced glutathione. The mixture was centrifuged at 20,000 x g for 45 minutes and the pellet was discarded. The supernatant was centrifuged at 100,000 x g in a Beckman Model L2-65 Ultracentrifuge for 2.5 hours. The top two-thirds of the supernatant was removed and placed on a 1.5 x 15 cm DEAE column that had been equilibrated with pH 7.5, 0.01 M TM containing 0.006 M 2-mercaptoethanol or GSH at 4^{o}. The sample was washed with 50 mls of the buffer and eluted with 100 mls of 0.35 M NaCl in the TM buffer. Four ml fractions were collected and the optical densities at 280 mu were read. The peak tubes were dialyzed against pH 7.5, 0.01 M TM buffer containing 0.006 M 2-mercaptoethanol or GSH and 0.06 M KCl. After dialysis for overnight, the synthetase was frozen in convenient small aliquots at -20^{o}.

Standard Incubation Assays with Membrane Filters

The standard incubation assay was an in vitro assay to determine the amount of amino acid attached to their respective tRNA. No ribosomal material was added, so that the end point was the radioactive amino acid-tRNA complex.

Each assay contained the following in a 0.5 ml reaction mixture: 200 ug tRNA in 0.1 M TM, pH 7.5 buffer; ATP, 5.0 u moles; $MgCl_2$, 5.0 u moles; KCl, 5.0 u moles; 0.2-1.0 uCi of radioactive amino acid; 100 ug aminoacyl synthetase; and double distilled water to make 0.5 mls. The above items were added in the following order: tRNA; stock solution of ATP, $MgCl_2$, KCl; radioactive amino acid; water; and finally the synthetase. The ATP and GSH solutions must be carefully adjusted to pH because these substances are quite acidic. The reaction tubes were heat treated prior to the reaction and sterile conditions were maintained throughout the assay. The tubes were incubated at 37^{o} for 30 minutes which allowed complete saturation of the tRNA available. The radioactive aminoacylated tRNA was precipitated with cold 10% TCA and iced for 10 minutes, after which the acid insoluble material was quantitatively transferred and suction filtered onto membrane filters (27 MM, type B-6, Bac-T-Flex membrane filters, Carl Schleicher & Schuell Co., Germany). The filters were washed with 10% TCA to remove unbound amino acids, then air dried and counted as previously described.

Reverse Phase Chromatography of Aminoacylated tRNA

This method was used to determine whether the same tRNA species were accepting both ^{14}C-methionine and ^{75}Se-selenomethionine by comparing the reverse phase chromatograms of the double labeled aminoacylated tRNA.

The reverse phase column support described by Weiss and Kelmers (122) was prepared by Dr. Peter Scott (92). The support was acid washed, silanized Chromosorb W (Varian Aerograph, Walnut Creek, California) coated with Freon 214 (Dupont) and methyl tricaprylyl ammonium chloride (Aliquat 336, General Mills). It was necessary to degas the support under vacuum before pouring the 0.7 x 55 cm column to prevent air bubbles from forming in the column.

The aminoacylation reaction was carried out separately for equal molar quantities of selenomethionine and methionine. Each reaction contained the same amount of material when comparisons were made. The amount of materials used varied between experiments with each reaction tube containing the following: 100-700 ug tRNA, 1-10 uCi and 20-200 *mu* moles of selenomethionine or methionine, 100 ug of aminoacyl synthetase, plus ATP, $MgCl_2$, KCl and water as previously described. After the incubations were completed, the separate reactions were pooled and an equal volume of 2M sodium acetate (NaAc), 0.1 M NaCl, pH 4.50 was added. Twice, equal volumes of phenol (redistilled) saturated with 0.05 M NaAc, 0.1 M NaCl, pH 4.5 were used to treat the reaction mixture in order to free the aminoacylated tRNA from the protein. At this pH the hydrolysis of attached amino acids is mimimal. With

each phenol extraction and centrifugation the upper aqueous phase containing the unbound labeled amino acids, aminoacylated tRNA, and non-aminoacylated tRNA was saved; whereas the phenol layers and the denatured protein between the phases were discarded. The phenol in the aqueous phase was removed by several repeated extractions with ethyl ether. The nucleic acids were ethanol precipitated as usual, then collected after centrifugation at 20,000 x g for 20 minutes. This precipitation step was repeated again in order to reduce the amount of radioactivity and optical density at 260 mu at the breakthrough peak early in the chromatogram.

The precipitate was dissolved in 2-3 mls of starting buffer and applied to the column which had been equilibrated with this buffer. The column was run at room temperature with a linear gradient of 100 mls each of 0.1 M NaCl, 0.05 M NaAc, 0.005 M $MgCl_2$, pH 4.5 to 1.0 M NaCl, 0.05 M NaAc, 0.005 M $MgCl_2$, pH 4.5. A Buchler polystaltic pump was used to insure an even flow rate of 0.5 mls per minute. After the gradient had completed, the column was washed with another 100 mls of the 1.0 M NaCl buffer. The gradient was checked by a conductimetric method using a diluted aliquot from every tenth fraction. Fractions of one ml were collected in Autogamma tubes and counted for ^{75}Se activity, read at 260 mu on the Beckman-Gilford instrument for nucleic acids, and then the whole fraction counted in the Triton X-toluene scintillation fluid for ^{14}C activity.

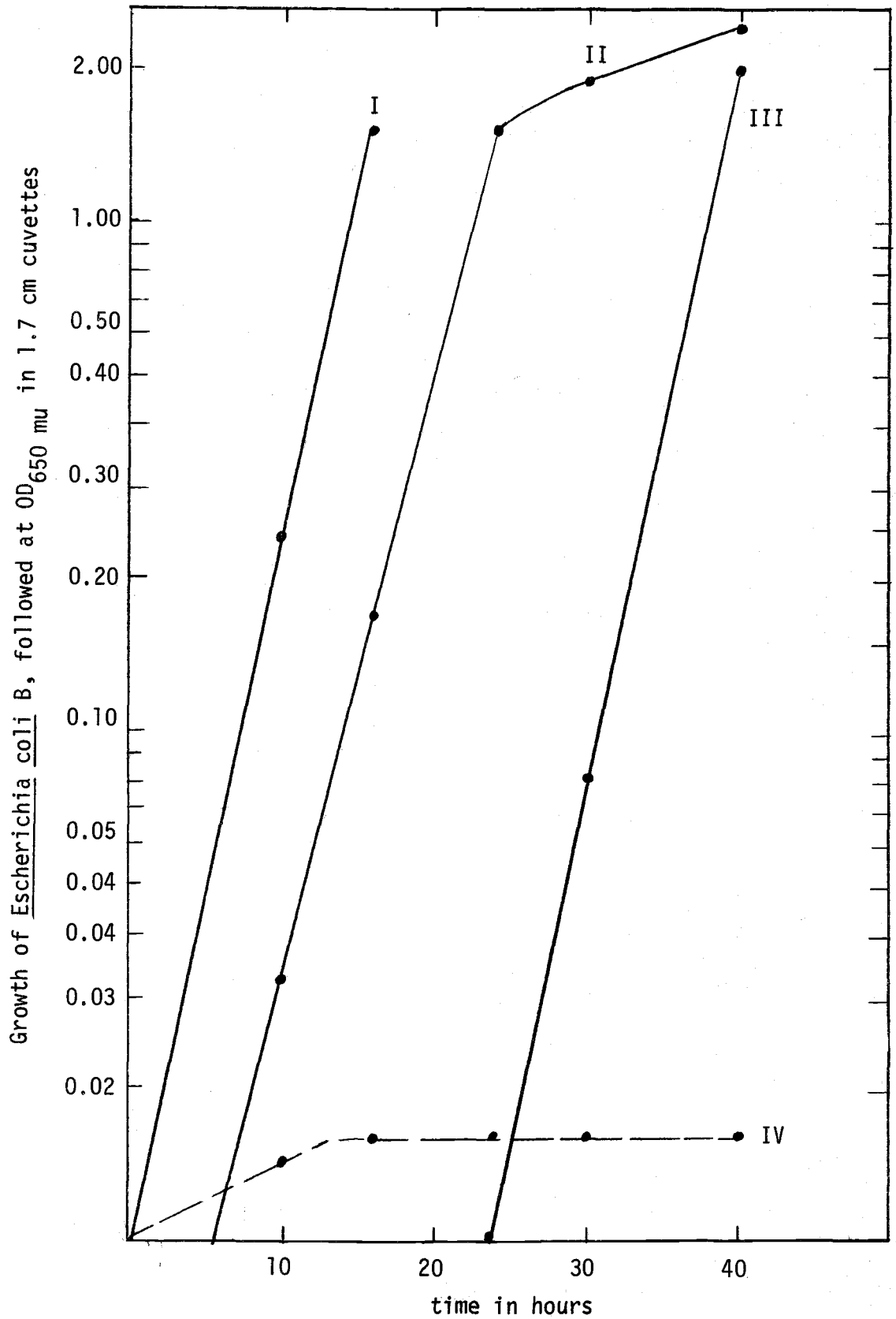
RESULTS AND DISCUSSION

Growth and Selenium Adaptation of Escherichia coli

Escherichia coli B were grown on consecutively increasing amounts of selenate in a low, 0.2 mM, sulfate media. Growth was exponential, but with increasing selenate there were increasing lag phases before exponential growth started, Figure 17. Very little growth occurred without sulfate or with selenite. An inoculum was taken from flask III, Figure 17 and grown in other flasks containing 0.8-2.4 mM selenate. There were decreased growth rates with these increasing selenate concentrations. In these experiments when the $OD_{650mu,cm}$ reached 0.5-0.6 the solutions turned red due to colloidal selenium, and growth could no longer be followed at 650 mu. Selenium adapted cells were harvested from 7.5 liters of solution containing 4 mM selenate. This culture was started from one grown on 2.4 mM selenate medium. Non-selenium adapted or normal Escherichia coli were grown on 0.8 mM sulfate.

Figure 17. Growth of normal and selenium adapted Escherichia coli B in salt and glucose media.

I. Normal growth in 0.2 mM sulfate.
II. Growth in 0.04 mM selenate, 0.2 mM sulfate.
III. Growth in 0.4 mM selenate, 0.2 mM sulfate.
IV. Growth with no sulfate or selenate; or growth with 0.04 mM selenate, but no sulfate.


Growth of Escherichia coli B, followed at OD650 mu in 1.7 cm cuvettes
2.00
1.00
0.50
0.40
0.30
0.20
0.10
0.05
0.04
0.04
0.03
0.02
I
II
III
IV
10
20
30
40
time in hours

Aminoacylated tRNA Assays: Plating on Membrane Filters

Aminoacyl synthetase (AAS) and tRNA were partially purified from both the selenium adapted (+Se) and normal (-Se) Escherichia coli. Figures 18 and 19 show the tRNA and synthetase purifications from normal E. coli. These purifications did not appear to be different for the material from selenium adapted cells.

Plating experiments were performed to determine optimum reaction conditions. Both synthetase and tRNA were necessary for binding methionine and selenomethionine, Table 14. Figures 20 and 21 are from another experiment that demonstrates that both synthetase and tRNA were necessary for binding selenomethionine. An excess of synthetase inhibited binding of selenomethionine probably due to nuclease activity commonly found in preparations of this type. In these plating assays the baseline for incubations without enzyme or tRNA or with heated (95^{o}, 5 minutes) enzyme and tRNA was also quite high. These baselines for ^{14}C-methionine or ^{75}Se-selenomethionine usually ran from 150-300 cpm or about 25-50% of the gross cpm, Table 14, footnote. Carnegie has also demonstrated baseline reactions of 10-200% of the enzymatic aminoacylation reactions for various amino acids with tRNA (11).

Baselines using ^{14}C-leucine or ^{14}C-lysine contained only 20-30 cpm. Precipitation of the assays with 0.25 M lead acetate instead of 10% TCA worked very well, but did not alleviate the high baseline problem. A time course experiment indicated that selenomethionine and methionine were instantaneously bound and only slowly increased in 0-30 minute incubations. Carnegie has also demonstrated very rapid binding of amino acids in his system (11).

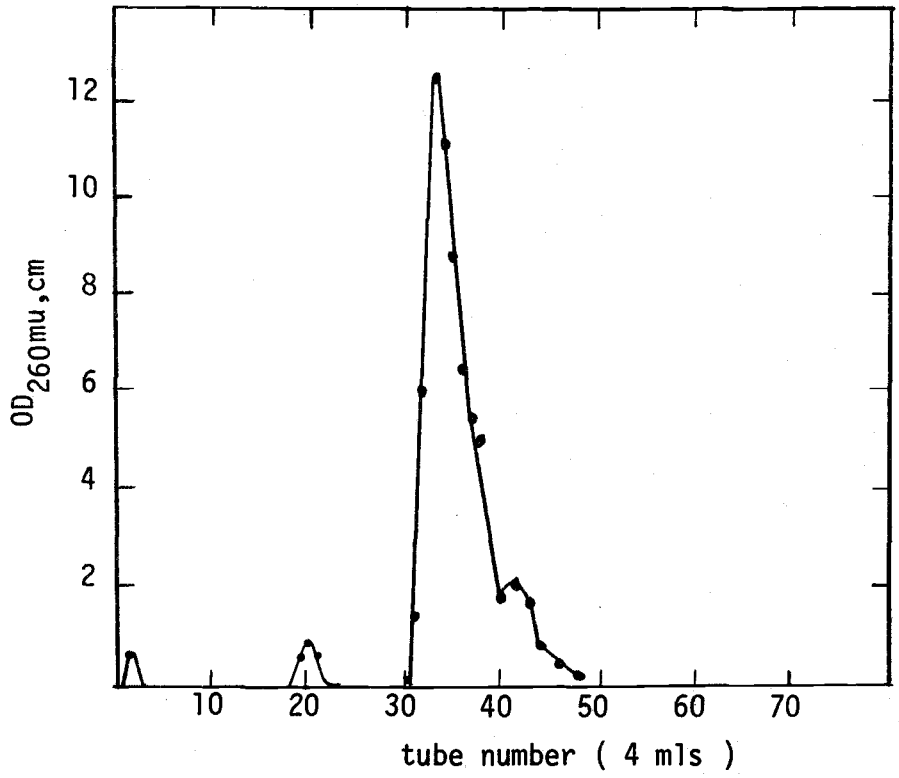


Figure 18. Purification of tRNA from Escherichia coli B on a column of DEAE-cellulose. The tRNA was washed on the 2.5 x 20 cm column with 0.2 M NaCl in 0.1 M Tris,0.005 M $MgCl_2$ (TM) for the first 10 tubes, then the NaCl concentration was increased to 2.0 M to elute the tRNA. Peak tubes 30-45 were pooled for use in subsequent amino-acylation reactions.

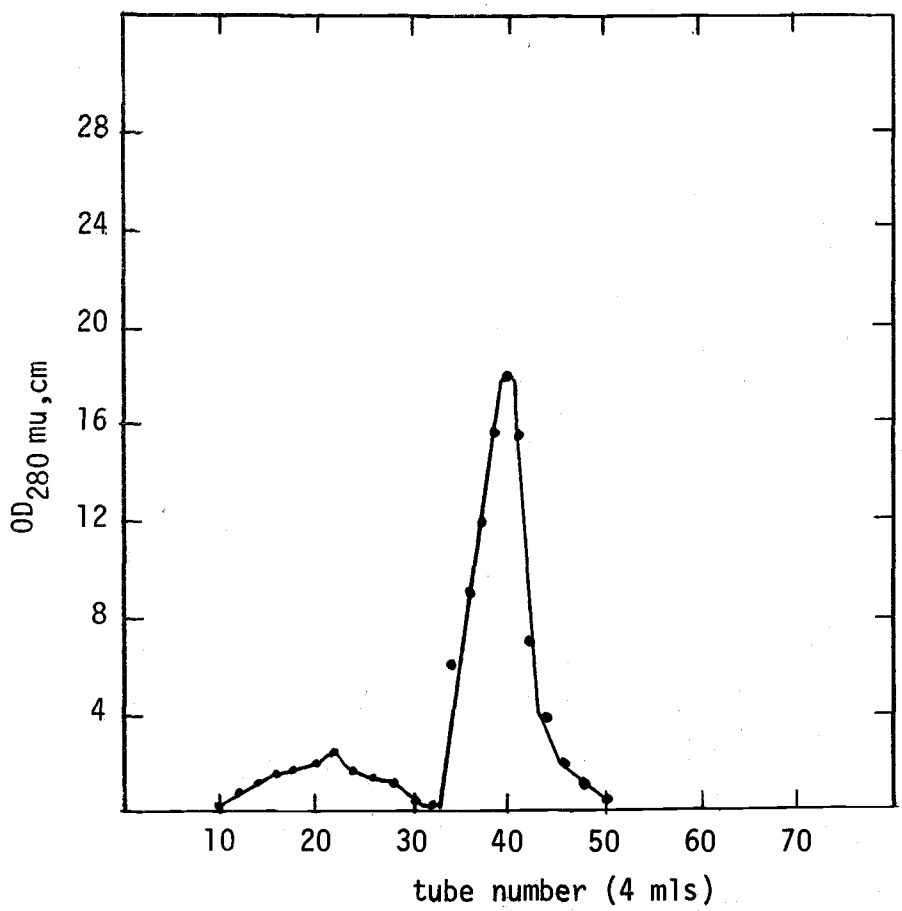


Figure 19. Purification of aminoacyl synthetase from Escherichia coli B on a column of DEAE-cellulose. The 1.5 x 15 cm column was equilibrated with pH 7.5, 0.01 M TM buffer containing 0.006 M reduced glutathione. The sample was washed with 50 mls of the TM buffer, then eluted with 0.35 M NaCl in the TM buffer. Peak tubes 32-45 were then pooled for use in subsequent aminoacylation reactions.

Table 14. Aminoacyl synthetase (AAS) assay[a] with selenium adapted (+Se) tRNA.

description	uu moles of Semet or met incubated	uu moles incorporated		incorporated
		^{75}Semet	^{14}C-met	Semet/met
+Se AAS, dialyzed	48000	108	52	2.1
-Se AAS, dialyzed	48000	136	37	3.7
+Se AAS, not dialyzed	680	5		-
	1360	22		-
	48000	195	54	3.6
	96000		96	-
+Se AAS, no RNA[b]	48000	0	0	-
no enzyme[c]	48000	(0)	(0)	-

[a]Each tube contained 5 u moles of ATP, KCl and $MgCl_2$, 100 ug AAS with GSH, 120 ug tRNA, 0.5 uCi ^{75}Se-selenomethionine (^{75}Semet) or methionine(methyl-^{14}C) (^{14}C-met). Incubations were 15 min.

[b]This assay was used as the background control: ^{75}Semet, 200 cpm or 70 uu moles bound; ^{14}C-met, 150 cpm or 13 uu moles bound. These values were substracted from the incubation results before being listed in the above table.

[c]Binding of the reactions containing no enzyme was about half of the reactions containing no RNA. These values listed in the above table are therefore less than zero.

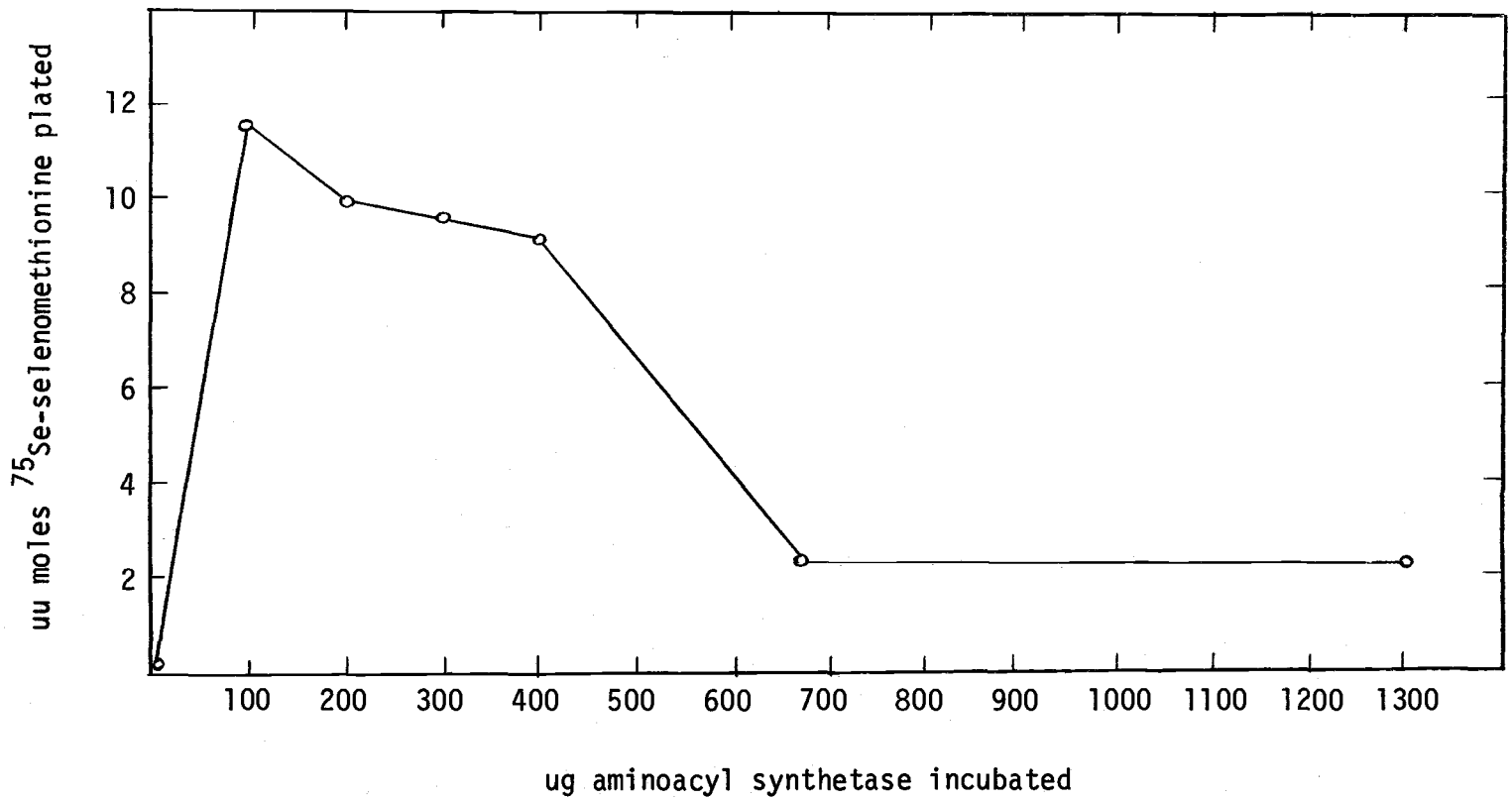


Figure 20. Transfer RNA aminoacylation of selenomethionine with increasing amounts of aminoacyl synthetase assayed by plating on membrane filters. The incubation contained the standard assay materials and a total of 680 uu moles of selenomethionine and 150 ug tRNA.

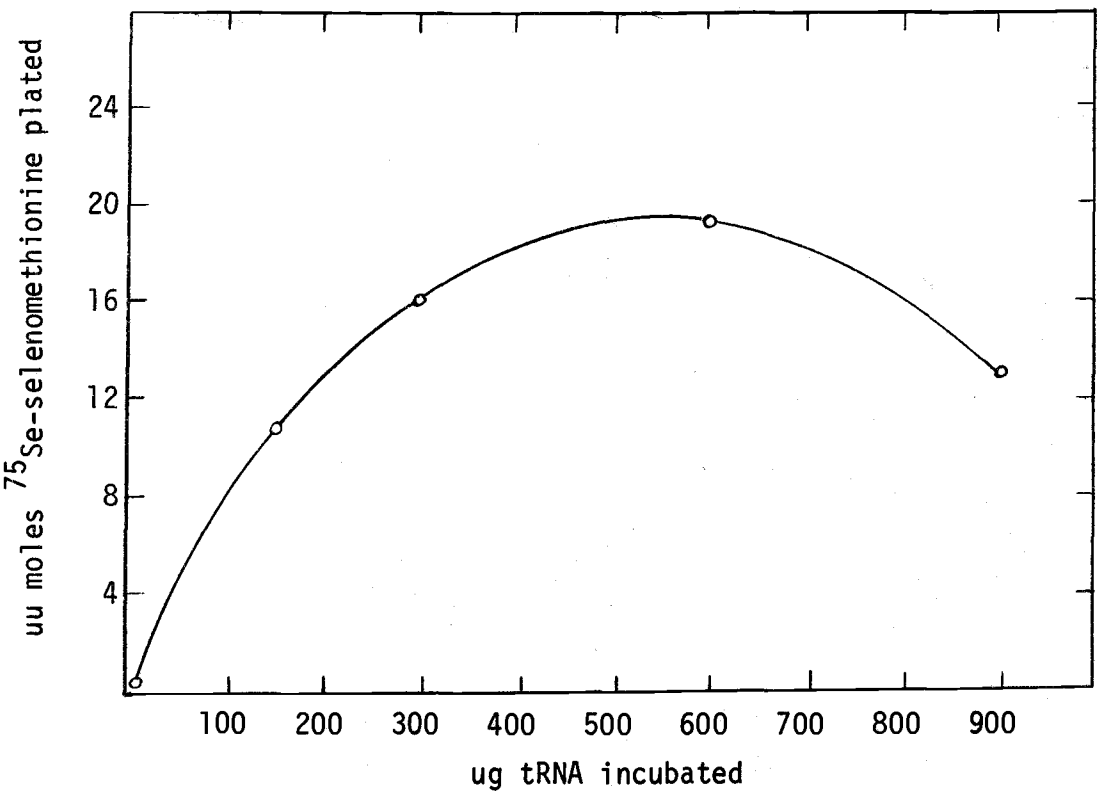


Figure 21. Aminoacylation of tRNA with selenomethionine with increasing amounts of tRNA. The incubation contained the standard assay materials including 100 ug of synthetase and 680 uu moles of selenomethionine.

Binding of both methionines were compared at different concentrations and with increasing concentrations of each other, Figures 22 and 23. The amount of methionine bound increased sharply and then plateaued when incubated with increased amounts of methionine. The selenomethionine assays showed no such saturation kinetics when incubated with increasing amounts of selenomethionine. The amount of bound methionine was maintained at a constant level when increasing amounts of selenomethionine was incubated with it. However, methionine appeared to increase the binding of selenomethionine.

All of the selenium adapted synthetase and tRNA activities were compared to the normal synthetase and tRNA activities to determine whether there was a preference for methionine or selenomethionine in either of the systems. Selenium adapted synthetase and tRNA showed the lowest preference for selenomethionine whereas there was no trend for methionine, Table 15. In almost every case selenomethionine was bound in greater amounts than methionine, Tables 14 and 15. In one experiment, after the incubation was completed, the pH was raised to 11.0 and incubated again for 30 minutes. This technique has been reported to release bound amino acids from the tRNA (23). Methionine but not selenomethionine was reduced by this pH 11.0 post incubation. Selenomethionine was actually bound to a higher degree, Table 15.

Leukemic chicken myeloblast aminoacyl synthetase and tRNA was very active with uniformly labeled ^{14}C-amino acid mixture and also with selenomethionine and methionine. The myeloblast synthetase worked well with the selenium adapted E. coli tRNA, whereas the myeloblast tRNA worked to a lesser extent with selenium adapted E. coli synthetase,

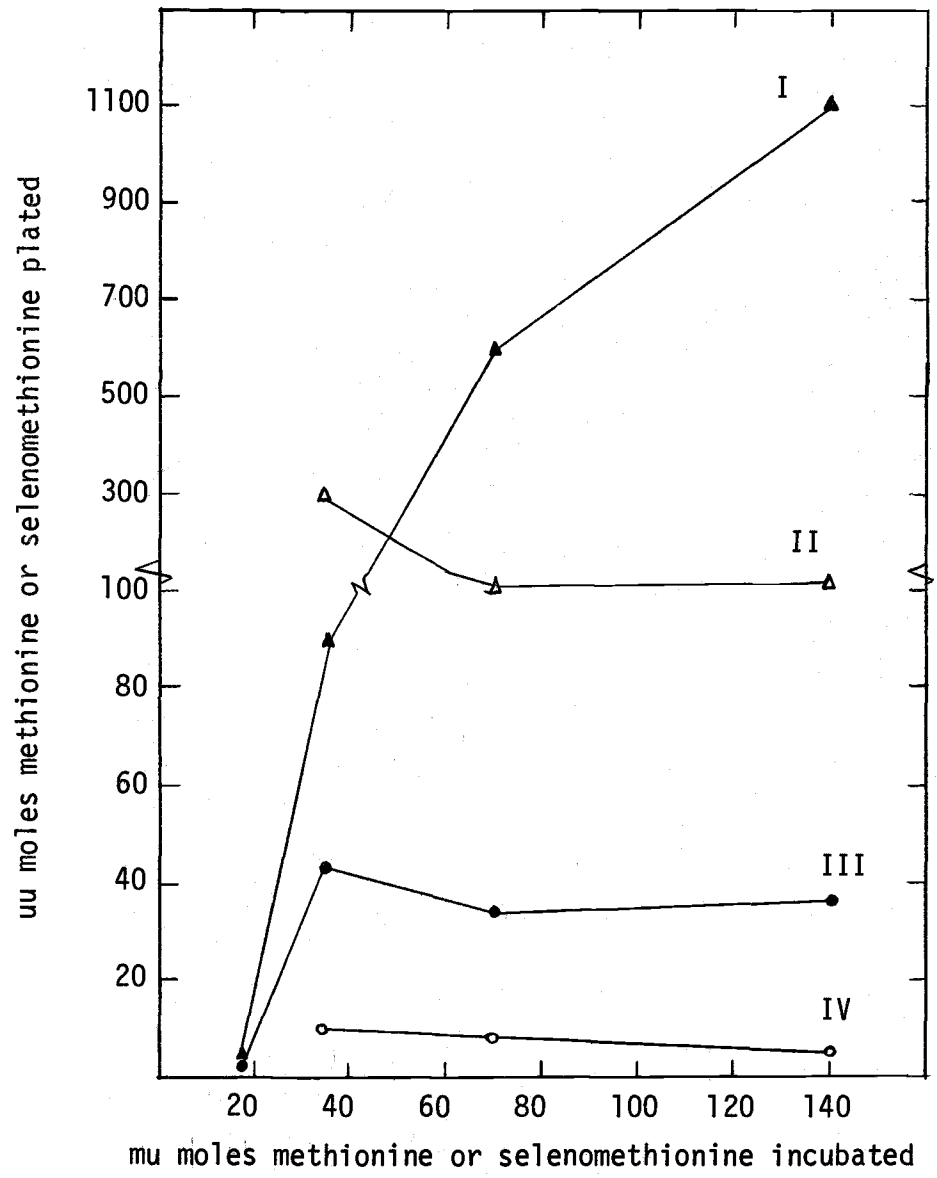


Figure 22. Aminoacylation of tRNA with methionine and selenomethionine: Effect of substrate and analog concentration.

I. Selenomethionine bound with increasing (selenomethionine).
II. Selenomethionine bound with increasing (methionine).
III. Methionine bound with increasing (methionine).
IV. Methionine bound with increasing (selenomethionine).

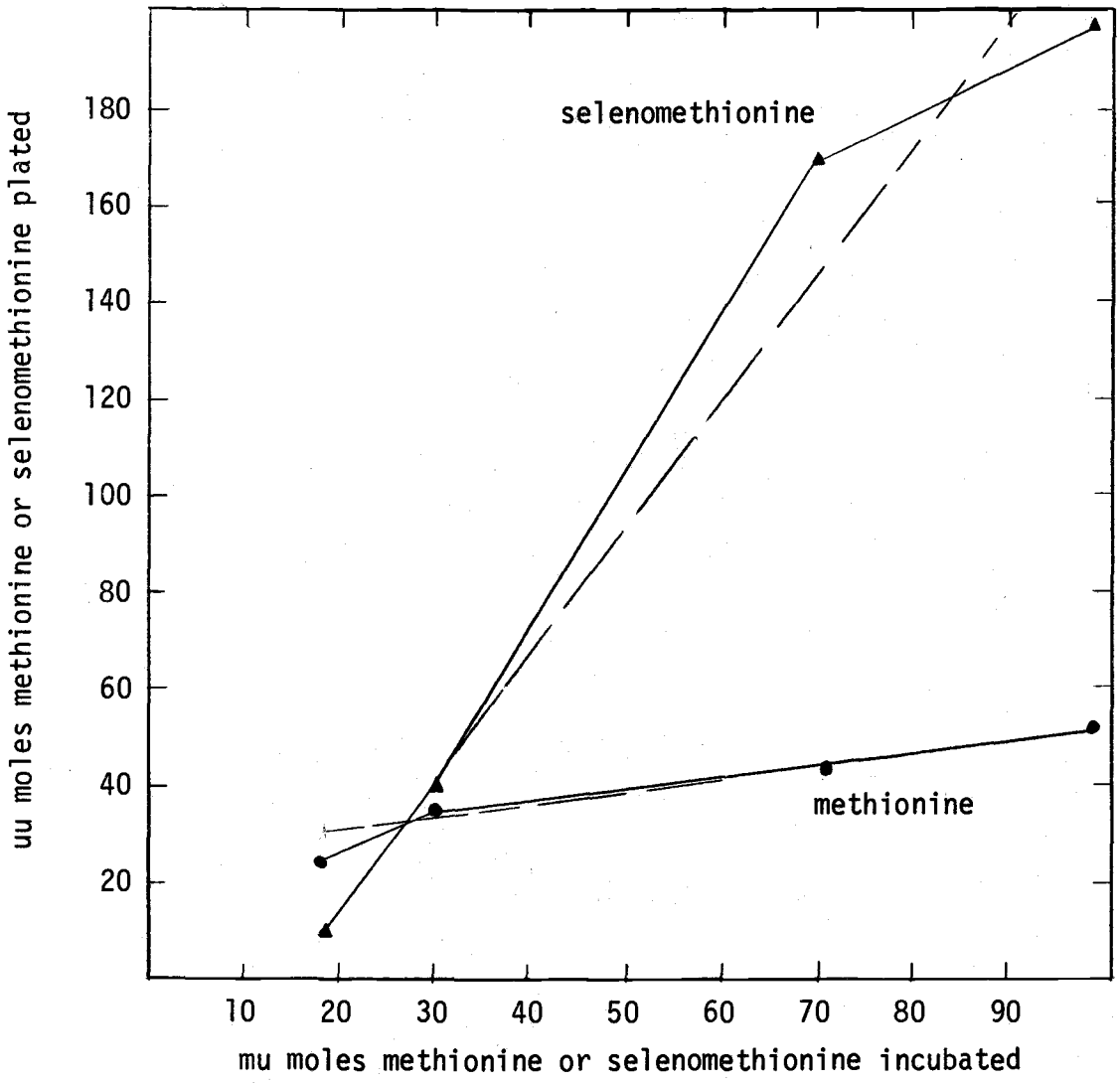


Figure 23. Aminoacylation of tRNA with methionine and selenomethionine: effect of substrate concentration repeated. Dashed lines are averaged lines for the points; solid lines are point connections.

Table 15. Specificity of aminoacyl synthetase and tRNA from normal and selenium adapted Escherichia coli B.

aminoacyl synthetase	tRNA[a]	uu moles plated[b]	
		^{75}Semet	^{14}C-met
+Se	+Se	4.4	3.6
+Se	-Se	0.8	7.7
-Se	+Se	41.2	4.4
-Se	-Se	27.4	1.6
+Se	+Se (heated 95°)	0.0	0.0
+Se	+Se (pH 11.0)	41.0	1.0

[a]+Se indicates material from selenium adapted cells, -Se indicates material from normal Escherichia coli B. 100 ug of synthetase and 120 ug of tRNA were used for each assay.

[b]17.6 mu moles of each methionine incubated for each respective assay.

Table 16. Myeloblast (MB)[a] aminoacyl synthetase and tRNA assay for methionine and selenomethionine accepting activities.

aminoacyl synthetase	tRNA[b]	^{14}C amino acid mixture plated, cpm	uu moles plated	
			^{75}Semet	^{14}C-met
MB	MB	6,000	-	-
MB	+Se	1,700	-	-
+Se	MB	610	-	-
MB	MB	--	81.0	19.7

[a]Myeloblast material was received from J. W. Carnegie (12). ^{14}C amino acid mixture, 0.25 uCi/tube also from Carnegie.

[b]MB denotes myeloblast material, +Se denotes material from selenium adapted E. coli.

Table 16. The myeloblast system again demonstrated that selenomethionine was bound to a greater extent (4X) than methionine. The selenium adapted synthetase may not have been interchangeable in the myeloblast system or it may have been due to the low activity of the preparation that low yields were obtained with the myeloblast tRNA.

It has been noted by some investigators that selenium adapted cells either produce less enzymes or else they have been inactivated by the presence of the high selenium concentrations during growth or during cell lysis processes (15, 38). This possibility was further examined by again growing and obtaining the aminoacyl synthetase fraction from selenium adapted and normally grown cells. Special attention was given to thoroughly washing the cells, but no substantial increase in activity was found in these preparations, Table 17. From these new preparations the +Se synthetase was almost inactive. Table 17 also demonstrates the requirement of ATP for the aminoacylation of methionine and selenomethionine with tRNA. Both methionines inhibited the binding of each other. An amino acid mixture containing 0.1 u moles of all the amino acids listed in Table 18 except the methionines, inhibited both selenomethionine and methionine aminoacylation. The inhibition of the methionines by another amino acid was interesting because each amino acid supposedly has its own set of synthetases and tRNA. This experiment was elaborated upon in Table 18, using either one or two amino acids per incubation to find which one or ones were inhibiting the methionine binding. Upon a five minute preincubation of the amino acids before the labeled methionines were added, some reduction was seen with most amino acids, but tyrosine, methionine and

Table 17. New preparations of aminoacyl synthetase from selenium adapted (+Se) and normal (-Se) Escherichia coli B.

description		uu moles incorporated		% of control	
		^{75}Semet	^{14}C-met	^{75}Semet	^{14}C-met
tRNA	synthetase[a]				
-Se	-Se	29.4	17.6	100	100
+Se	+Se	6.8	0.0	-	-
-Se	+Se	6.0	0.0	-	-
+Se	-Se	23.5	14.1	-	-
-Se	-Se, no ATP	11.3	9.0	38.4	51.0
-Se	-Se, 3X analog	15.2	4.2	51.5	23.8
-Se	-Se, 3X substrate	55.0	12.5	187	71.0
-Se	-Se, amino acid mixture[b]	14.8	1.9	50.0	10.8

[a]100 ug of both tRNA and synthetase were used in each assay.

[b]amino acids listed in Table 18, except methionine and selenomethionine.

selenomethionine were the only amino acids to completely inhibit the binding of the selenomethionine, Table 18. The ability of preincubation, but not simultaneous incubation of selenomethionine, methionine and tyrosine to completely inhibit the selenomethionine binding was probably related to the rapid binding of selenomethionine (and methionine) as seen with the time course experiment. The ability to completely saturate the system with preincubations of these certain amino acids indicated a definite binding capacity of the system.

Table 18. Effect of other amino acids on selenomethionine aminoacylation.

description[a]	net cpm	% of control
preincubation, 5 minutes		
100 mu moles arg, asp	379	42
" " " his, gly	484	54
" " " ileu, leu	596	66
" " " lys, phe	384	43
" " " ser, thr	500	56
" " " try, val	817	91
" " " ala, cys	996	110
" " " glu, pro	403	45
" " " tyr	0	0
" " " met	0	0
50 mu moles Semet	0	0
" " " met	0	0
" " " met, tyr	0	0
control, no other amino acids	900	100
no preincubation		
50 mu moles Semet	362	40
50 mu moles met	547	61
50 mu moles tyr	663	74
10 mu moles tyr	562	63

[a]Incubations were with 0.5 uCi, 1.0 mu moles L-selenomethionine-^{75}Se. All amino acids were in the L-configuration.

An experiment with E. coli tRNA and pea seedling root synthetase showed a greater binding of selenomethionine than methionine, although the plant enzyme was fairly inactive, Table 19. This experiment also indicated that methionine was bound to a lesser extent than lysine, leucine and selenomethionine with the E. coli synthetase and E. coli t RNA. During purification of the plant synthetase, five tubes on both sides of the protein peak were assayed for lysine, leucine, methionine and selenomethionine activity, Figure 24 (91). The main peak activity occurred at the same fraction for both methionines, whereas the lysine activity peak occurred earlier, and the leucine activity was diffuse.

Table 19. Escherichia coli and pea seedling root[a] aminoacyl synthetase with E. coli tRNA.

amino acid, amount incubated	total uu moles bound	
	E. coli synthetase	pea root synthetase
^{14}C-lysine, 0.4 mu moles/0.1 uCi	50	0.4
^{14}C-leucine, 0.3 mu moles/0.05 uCi	67	0.3
^{14}C-methionine, 4.4 mu moles/0.25 uCi	33	4.1
^{75}Se-selenomethionine, 13.8 mu moles/0.25 uCi	1060	113
^{14}C-methionine, 4.4 mu moles/0.25 uCi		
" " " (-enzyme)	6.4	--
" " " (-tRNA)	4.4	--

[a]Pea seedling root (Alaskan variety) material was received from Dr. P. C. Scott (91).

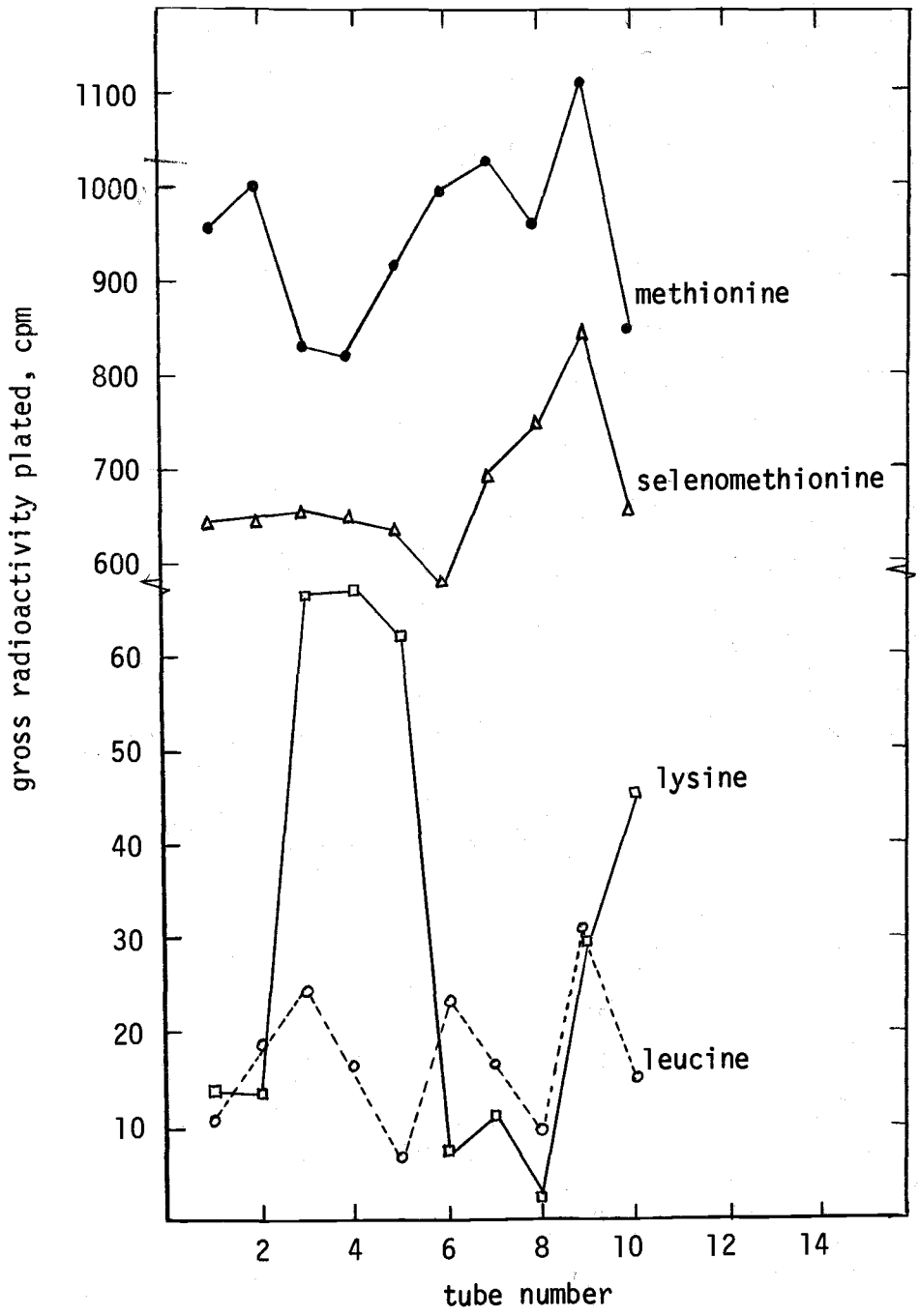


Figure 24. Protein peak tubes from a DEAE-cellulose column assayed for distribution of plant aminoacyl synthetase activities toward lysine, leucine, methionine, and selenomethionine (91).

Aminoacylated tRNA Assays: Reverse Phase Columns.

Selenomethionine and methionine were separately incubated in the cell free system and then assayed on the reverse phase column in order to determine whether they were both attaching to the same tRNA species. The initial assay used 208 ug (-Se) E. coli tRNA and 100 ug (-Se) E. coli aminoacyl synthetase per incubation. This was a triple labeled experiment with separate incubations of 10 uCi, 0.186 u mole methionine(methyl-^{14}C); 20 uCi, 0.0195 u mole ^{3}H-isoleucine; and 7.7 uCi, 0.0136 u mole ^{75}Se-selenomethionine. Selenium-75 counting efficiency in the gamma counter was 49.0% and its crossover in liquid scintillation counting was 17.3% in the tritium channel and 23.3% in the carbon-14 channel. Tritium crossover in the carbon-14 channel was 1.0% and the carbon crossover in the tritium channel was 11.4%. The column yielded two selenomethionine peaks containing 28.7 and 18.0 uu moles respectfully (46.7 uu moles total). Whereas only one peak was found for methionine, 52.5 uu moles; and only one peak was found for isoleucine, 19.1 uu moles. The methionine peak exactly overlapped the first selenomethionine peak and the isoleucine peak occurred one tube ahead of the second selenomethionine peak, Figure 25. There appeared to be no random binding of the tRNA by the selenomethionine, leucine, or methionine as evidenced by the low background counting rates in the rest of the chromatogram.

The second reverse phase column contained material incubated separately with each incubation containing 420 ug (-Se) tRNA, 100 ug (-Se) synthetase, and equal molar concentrations of selenomethionine

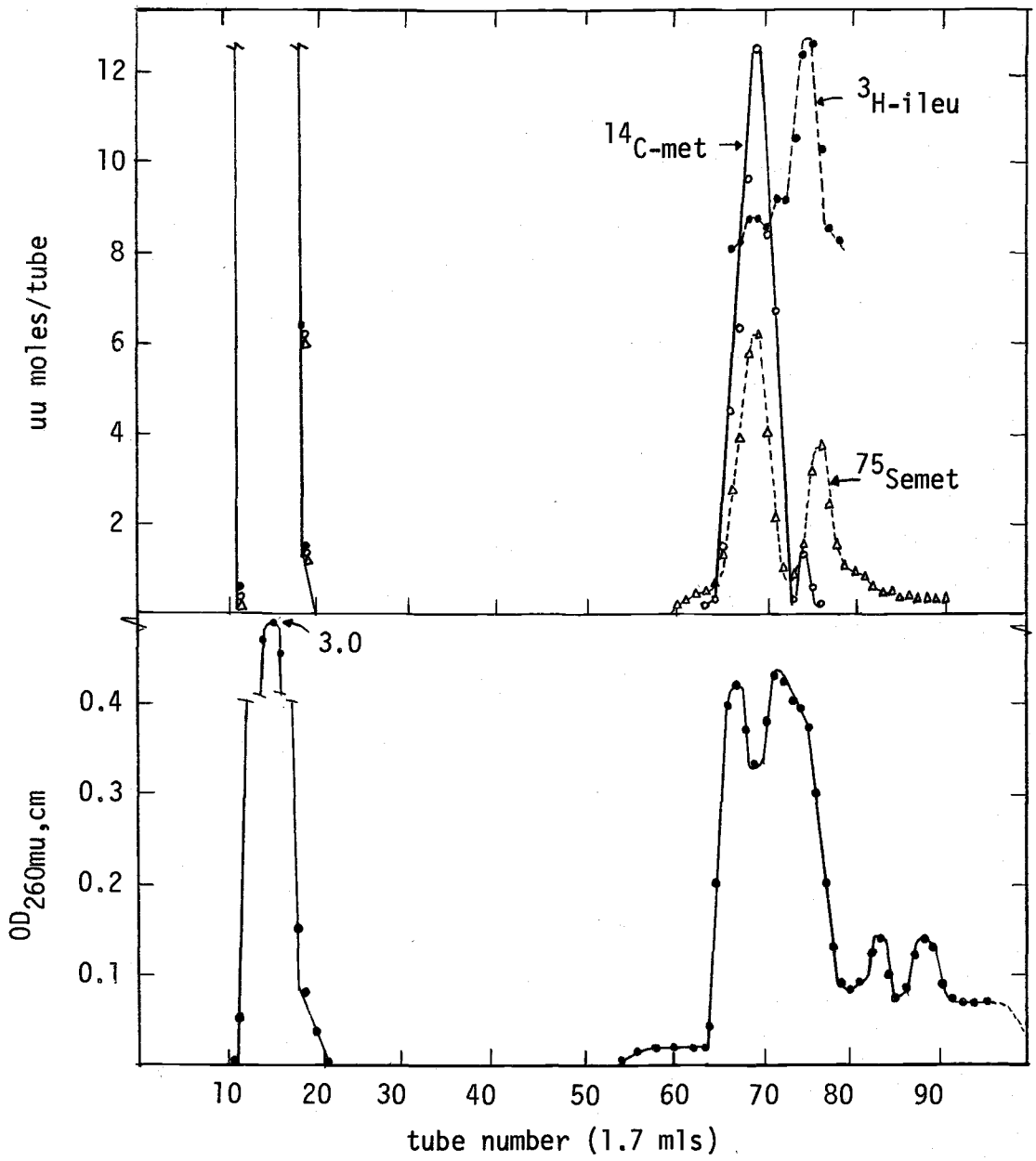


Figure 25. Reverse phase column assay of selenomethionine-, methionine-, and isoleucine-aminoacylated tRNA species. The initial peak contains unbound aminoacids and low molecular weight nucleic acids. The gradient is from 1-10X as previously described in the experimental section.

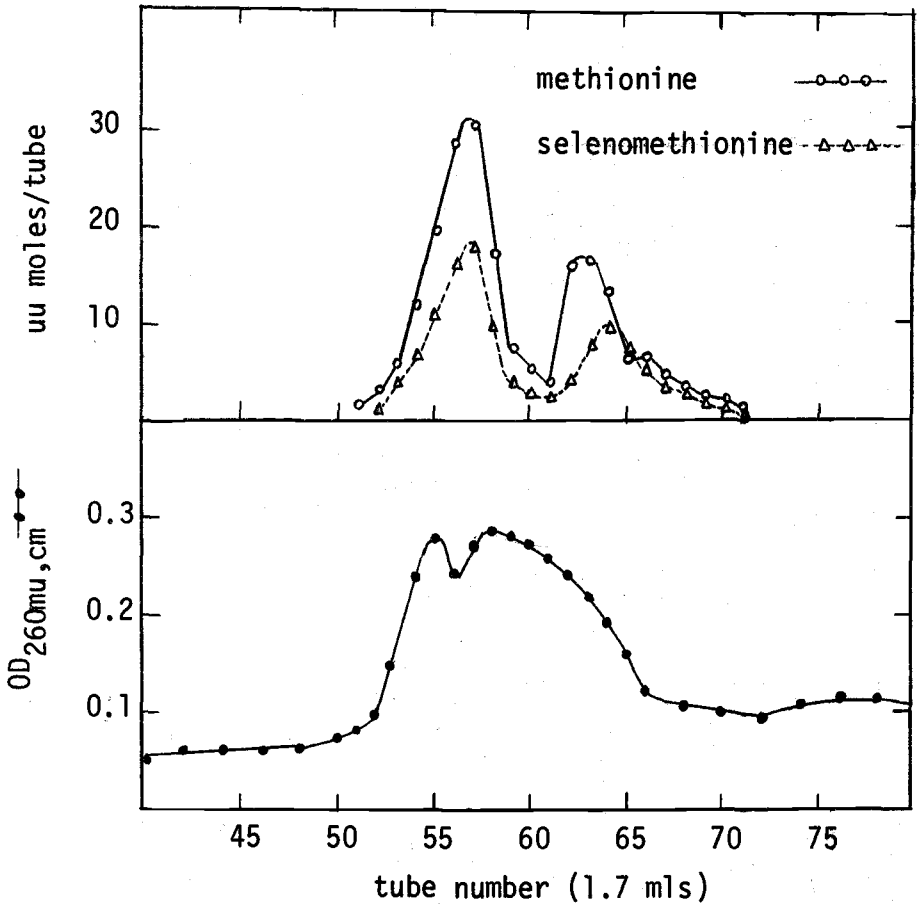


Figure 26. Reverse phase column assay of selenomethionine-, methionine-aminoacylated tRNA species.

and methionine, 10 uCi, 186 mu moles. This column separated two peaks for both selenomethionine and methionine. The peaks contained 69.3 and 43.5 uu moles selenomethionine,and 126 and 74 uu moles methionine for the first and second peaks respectfully, Figure 26. The first peaks coincided, but the second selenomethionine and methionine peaks were separated by one fraction, Figure 26. These results agree well with the literature in that one large tRNA peak followed by one or two smaller peaks have been observed for methionine accepting activity (29, 71, 96, 122, 129).

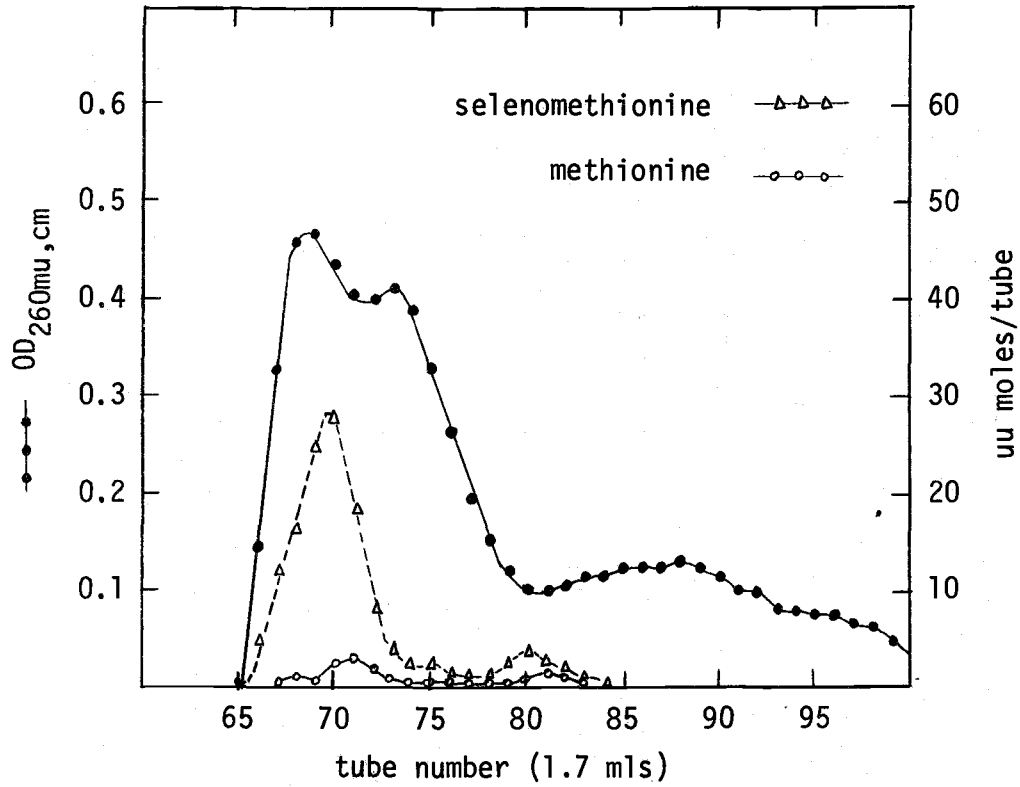


Figure 27. Reverse phase column assay for selenomethionine inhibition of methionine. The reaction was incubated for 10 minutes with labeled selenomethionine and then for 20 minutes longer with labeled methionine.

Another experiment using the same quantities of material used in the previous column was performed. In order to show analog inhibition with this column assay procedure, the selenomethionine was incubated for 10 minutes with the synthetase and tRNA, then the methionine was added and the reaction was allowed to proceed for 20 minutes longer. This column yielded two peaks for selenomethionine, 127 and 21 uu moles; and two peaks for methionine, 13 and 4.3 uu moles; Figure 27. Selenomethionine therefore inhibited the binding of methionine about 90% under these conditions. A similar column demonstrated the inhibition of selenomethionine aminoacylation by methionine.

Several preparations of selenium adapted aminoacyl synthetase

gave low aminoacylation of the methionines when assayed by the reverse phase column. The selenium adapted (+Se) E. coli tRNA (208 ug) and unadapted E. coli aminoacyl synthetase (100 ug),when incubated separately with selenomethionine and methionine, gave two peaks for selenomethionine, 122 and 53 uu moles; and two peaks for methionine, 86 and 40 uu moles; with the two peaks corresponding in position, Figure 28.

The reaction was shown to be enzymatic when tRNA showed no selenomethionine or methionine attachment on the reverse phase column when incubated with heat treated (3 minutes, 90°) synthetase.

Table 20. Amounts of amino acids bound per mg tRNA from different experiments.

data from: (assay method)	mu moles amino acid/mg tRNA			synthetase	tRNA
	Semet	met	ileu		
Table 14 (plating)	2.03	0.74	--	+Se	+Se
Figure 25 (column)	0.225	0.265	0.092	-Se	-Se
Figure 26 (column)	0.267	0.357	--	-Se	-Se
Figure 28 (column)	0.830	0.600	--	-Se	+Se
von Ehrenstein (23) (plating)	--	0.92	0.95	-Se	-Se

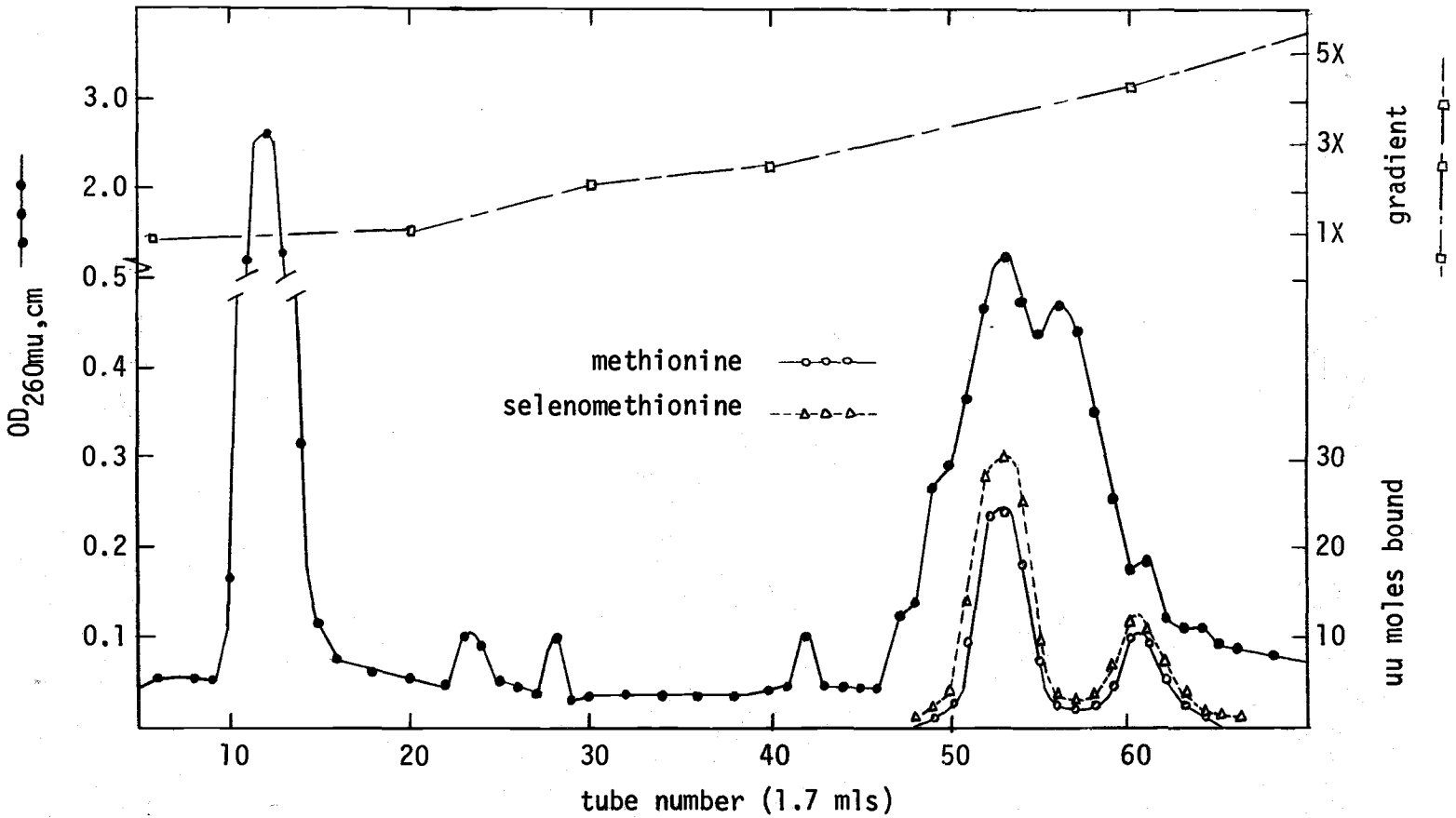


Figure 28. Reverse phase column assay of selenomethionine and methionine aminoacylation using tRNA from selenium adapted Escherichia coli.

Table 20 is a summary of experiments assayed by either plating on membrane filters or assaying on reverse phase columns. Data from von Ehrenstein (23) for methionine agreed well for plating experiments, but the same system used for the reverse phase column assay gave lower binding. Transfer RNA from selenium adapted E. coli appeared to be the most active or have a higher specific activity when assayed on the column, but still gave slightly lower binding than the von Ehrenstein data. Selenomethionine gave considerably more binding than methionine with the plating experiments, but the two methionines were bound to about the same degree in the column assay.

SUMMARY AND CONCLUSIONS

Although Penner had previously indicated that the label from ^{75}Se-selenomethionine was associated with hemoglobin when injected into human patients, his work was not quantitated or compared to methionine (82). In this thesis rates of selenomethionine and methionine incorporation into rabbit reticulocyte hemoglobin was not significantly different, although selenomethionine activity was usually somewhat higher. Therefore there was no interruption of reticulocyte protein synthesis by selenomethionine as has been demonstrated for other analog inhibitors (85). There were no differences in α or β chain specific activities of the globin. Both methionine and selenomethionine showed reduced activities when ferrous iron and other amino acids were left out of the incubation mixture. The K_m and V_{max} values were both larger for selenomethionine, but only the K_m value was significantly larger. Both methionines inhibited each other in their incorporation into the reticulocyte hemoglobin.

Tryptic peptide chromatograms of labeled globin showed a similar but not identical elution of methionine and selenomethionine labeled peptides. Amino acid analyses of the methionine labeled peptides confirmed the presence of methionine along with other amino acids known to be in these peptides. The absence of methionine in selenomethionine labeled α chain peptides has not precluded the identity of the two labeled peptides, but was probably a difference in their chromatographibility. There was an equal amount of the two methionines in both the α and β peptides.

Hemoglobin labeled with methyl labeled methionine and selenium labeled selenomethionine was similarily reduced in both activities upon sulfitolysis. Sulfitolysis reduced both activities in the labeled hemoglobin to a significant degree. Dithiothreitol reduced these activities to a smaller extent. Heating led to a small reduction in the ^{75}Se activity, but not in the ^{14}C activity. Because the label was shown to remain in the methionines, sulfitolysis and other treatments were concluded to remove activity from the methionine and selenomethionine residues in the globin.

Aminoacyl synthetase and tRNA from selenium adapted or normal Escherichia coli was not noticeably different from unadapted material when assayed for selenomethionine and methionine accepting abilities. The adapted enzyme was not as active, probably due to inorganic selenium inhibiting the active sulfhydryl groups on the synthetases. Although virus infected host tRNA sometimes changes (11), selenium adaptation did not appear to alter the tRNA for methionine or selenomethionine accepting abilities. Therefore selenium adaptation probably only involved a mechanism to prevent selenium from entering the cells as postulated by Shrift et al. (100, 101). ATP was stimulatory for both methionines' aminoacylation, but a pH 11.0 incubation released only methionine and not selenomethionine. Selenomethionine and methionine competitively inhibited each other. This competition was complete only if the analogs were preincubated before the radioactive amino acid was added. This was probably related to the rapid binding of both methionines that was noticed in previous experiments. McConnell

and Roth have shown instantaneous binding of selenoamino acids on rat liver ribosomes which was dependent on ATP (61). Increasing amounts of methionine saturated the amount of methionine bound, however increasing amounts of selenomethionine resulted in increased binding or aminoacylation in the plating experiments. Increased enzyme inhibited the selenomethionine binding, whereas increased tRNA increased selenomethionine binding to a plateau level.

Myeloblast synthetase and tRNA, plant synthetase with E. coli tRNA, and E. coli synthetase and tRNA all attached selenomethionine 2-4 times better than methionine in the plating experiments. Plant synthetase chromatographed similarly for both methionines. Both selenomethionine and methionine showed an unusually high background binding in this tRNA aminoacylation when assayed by plating on membrane filters, but not when assayed by reverse phase chromatography. On the reverse phase profile there was some small difference in the position of the second ^{75}Se and ^{14}C peaks which may have been due to differences in the chromatography of sulfur and selenium, and not due to different tRNA species. A recent abstract by Hoffman, McConnell and Carpenter using slightly different techniques for aminoacylation measurements have indicated a 40% higher K_m for selenomethionine than methionine, but did not indicate number or differences in chromatography of selenomethionine or methionine aminoacylated tRNA species (35). As assayed by reverse phase chromatography in this thesis, selenomethionine and methionine were apparently attached to the same tRNA species and in equal quantities. There was no discernible difference in the tRNA from selenium adapted or normal Escherichia coli when assayed for

aminoacylation with selenomethionine and methionine by reverse phase chromatography.

Although some differences were observed, selenomethionine and methionine appeared to be equal in manner and quantity in all observed phases of protein synthesis.

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Abbreviations used throughout the text

A^o, angstroms, 10^{-10}meters
AAS, aminoacyl synthetase
Ac, acetate
ATP, adenosine-5'-triphosphate
cm, centimeter
CMC, carboxymethyl cellulose
^{14}C-met, methionine(methyl-^{14}C)
cpm, counts per minute
DEAE, diethylaminoethyl cellulose
DNA, deoxyribonucleic acid
dpm, disintegrations per minute
DTT, dithiothreitol
evap., evaporated
g, gram
GSH, reduced glutathione
GSSG, oxidized glutathione
Km, Michaelis enzyme constant
m, milli or 10^{-3}
M, molarity
min, minutes
ml, milliliter
mu, millimicro or 10^{-9}; 260 mu, wavelength 260 x 10^{-9} meters
NADP, nicotine-adenine dinucleotide phosphate
no., number
ppm, parts per million
PPi, pyrophosphate
OD, optical density
RSeH, selenhydryl or selenol compound
RSH, sulfhydryl or thiol compound
^{75}Semet, selenomethionine-^{75}Se
+Se, selenium adapted
-Se, normal or not selenium adapted
sRNA, soluble ribonucleic acid
TCA, trichloroacetic acid
TM, tris-magnesium buffer
tRNA, transfer RNA
tris, tris(hydroxymethyl)aminomethane
u, micro or 10^{-6}
uCi, microcurie, 2.22 x 10^6 dpm
uu, micromicro or 10^{-12}
v, enzyme reaction velocity
Vmax, maximum velocity of an enzyme reaction
x, dimension
X, buffer strength in relative units
x g, centrifugation force in gravity units

APPENDIX

ADDENDUM

RIBONUCLEASE FREE tRNA AND AMINOACYL SYNTHETASE FROM ESCHERICHIA COLI B AND THE AMINOACYLATION OF SELENOMETHIONINE AND METHIONINE

A further study was undertaken for the aminoacylation of selenomethionine and methionine with nuclease free aminoacyl-tRNA synthetase and tRNA. Certain parameters for the reaction were also to be determined, including ATP/Mg++ and Na+/K+ ratios and pH optimum.

Preparation of synthetase and tRNA free of nuclease

The synthetase was prepared with reference to Meunch and Berg (130) as described in Figure 29. Synthetase activity was observed only in the peak resulting from the change of salt concentration and none was found in the breakthrough peak, Table 21.

Table 21. Synthetase activity from the DEAE-column, Figure 29.

	uu moles selenomethionine plated/100 ug tRNA
breakthrough peak, tube 13 (900 ug protein/assay)	
complete[a]	0.5
no tRNA	1.0
no ATP	1.9
peak resulting from salt change, tube 100 (370 ug protein/assay)	
complete	31.6
no tRNA	1.8
no ATP	35.4

[a]Reaction conditions: ATP, 1 u mole; $MgCl_2$, 2 u moles (ATP/Mg = 1/2); 50 uu moles selenomethionine/0.1 u Ci; volume 0.3 mls; pH 7.5; 0.01 M tris; 1 u mole KCl; 100 ug tRNA (before $NaClO_4$-phenol step).

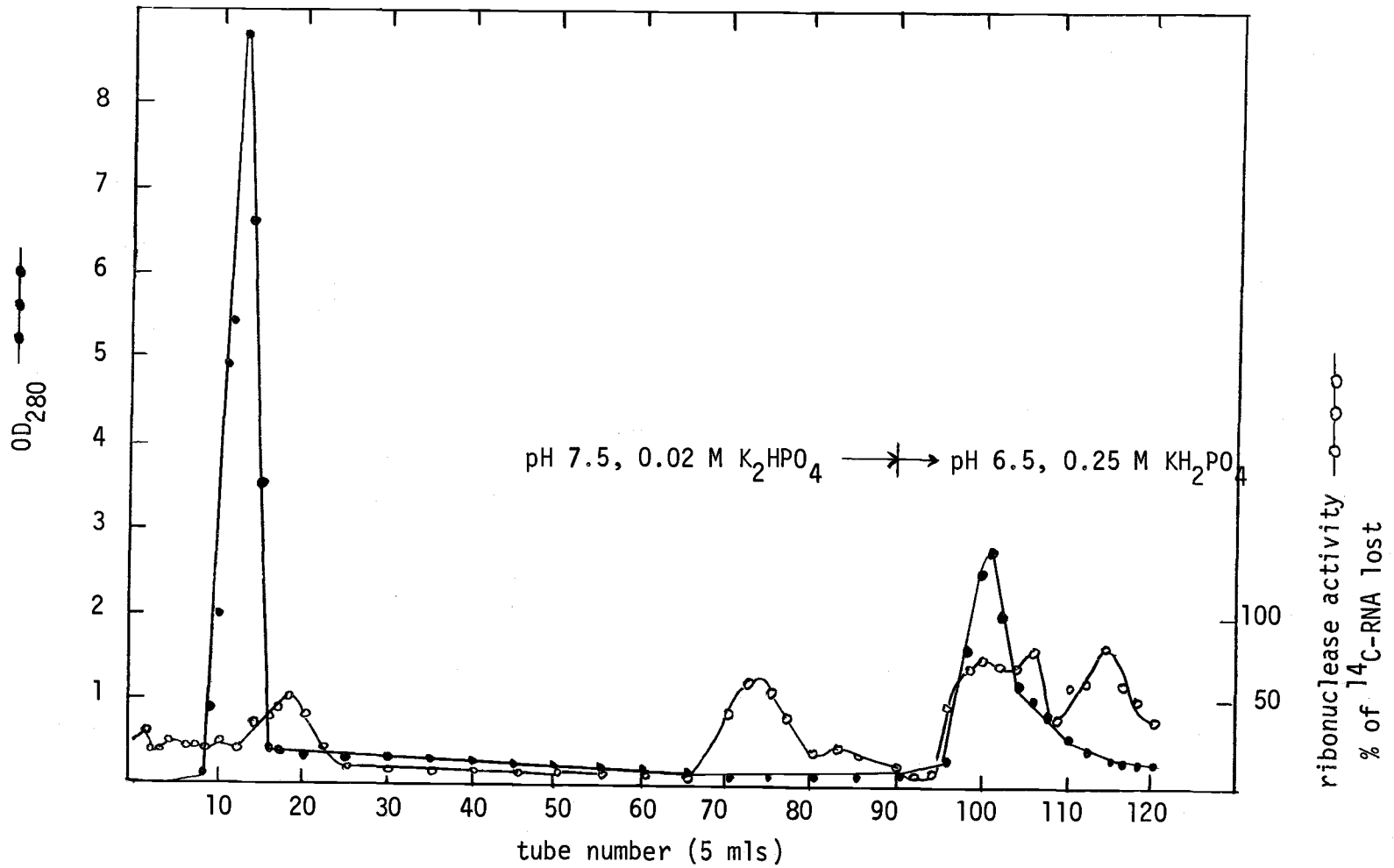

Figure 29. Elution of aminoacyl synthetase of Escherichia coli B on a column of DEAE-cellulose. The 1.5 x 15 cm column was equilibrated with pH 7.5, 0.02 M potassium phosphate containing 10% glycerol, 0.001 M $MgCl_2$ and 0.02 M mercaptoethanol. At tube 90 the synthetase was eluted with 0.25 M potassium phosphate, pH 6.5 containing the glycerol, Mg++ and mercaptoethanol. Used tubes 97-110.

Ribonuclease was observed throughout the column eluant as assayed by a 10 minute incubation of ^{14}C-uridine labeled RNA (2000 cpm/0.1 ug ribosomal RNA). The ribonuclease activity was determined by loss of 10% TCA insoluble material on membrane filters.

The synthetase preparation was dialyzed for several days against 15% polyethylene glycol (average molecular weight, 6000), 0.02 M beta mercaptoethanol, 10% glycerol, 0.005 M $MgCl_2$, 0.001 M potassium phosphate, pH 6.8. This procedure concentrated the synthetase preparation by about six fold. Assay of the enzyme preparation for ribonuclease activity now showed no ribonuclease activity towards the ^{14}C-RNA, Figure 30.

Another test for ribonuclease activity was to test the degree of aminoacylation in a 10 minute assay with the complete system after preincubation of the enzyme and tRNA or tRNA for periods up to one hour. Figure 31 shows that the enzyme plus tRNA showed no loss of activity. Its increase of activity possibly could be an esterase present in the crude synthetase preparation, i. e., hydrolysis of charged tRNA species making more tRNA available for acylation. Preincubation of the tRNA showed a drastic loss of acceptor activity. This tRNA had been prepared as previously described except that it was eluted from the DEAE-column with 1.0 not 2.0 M NaCl. It also had not been dialyzed. In order to removed nuclease from this tRNA, the preparation was shaken for one hour with 0.2 volumes of 5 M $NaClO_4$, 0.5 volumes of phenol saturated with 0.1 M TM, pH 7.5 and 0.1 volumes of chloroform(131). The mixture was centrifuged and the aqueous phase was phenol extracted, ether extracted, and then dialyzed for two days against 0.01 M TM, pH 7.5. The

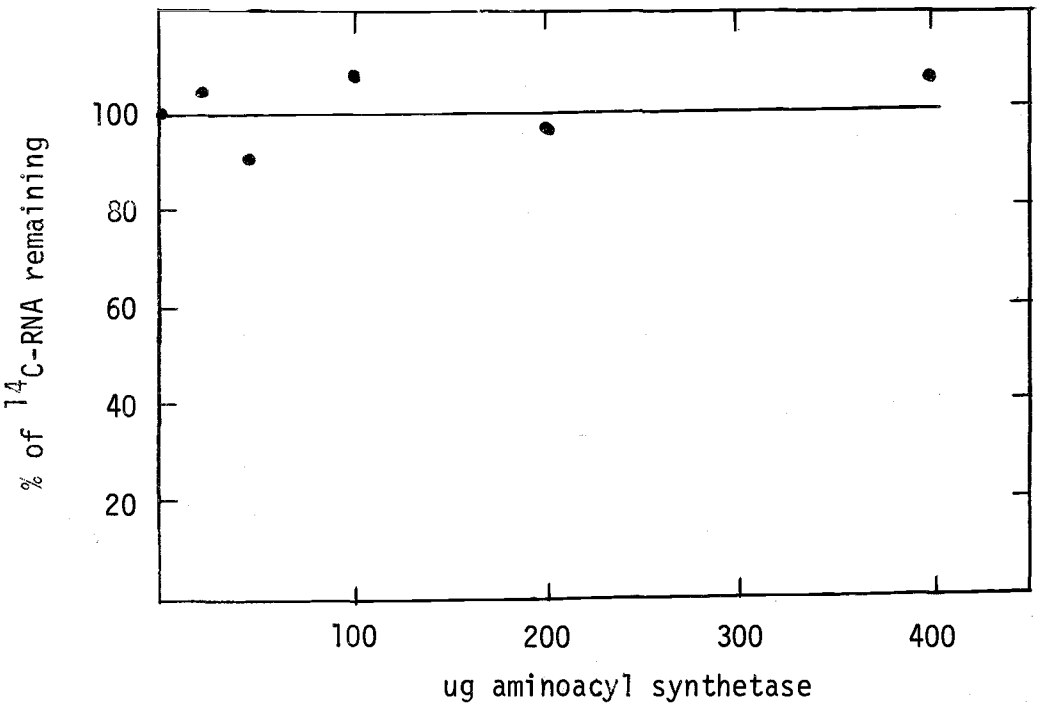

Figure 30. Test for nuclease in aminoacyl synthetase preparation (after dialysis against 15% polyethylene glycol 6000). Each assay contained 2000 cpm of ^{14}C-uridine labeled ribosomal RNA (0.1 ug). Incubations were for 10 minutes at 37°, then precipitated with 10% TCA and plated on membrane filters.

tRNA was then alcohol precipitated and recovered in a small volume of the buffer. Upon subsequent preincubation of this $NaClO_4$-phenol treated tRNA, no loss of activity was noted, Figure 31.

Increased amounts of enzyme preparation also were initially linear and then plateaued when tRNA became limiting. No decrease of activity was noted as previously shown in Figure 20. This also suggests that ribonuclease was freed or inhibited in the preparation. Amino-acylation of selenomethionine was linear with increased amounts of tRNA with an excess of enzyme and plateaued with limiting enzyme, Figures 32 and 33.

Incidentally the OD_{260}/OD_{280} of the tRNA before $NaClO_4$-phenol treatment was 2.20, and 2.01 after treatment. The enzyme ratio was 0.98.

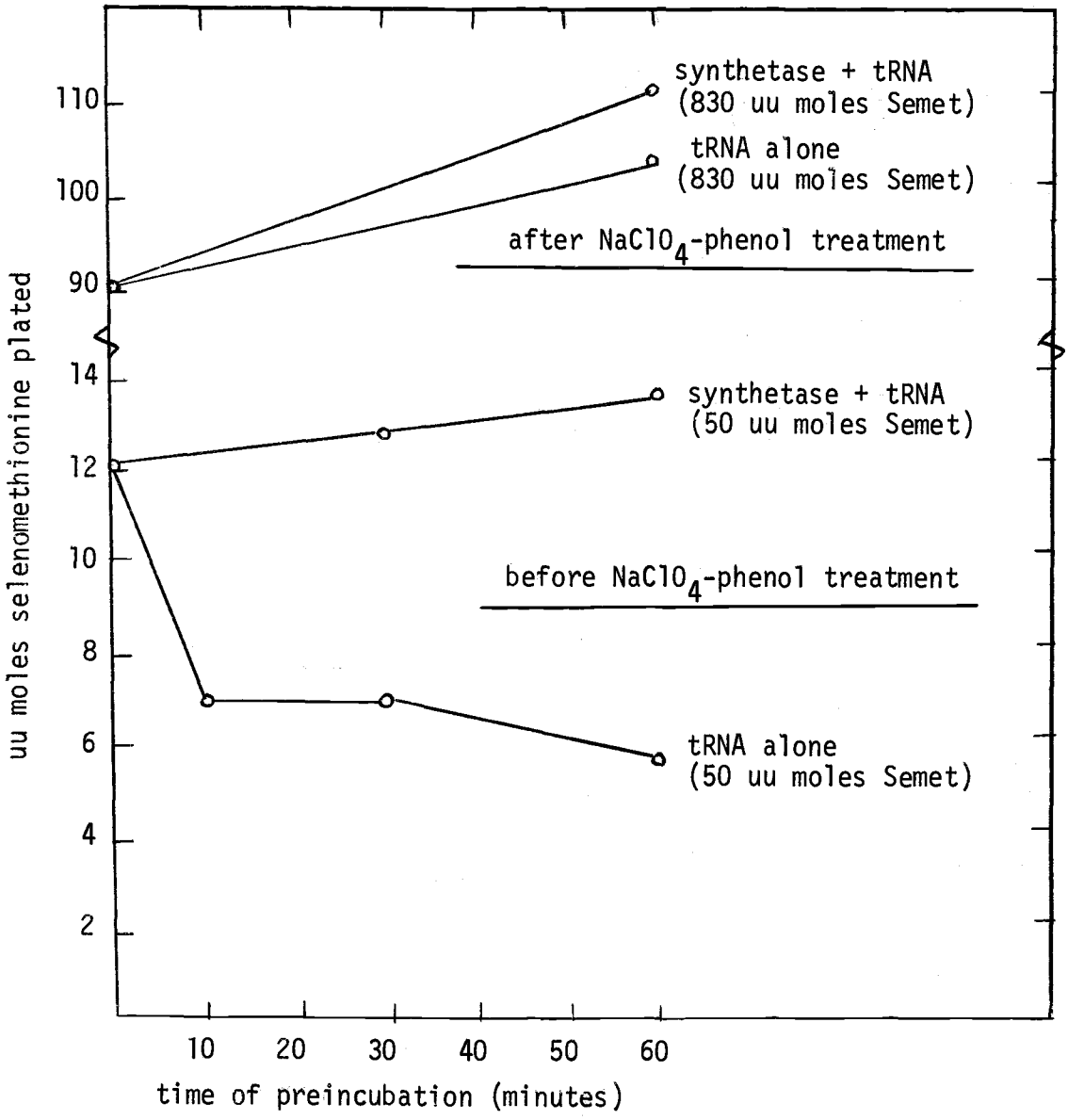

Figure 31. Effect of preincubation on aminoacylation of selenomethionine before and after treatment of tRNA with $NaClO_4$ and phenol. After preincubations at 37° C, assays were carried out as usual: ATP/Mg = 1/2, other conditions as in Table 21.

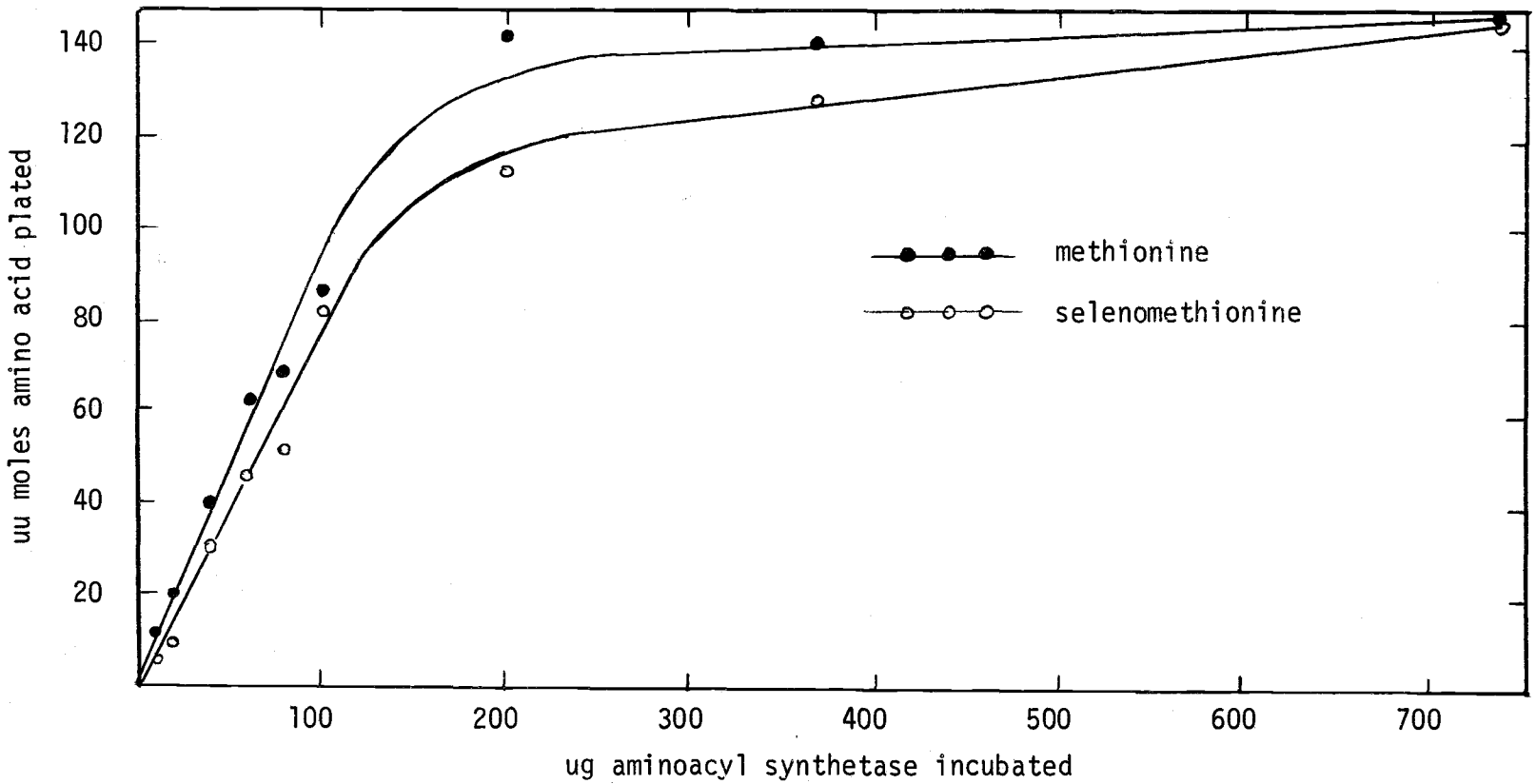

Figure 32. Aminoacylation of methionine and selenomethionine with increasing amounts of nuclease free synthetase. The incubation conditions were: 830 uu moles of selenomethionine or methionine; ATP/Mg++, 0.15/1.0; 100 ug tRNA; other conditions as in Table 21.

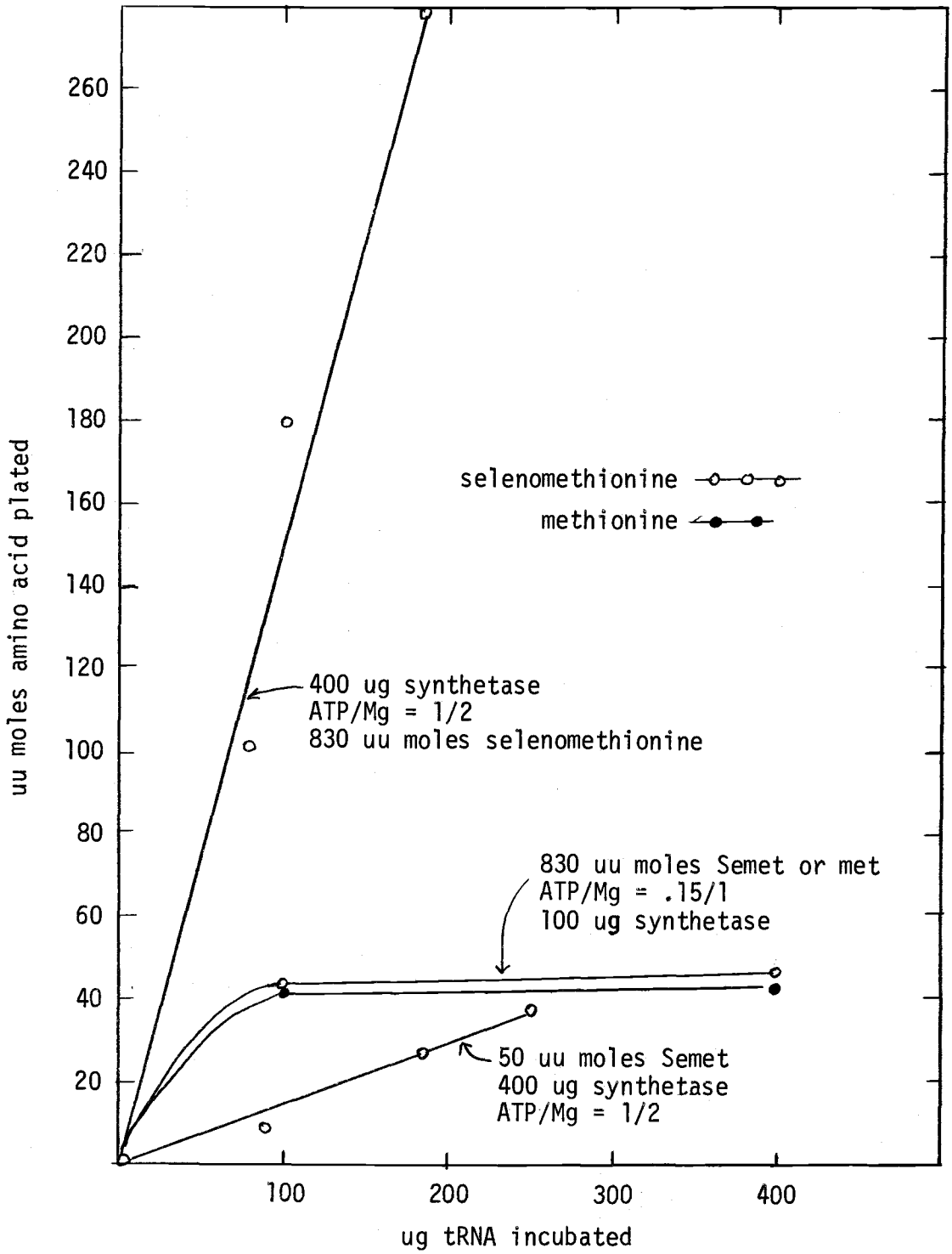

Figure 33. Aminoacylation of selenomethionine and methionine with increasing tRNA with excess and limiting enzyme.

Optimum Reaction Conditions: ATP/Mg++ Ratios

With the ribonuclease free system the optimum ATP/Mg ratios or concentrations were determined. The system showed a complete dependence on ATP after the tRNA was $NaClO_4$-phenol treated and exhaustively dialyzed. All assays were as listed in Table 21 except that the tRNA was $NaClO_4$-phenol treated.

Table 22. A typical assay.

description of assay	cpm		uu moles plated[a]	
	^{75}Se	^{14}C	Semet	met
complete[b]	8475	3620	40.8	42.0
no enzyme	1521	44	0	0
no tRNA	1530	46	0	0
no ATP	1505	13	0	0
^{75}Semet or ^{14}C-met alone	1200	0	-	-

[a]Amount of material aminoacylated by the plating technique was determined by subtracting the reaction blank containing no ATP.

[b]Complete assay: methionine or selenomethionine, 830 uu moles/0.1 uCi; ATP/Mg++ = .15/1.0 (u moles/u moles); 370 ug enzyme, 100 ug tRNA; 0.3 mls volume; 37°C; 10 minutes.

As illustrated by Table 22 and Figures 34 and 35, the amount of selenomethionine and methionine aminoacylated varied drastically with ATP concentration when the Mg++ concentration was limiting (1 u mole Mg++/0.3 mls reaction mixture). The ATP/Mg ratios were less dramatic on the aminoacylations when the Mg++ concentration equalled 2 u moles in the 0.3 mls reaction mixture. The optimum ATP/Mg ratio found by Littauer et al. for methionine was found to be 0.2 u moles/1.0 u moles

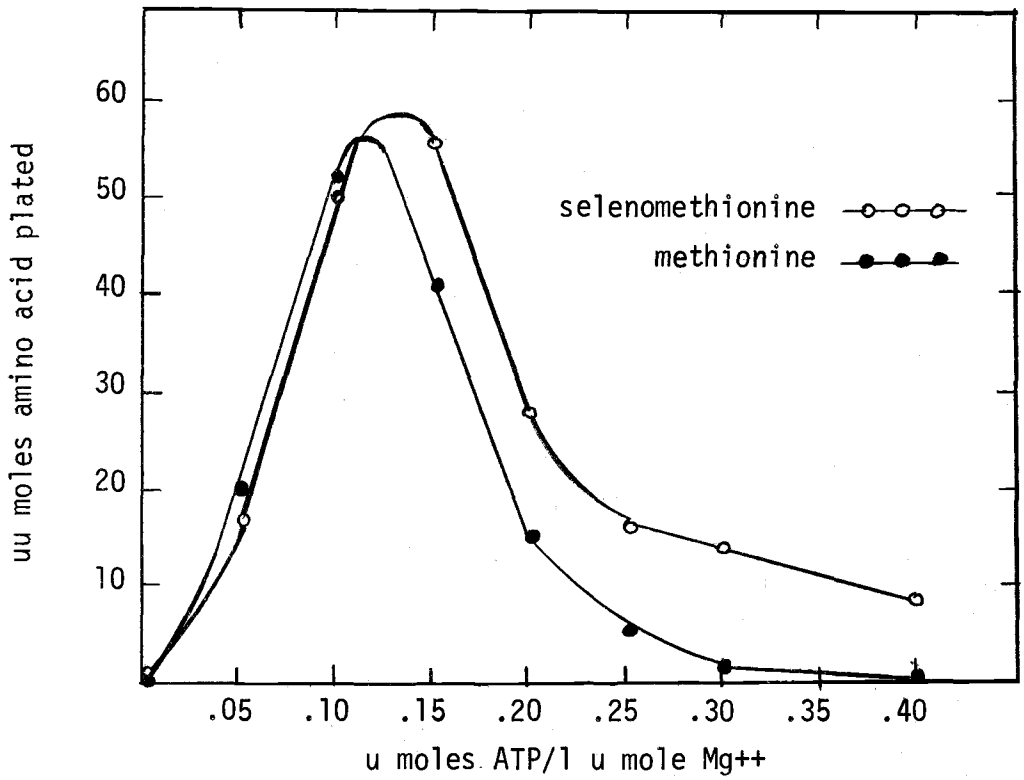

Figure 34. ATP/Mg ratios with Mg limiting: effect on aminoacylation of methionine and selenomethionine. Reaction conditions: 100 ug tRNA and synthetase; 0.3 mls volume; 830 uu moles methionine or selenomethionine; others as in Table 21.

for a reaction mixture of 0.1 mls(131).

Table 23. ATP/Mg++ ratios, conditions as in Table 22.

$ATP/MgCl_2$ (u moles/u moles)	uu moles aminoacylated	
	Semet	methionine
0.15/1	55	40
0.15/2	67	109
reproducibility 1/2	74.5	64.1
reproducibility 1/2	75.8	87.5
reproducibility 1/2	72.1	84.8
2/2	78.1	86.2
6/2	80.0	70.0
1/6	68.3	67.5
5/6	84.0	78.0

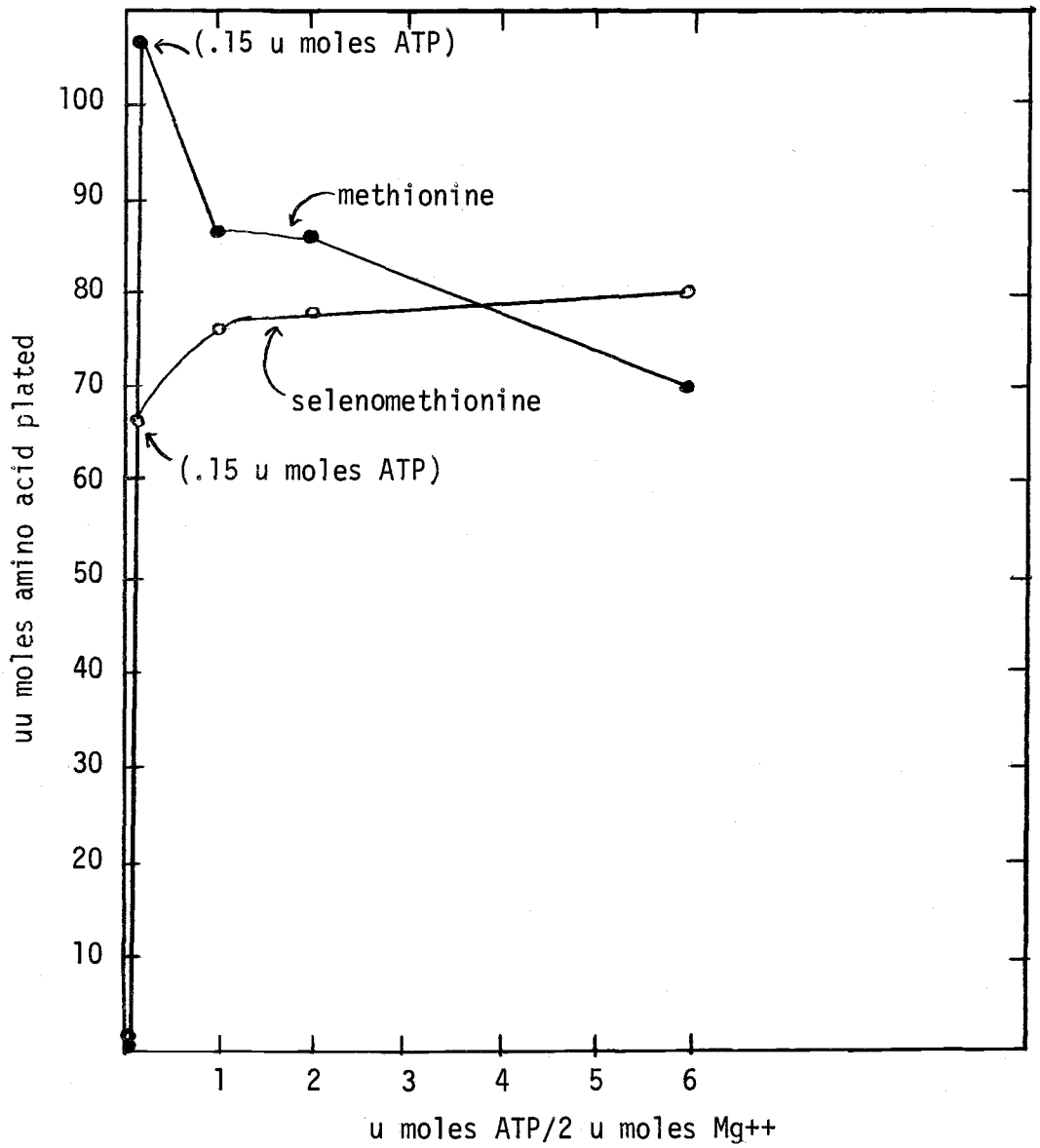

Figure 35. ATP/Mg ratios with sufficient Mg: effect on aminoacylation of methionine and selenomethionine. Reaction conditions: 100 ug tRNA and synthetase; 0.3 mls volume; 1660 uu moles methionine or selenomethionine; others as in Table 21.

Time and pH Optimas

The pH optima appeared to be between 7.5 and 8.0 when the pH was adjusted at 25°C, but since the incubations were at 37°C using 0.1 M Tris buffer the pH optima was probably between 7.2 and 7.7, Figure 36. For this experiment the pH's were individually checked after the incubation of the tubes, then the TCA was added. For all reactions other than for this Figure 36, the pH was 7.5 at 25°C or about pH 7.2 at 37°C.

The time course with this material showed a definite zero amino-acylation at zero time as opposed to instaneous acylation as observed in the previous experiments for time course. The acylation of seleno-methionine appeared to be much more rapid than methionine. The methionine was plateaued in 10 minutes whereas the selenomethionine was plateaued in 3-5 minutes, Figure 37.

Methionine and Selenomethionine Concentration

Results from plating aminoacyl reactions showed that selenometh-ionine did not plateau whereas methionine did when the two methionine's concentrations were increased. This was apparent when the stoichiometry of the reactions did not coincide using larger amounts of the radio-active amino acids, Figure 38. This effect was seen at a lower concentration level than before for the selenomethionine, Figures 22 and 23. The binding of selenomethionine was dependent on ATP through-out the range of the curve as blanks of no enzyme, no tRNA or no ATP did not increase with the increasing selenomethionine concentration. The methionines did show competitive inhibition for each other, Table 24.

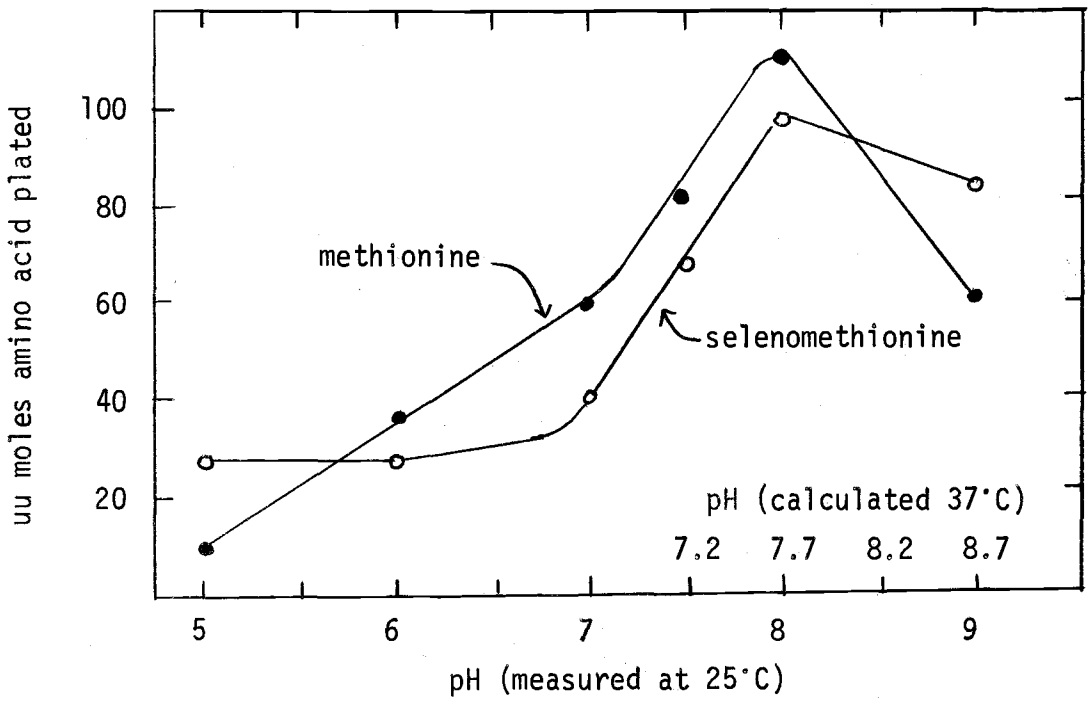

Figure 36. Effect of pH on aminoacylation of methionine and selenomethionine. Conditions: ATP/Mg = 0.15/1; 830 uu moles methionine or selenomethionine; others as in Table 21.

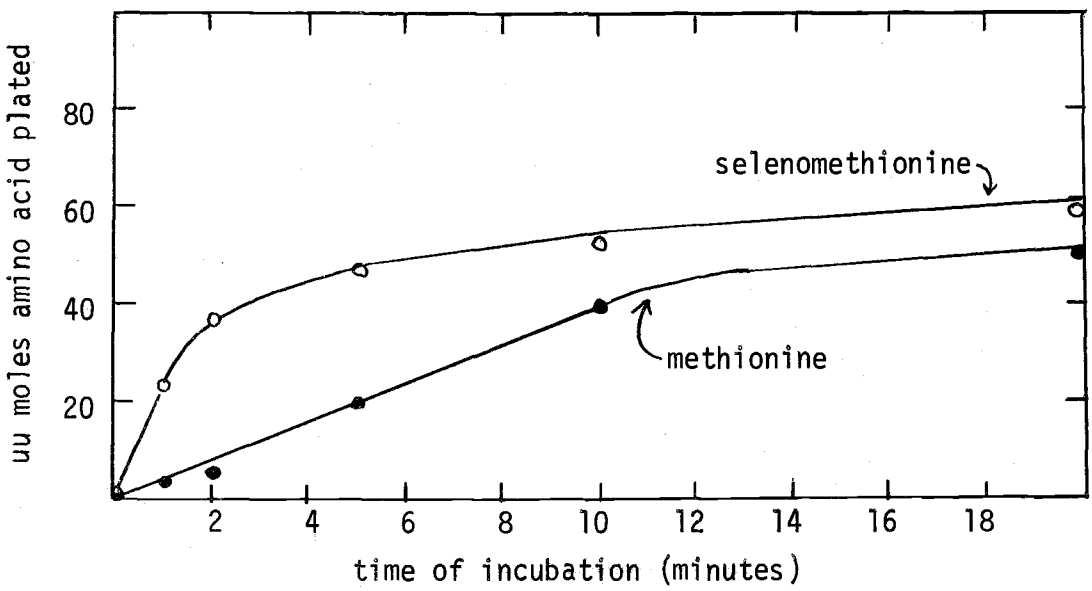

Figure 37. Time course of aminoacylation of methionine and selenomethionine. Conditions: as in Figure 36.

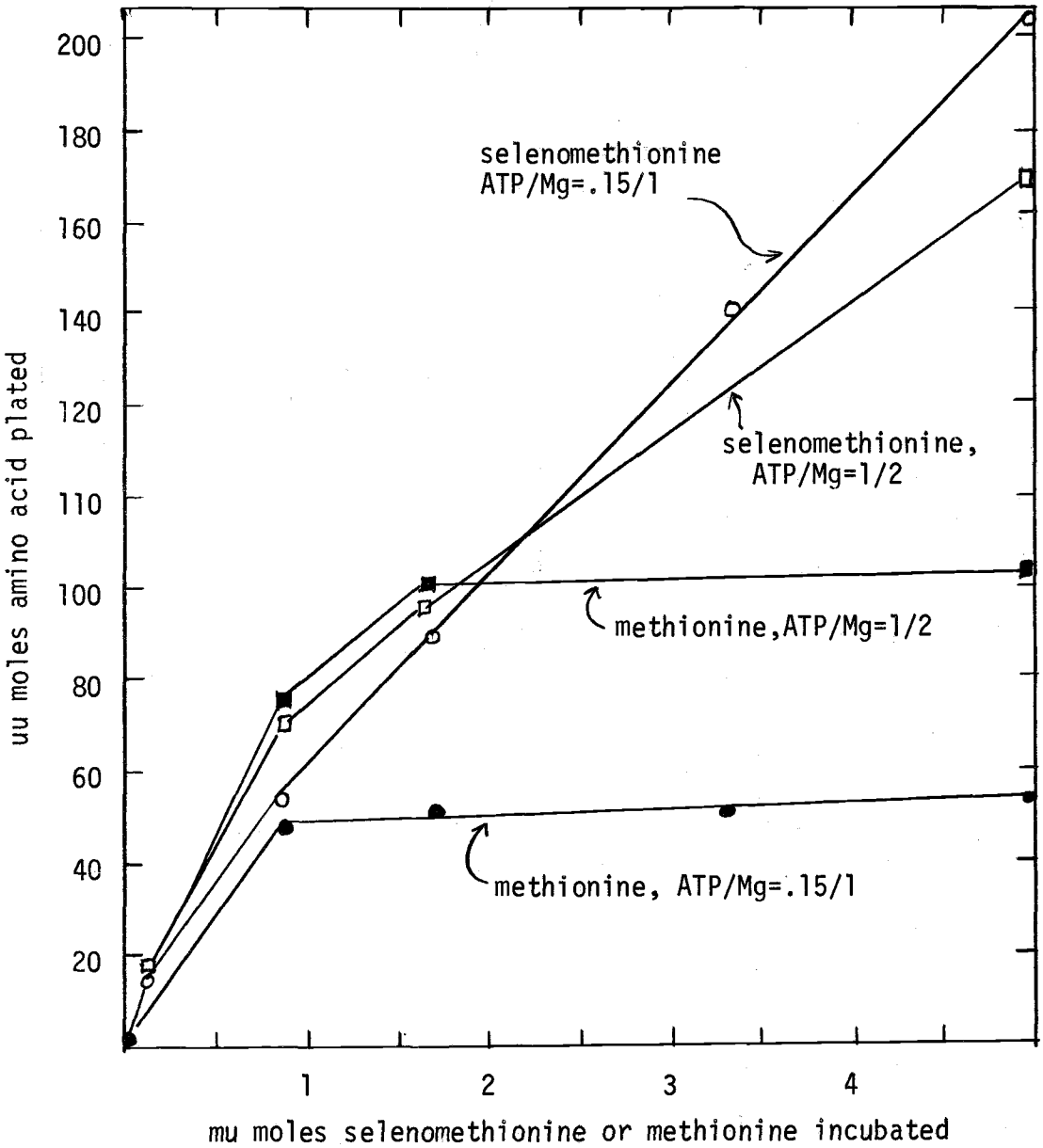

Figure 38. Aminoacylation of tRNA with methionine and selenomethionine: Effect of substrate concentration at two ratios of ATP/Mg.

Table 24. Analog and concentration effects of selenomethionine and methionine.

mu moles incubated[a]		% of controls[b]	
selenomethionine	methionine	selenomethionine	methionine
0	0.83	--	87
0	1.66	--	100
0	3.32	--	100
0	6.67	--	100
0.83	0	85	--
1.66	0	100	--
3.32	0	195	--
6.66	0	280	--
1.66	5.00	36	--
5.00	1.66	--	35
1.66	1.66	103	56

[a]Incubation conditions: 100 ug synthetase and tRNA; ATP/Mg = 1/2.

[b]Controls: 1.66 mu moles methionine or selenomethionine incubated resulted in a net of 100 uu moles methionine and 96 uu moles selenomethionine aminoacylated.

Reverse Phase Chromatography of Ribonuclease Free Aminoacylations

Reverse phase chromatograms are very sensitive methods of analysis for differences in tRNA species. The apparent number of tRNA species for an amino acid as observed by this method can be altered by the presence of ribonuclease. Previous work in this thesis reported two methionine tRNA species, whereas other workers with similar columns report one or two or more peaks for methionine tRNAs.

With nuclease free material the two reverse phase columns yielded only one peak for methionine and selenomethionine which were identical in stoichiometry and position. The first column run on combined tRNA

from separate incubations of selenomethionine (5.0 mu moles/incubation) and methionine (1.67 mu moles/incubation) showed that there was no difference in degree of aminoacylation due to the concentration differences of the methionines in the reaction mixtures as indicated by plating experiments, Figures 22, 23 and 38 and Table 24. The peak for this incubation contained 119 uu moles of selenomethionine and 110 uu moles of methionine, or a yield of 1.19 mu moles selenomethionine/mg tRNA and 1.10 mu moles methionine/mg tRNA. These Reaction conditions were the same as in Figure 39.

The second column was performed on aminoacylated tRNA from incubations of equal molar concentrations of incubated methionine and selenomethionine, Figure 39. Again equal amounts of selenomethionine and methionine were aminoacylated. The methionines were probably aminoacylated to the same degree as in the first column. However more material was probably lost during two ethanol precipitations for the second column since only one ethanol precipitation was made for the first column.

Since the tRNA and synthetase were obtained from the same species of Escherichia coli (B), the difference in the number of tRNA species observed could be attributable to the presence of ribonuclease in the first preparations.

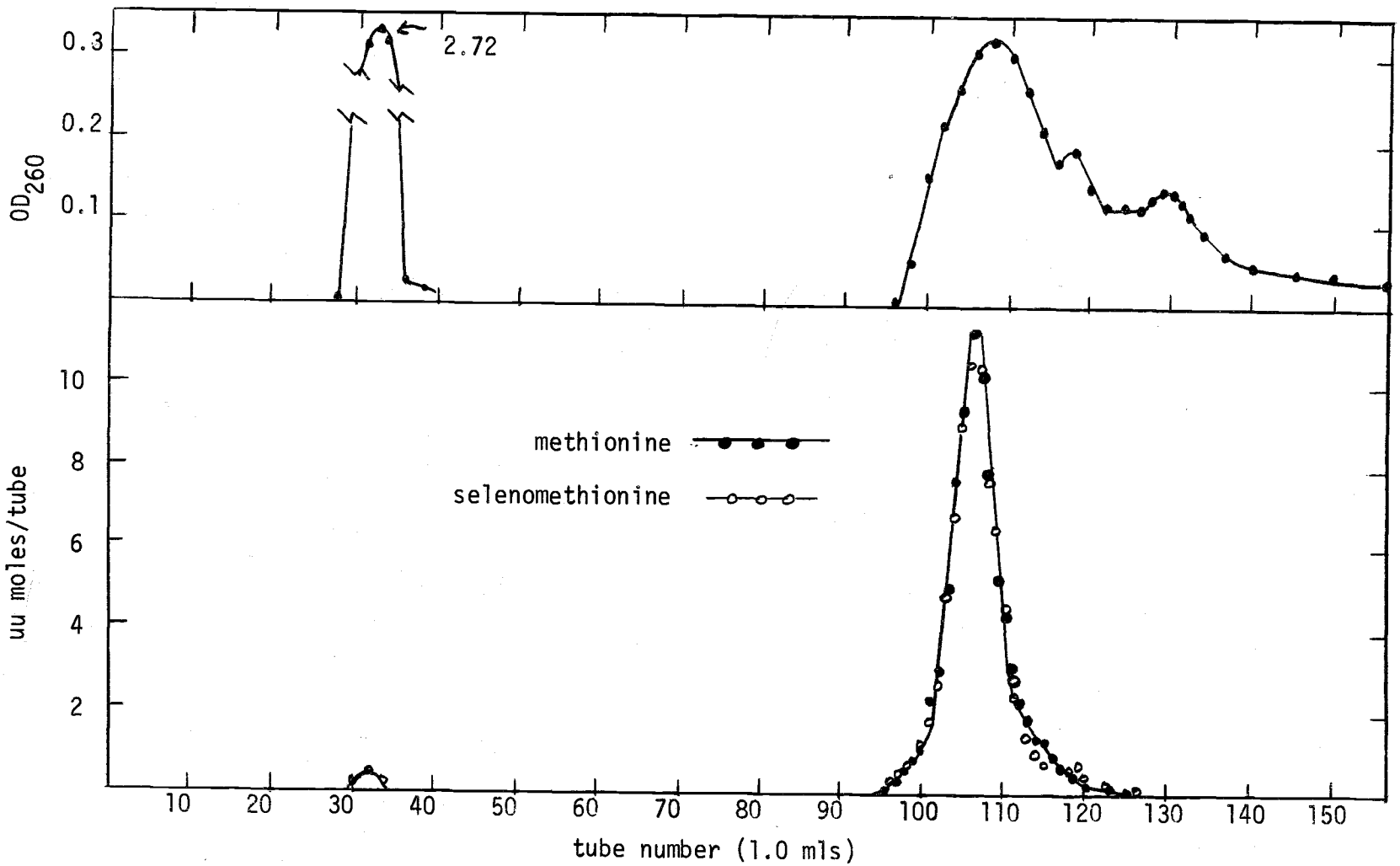

Figure 39. Reverse phase column assay of selenomethionine- and methionine aminoacylated tRNA species using nuclease free enzyme and tRNA. Incubation conditions: separate incubations for 1.66 mu moles selenomethionine and methionine; 100 ug enzyme and tRNA; ATP, 1 u mole; Mg++, 2 u moles; 37°; 10 min.

Hydrolysis and Reaminoacylation of Methionine and Selenomethionine Charged tRNA

As a final test for freedom from ribonuclease methionine and selenomethionine charged tRNA was hydrolyzed and reaminoacylated to see if the same degree of aminoacylation existed both times. Three sets of tubes were incubated as normal: ATP/Mg=1/2, selenomethionine or methionine, 1.66 mu moles, 100 ug enzyme and tRNA. One set contained the selenomethionine assays and the other set contained the methionine assays. The first pair was TCA precipitated and assayed after the incubation. The second and third sets were combined to make two tubes so that treatments to the materials would be identical. These reactions were ethanol precipitated, phenol extracted, ether extracted and ethanol precipitated again. The precipitate was then dissolved in 0.5 mls of pH 8.8, 1.0 M TM buffer with the addition of one drop of $CHCl_3$. This hydrolysis was carried out for two hours at 37°C. Tube two was precipitated with 10% TCA to detect the degree of deacylation. Tube three was ethanol precipitated and dissolved in 0.01 M TM, pH 7.5 and assayed as the first time. There was loss of about 40% of the RNA during these precipitations and extractions as checked by the OD_{260}, so the amount of selenomethionine and methionine reaminoacylated was corrected to 100 ug tRNA.

The reason that selenomethionine appeared to bind better than methionine was that the two methionines were added in the same reaction tube, see Table 24 for explanation. The previous experiment on hydrolysis of selenomethionine, Table 15, probably failed because the material was not ethanol precipitated before the raised pH treatment.

Table 25. Aminoacylation, hydrolysis and reaminoacylation of selenomethionine and methionine.

treatment	uu moles/100 ug tRNA	
	Semet	met
first aminoacylation	100	104
ethanol ppt, pH 8.8 hydrolysis, ppt.	6.0	1.0
ethanol supernatant after pH 8.8	88	--
ethanol supernatant + 10% TCA, filter	0.2	--
reaminoacylation	115	45.8

The ethanol supernatant was checked by adding 10% TCA after the pH 8.8 hydrolysis to check the possibility of some fragmented particles of tRNA-radioactive amino acid.

K^+/Na^+ Effects

Sodium ion is known to inhibit the aminoacylation of amino acids. Except for the tRNA elution, the reactions and reagents for enzyme and tRNA preparations were prepared from potassium salts. ATP solutions were prepared from the barium salt by precipitating the barium by the addition of excess potassium sulfate. As seen by Table 26, sodium ion does inhibit the aminoacylation of methionine and selenomethionine, but that this effect may be overcome by the addition of equal quantities of potassium ion.

Table 26. Sodium and potassium ion effects of the aminoacylation of methionine and selenomethionine.

u moles added		% of control[a]	
KCl	NaCl	Semet	met
0	0[a]	100	100
10	0	117	146
0	10	55	28
0	20	50	28
20	0	96	100
10	10	93	85

[a]Incubations: ATP/Mg++ = .15/1; enzyme and tRNA, 100 ug each; methionine or selenomethionine 830 uu moles.