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The In Vivo and In Vitro Metabolism of S-Adenosyl-L-Homocysteine by the Rat

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DETERMINATION OF THE PRESENCE AND PROPERTIES OF S-RIBOSYLHOMOCYSTEINASE FROM *ESCHERICHIA COLI*

by

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Chairman

Dean of the Graduate School

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INTRODUCTION

S-Adenosylhomocysteine is a resultant product in numerous transmethylation reactions involving S-adenosylmethionine. Some of these reactions are as follows: formation of N-methylnicotinamide from nicotinamide, epinephrine from norepinephrine, creatine from guanidinoacetic acid, choline from dimethylaminoethanol, and the C_{28} group of ergosterol (1). S-Adenosylhomocysteine is also formed as a result of the condensing action of adenosylhomocysteinase on adenosine and homocysteine. The metabolism of S-adenosylhomocysteine is therefore important since the forgoing information indicates the presence of significant amounts of this compound in biological systems, and since numerous investigators have suggested that S-adenosylhomocysteine may serve as a direct percursor of S-adenosylmethionine in mammals and in yeast.

Various studies have also indicated that S-adenosylhomocysteine is metabolized in microorganisms by enzymatic cleavage of the glycosidic bond of this compound which yields S-ribosylhomocysteine and adenine; the adenine moiety is used in the synthesis of purines and nucleotides. Some information concerning the metabolic fate of S-ribosylhomocysteine has been obtained. Preliminary

studies in this laboratory have suggested that the thioether linkage of S-ribosylhomocysteine is cleaved by cell-free extracts from Escherichia coli yielding free homocysteine and pentose. A suggested fate of the homocysteine moiety of S-adenosylhomocysteine, formed through the action of cleavage enzymes, is that of remethylation to methionine, a constituent of the methylsulfonium compound S-adenosylmethionine. Thus, an intimate link may exist between certain thioether compounds and physiologically important transmethylation reactions which indicates the significance of the metabolic fate of these thioether compounds.

This thesis is concerned with the investigation of the catalytic properties of an enzyme, from E. coli strain W, that cleaves the thioether linkage of S-ribosylhomocysteine. The reaction appears to be of the hydrolytic type resulting in the formation of homocysteine and possibly ribose. Free homocysteine has been quantitatively recovered as one of the reaction products; however, ribose as of yet has not been quantitatively recovered which suggests the presence of degradative enzymes still present in the partially purified extracts.

HISTORICAL REVIEW

S-Adenosylmethionine

The first experimental evidence of methyl group transfer was presented by DuVigneaud and coworkers (2) in 1939. They found that choline plus homocysteine could replace methionine in the diet of white rats. This metabolic relationship gave rise to the thought that a methyl group was transferred from choline to the sulfur atom of homocysteine which formed methionine. This discovery eventually led to a great amount of research which culminated in the establishment of transmethylation as a metabolic process and which yielded the discovery of S-adenosylmethionine.

In 1947 Borsook and Dubnoff (3) suggested that ATP was required for the transfer of methyl groups from methionine to guanidinoacetic acid. They also suggested that a methionine derivative rather that methionine was the immediate methyl donor in creatine formation. In continuing this work Cantoni (4,5) suggested the attachment of a phosphoric acid group to methionine for the formation of the derivative refered to by Borsook and Dubnoff (3). Later in 1953 Cantoni (6) indicated that the participation of ATP in transmethylation reactions involving methionine as the methyl donor was necessary for the formation of S-adenosylmethionine, an active intermediate form of methionine,

and that the formation of this intermediate was necessary for the utilization of methionine in this capacity. Cantoni (6) prepared S-adenosylmethionine enzymatically by incubating L-methionine and ATP with partially purified rabbit liver extract. The enzyme responsible for this catalytic reaction was termed methionine adenosyltransferase[1].

From the reaction mixture described above Cantoni (6) isolated S-adenosylmethionine by using various means of chemical extraction and characterized the compound using radioactively labelled methionine as substrate in the catalytic reaction. When he incubated ATP and methionine-S^{35} with methionine adenosyltransferase, the active intermediate formed contained S^{35}; likewise, use of methionine-2-C^{14} yielded the intermediate labelled with C^{14}. The structure of S-adenosylmethionine (Formula I) suggested by Cantoni (6) has been corroborated by total synthesis (7,8,9).

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[1]Trivial names, I.U.B., EC numbers, and systematic names of principal enzymes discussed are as follows: methionine adenosyltransferase, 2.4.2.13, ATP:L-methionine S-adenosyltransferase; guanidinoacetate methyltransferase, 2.1.1.2, S-adenosylmethionine:guanidinoacetate N-methyltransferase; adenosylhomocysteinase, 3.3.1.1, S-adenosyl-L-homocysteine hydrolase; homocysteine methyltransferase, 2.1.1.e, S-adenosylmethionine:L-homocysteine S-methyltransferase.

The biosynthetic mechanism of S-adenosylmethionine was studied by Cantoni and Durell (10); P^{32}-labelled ATP (adenine-ribose-P^{32}-P^{32}-P and adenine-ribose-P-P^{32}-P^{32}) was used. They demonstrated that the terminal phosphate of ATP emerged as inorganic ortho-phosphate, and the resulting active methionine was free of phosphate but contained adenosine derived from ATP. The reaction can be written as follows:

$$\text{L-Methionine + ATP} \xrightarrow{Mg^{++}} \text{S-Adenosyl-L-Methionine + Pyrophosphate + ortho-Phosphate}$$

Reaction 1

This is apparently the only known reaction in which the phosphates of ATP appear as a molecule of inorganic ortho-phosphate and a molecule of pyrophosphate.

This reaction also has been reported to occur in yeast by Schlenk and DePalma, and Mudd and Cantoni (11,12) and in other microorganisms by Tabor, Rosenthal, and Tabor (13). In 1959 Remy (14) demonstrated the presence of significant amounts of methionine adenosyltransferase in Escherichia coli B, E. coli $15T^{-}$, and Proteus vulgaris, and trace amounts in Neisseria catarrhalis and Pseudomonas aeruginosa.

The biosynthesis and isolation of S-adenosylmethionine has greatly facilitated the study of

adenosyl sulfonium compounds. Cantoni (15) first prepared S-adenosylmethionine by the method described in Reaction 1. However, certain disadvantages of this procedure, such as the need for purified liver enzyme and large amounts of ATP, have stimulated work on better and more efficient methods of preparation and isolation. Schlenk and DePalma (11) in 1957 developed a procedure for the biosynthesis and isolation from yeast. The process involved the stimulation in production of S-adenosylmethionine in metabolizing yeast cells by the addition of an excess of methionine. Improvements in the methods of extraction and purification have also been made, the latest being that of Schlenk, Zydek, Ehninger, and Dainko (16).

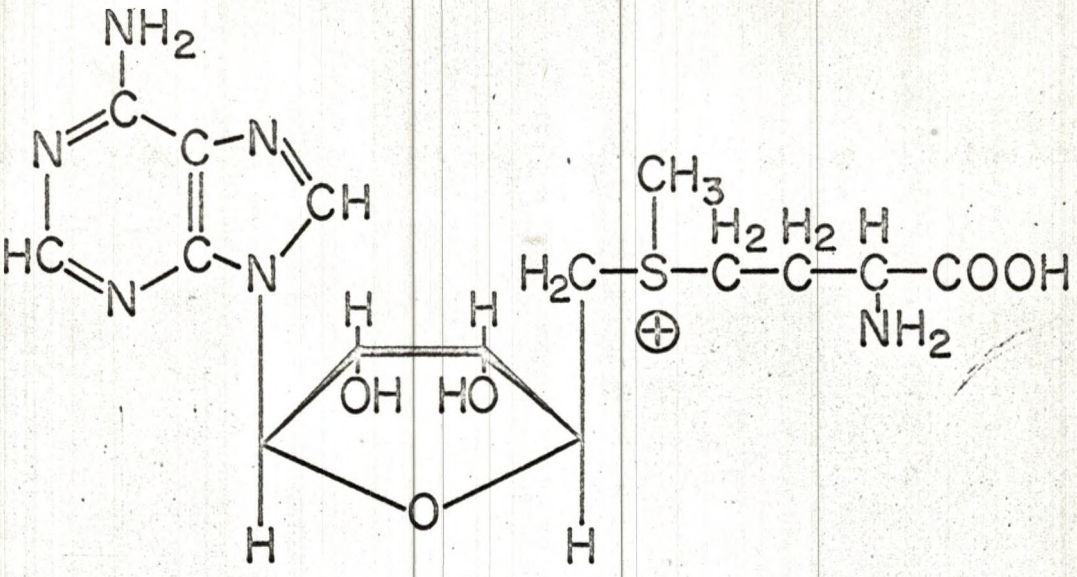
S-Adenosylhomocysteine

Investigation by Cantoni and Scarano led to the characterization of S-adenosylhomocysteine a demethylated product of S-adenosylmethionine (17). They prepared this compound by incubating guanidinoacetic acid and ^{35}S-adenosylmethionine with liver guanidinoacetate methyltransferase. After incubation the excess S-adenosylmethionine was precipitated with phosphotungstic acid and the supernatant fluid placed on a small column of Norite. After 100 ml of water had passed through the column, a small volume of 10% pyridine was

used as the eluting agent. Those fractions exhibiting radioactivity (S^{35}) were subjected to descending chromatography to remove contaminants such as guanidinoacetic acid, creatine, and pyridine. Only one spot on the chromatogram exhibited radioactivity, ultraviolet absorbancy, and a positive test for ninhydrin. This purified material had an ultraviolet absorption spectrum characteristic for adenine nucleosides with maximum absorption at 260 mμ. For each mole of adenine the compound contained one mole of pentose.

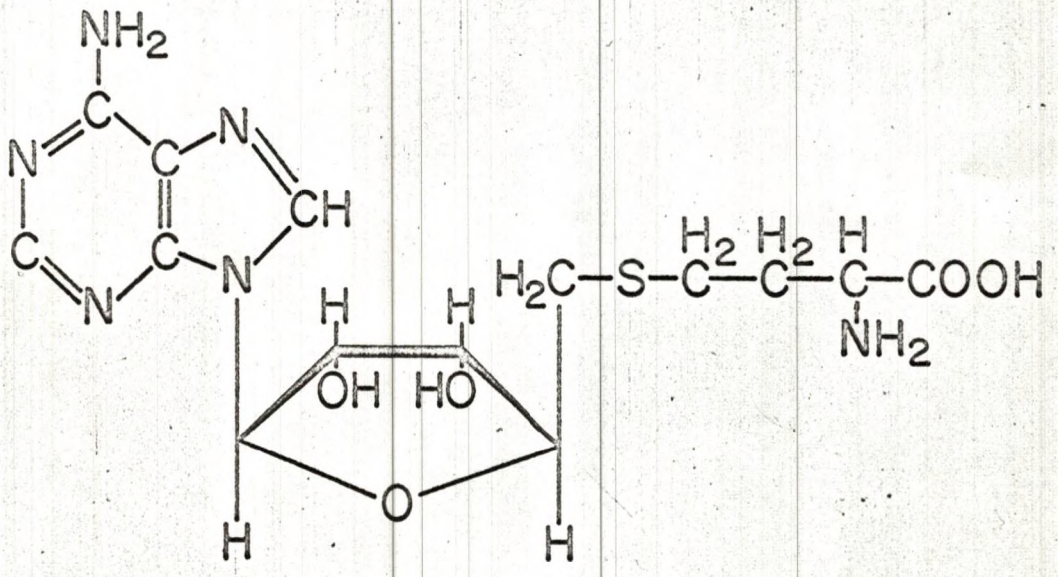
The structure of S-adenosylhomocysteine (Formula III) was unequivocally established through total synthesis by Baddiley and Jamieson (9).

In 1959 de la Haba and Cantoni (18) described the enzymatic synthesis of S-adenosylhomocysteine from adenosine and L-homocysteine by adenosylhomocysteinase from rat liver. They observed that this enzyme catalyzed the reversible hydrolysis of S-adenosylhomocysteine, and that the equilibrium of the reaction favored synthesis. However, hydrolysis was observed when the products of the reaction were removed enzymatically. They identified this compound chromatographically, by methylation to S-adenosylmethionine, by elemental analysis of the crystalline product, and by melting-point determinations of the compound and of its picrate derivative. The reaction described by



S-ADENOSYLMETHIONINE

Formula I



S-ADENOSYLHOMOCYSTEINE

Formula II

de la Haba and Cantoni (18) was as follows:

Adenosine + L-Homocysteine $\rightleftharpoons$ S-Adenosyl-L-Homocysteine + H_2O

Reaction 2

An analogous reaction for the synthesis of S-adenosylhomocysteine was also found to occur in yeast by Duerre and Schlenk (19). They supplemented growing cultures of Saccharomyces cerevisiae with L-homocysteine and observed an increase in production of S-adenosylhomocysteine. When they added L-homocysteine plus adenosine to the growing cultures no significant increase in the production of S-adenosylhomocysteine was observed over that amount produced with the addition of L-homocysteine alone. This merely indicated the availability of ample amounts of adenosine in the metabolic pool.

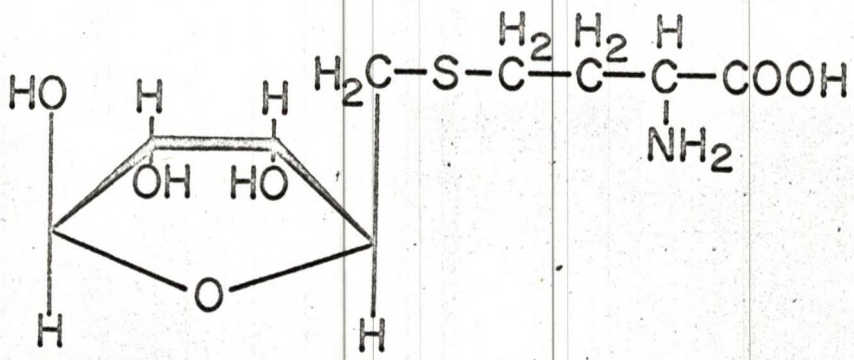
In 1962 Duerre (20) discovered that various modifications in the procedure of de la Haba and Cantoni (18) for the preparation of S-adenosylhomocysteine led to a pure crystalline compound. He also identified the hydrolytic decomposition products adenine and S-ribosylhomocysteine. The latter compound gave positive results in the quantitative ninhydrin test for amino nitrogen, in the orcinol test for pentose, and in the nitroprusside test

for sulfur. Since this compound was derived from S-adenosylhomocysteine, and since it possessed the functional groups mentioned above, Duerre (20) suggested the structure depicted in Formula III. The preparation of S-adenosylhomocysteine and S-ribosylhomocysteine will be described under"Materials and Methods".

Metabolism of S-Adenosylhomocysteine and S-Adenosylmethionine

As indicated earlier the function of S-adenosylmethionine as an important methyl donor has been clearly established in a variety of transmethylation reactions (1). In addition Borek and Srinivasan (21) found that S-adenosylmethionine functioned in the methylation of nucleic acids. When they incubated methyl-deficient transfer-RNA with a soluble extract of E. coli cells and S-adenosylmethionine-C^{14}-H^3, methyl groups were attached to the various bases in the methyl-deficient transfer-RNA to the level obtained with cell grown on a complete medium.

It has also been shown that this compound acts as a direct methyl donor in methionine biosynthesis through the methylation of homocysteine to methionine with cell-free extracts of bacteria, yeast, rat liver, and plant seeds (22,23,24,25). The reaction, catalyzed by homocysteine methyltransferase, is shown in Reaction 3. Studies on the mechanism of this reaction by Shapiro



S-RIBOSYLHOMOCYSTEINE

Formula III

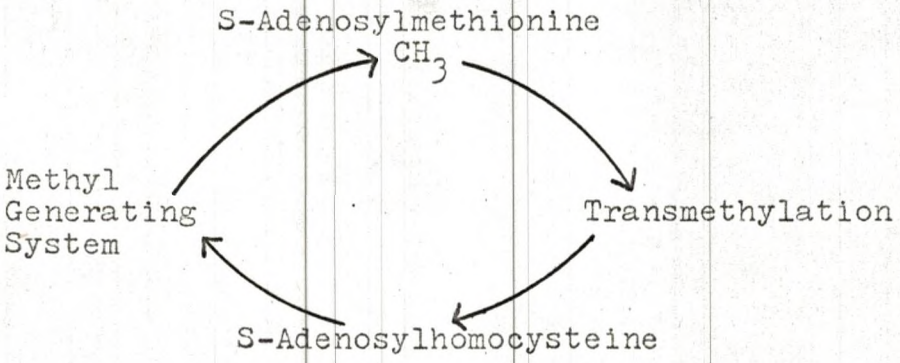
and coworkers (25) suggested that the net synthesis of methionine in S. cerevisiae could not occur from this reaction alone; however, net synthesis would occur if S-adenosylmethionine were regenerated from S-adenosylhomocysteine.

S-Adenosylmethionine + Homocysteine ⟶ S-Adenosylhomocysteine + Methionine

Reaction 3

Duerre and Schlenk (19) in 1962 postulated that S-adenosylmethionine could be regenerated from S-adenosylhomocysteine by transfer of a preformed methyl group from a biological methyl group reservoir or from a system that generates methyl groups. They found that when S-adenosylhomocysteine, labelled with C^{14} in the adenine moiety, was added to the culture medium of Candida utilis or S. cerevisiae, labelled S-adenosylmethionine could be isolated from the cells. Pig, Spence, and Parks (26) in 1962 also implicated S-adenosylhomocysteine as a methyl acceptor for regeneration of S-adenosylmethionine in yeast without prior degradation. The relation ship between transmethylation reactions involving S-adenosylmethionine and the regeneration of this compound from S-adenosylhomocysteine plus a C-1 unit suggests that these two compounds act as a methyl carrier

system or a coenzyme pair interrelated with the transmethylating enzymes and the methyl generating system (27), (Scheme I).



Scheme I

As suggested by Shapiro and coworkers (25) this scheme could account for the net synthesis of methionine in S. cerevisiae.

The enzymatic decomposition of S-adenosylhomocysteine by cell-free extracts of various bacteria was studied by Duerre (28) in 1962. He described various properties of an enzyme capable of hydrolytically cleaving the glycosidic bond of S-adenosylhomocysteine yielding adenine plus S-ribosylhomocysteine (Reaction 4).

S-Adenosylhomocysteine ⟶ S-Ribosylhomocysteine + Adenine

Reaction 4

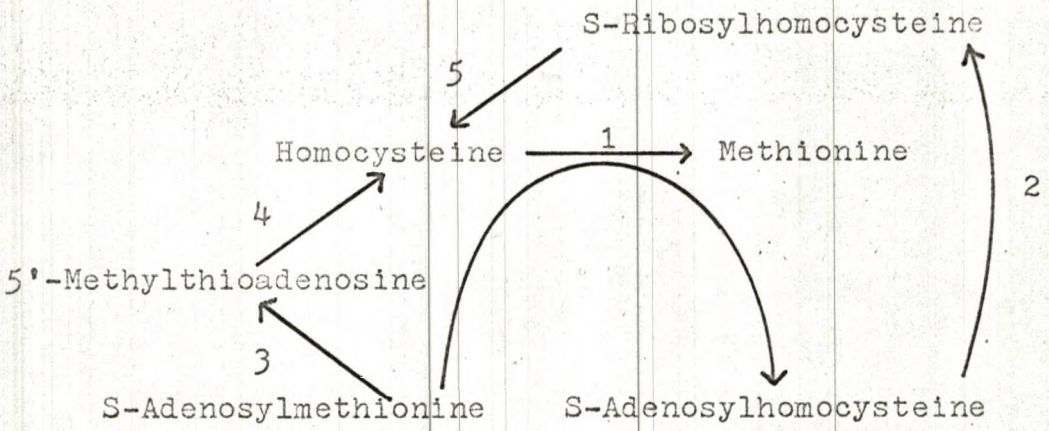
He demonstrated the presence of this enzyme in E. coli,

Aerobacter aerogenes, and Salmonella typhimurium and suggested that a thioether linkage in the substrate molecule was a prerequisite for the reaction since neither the usual purine or pyrimidine nucleosides nor the related sulfonium compounds or sulfoxides were split. He postulated that Reaction 4 may be important in the catabolism of products resulting from S-adenosylmethionine.

Shapiro (29) utilized two methionine mutants of A. aerogenes to study the metabolic relationship of S-adenosylmethionine and S-adenosylhomocysteine to methionine. Various combinations of S-adenosylmethionine, S-adenosylhomocysteine, S-ribosylhomocysteine, homocysteine, and methionine were added to the culture media and growth responses were recorded at constant time intervals. The fact that homocysteine plus S-adenosylmethionine supported almost maximal growth for these mutants suggested that transmethylation occurred via Reaction 3. Shapiro (30) correlated the results of his in vivo studies with those of the in vitro studies previously described or discussed below and suggested the interrelationships found in Scheme II.

Reactions 1 and 2 (Scheme II) were discussed earlier (25,28) respectively). Reaction 3 has been demonstrated to occur in the presence of cell-free extracts of A. aerogenes by Shapiro and Mather (31). They gave evidence that the enzymatic formation of homoserine

lactone and homoserine from S-adenosylmethionine also yielded the reaction product 5'-methylthioadenosine.



Scheme II

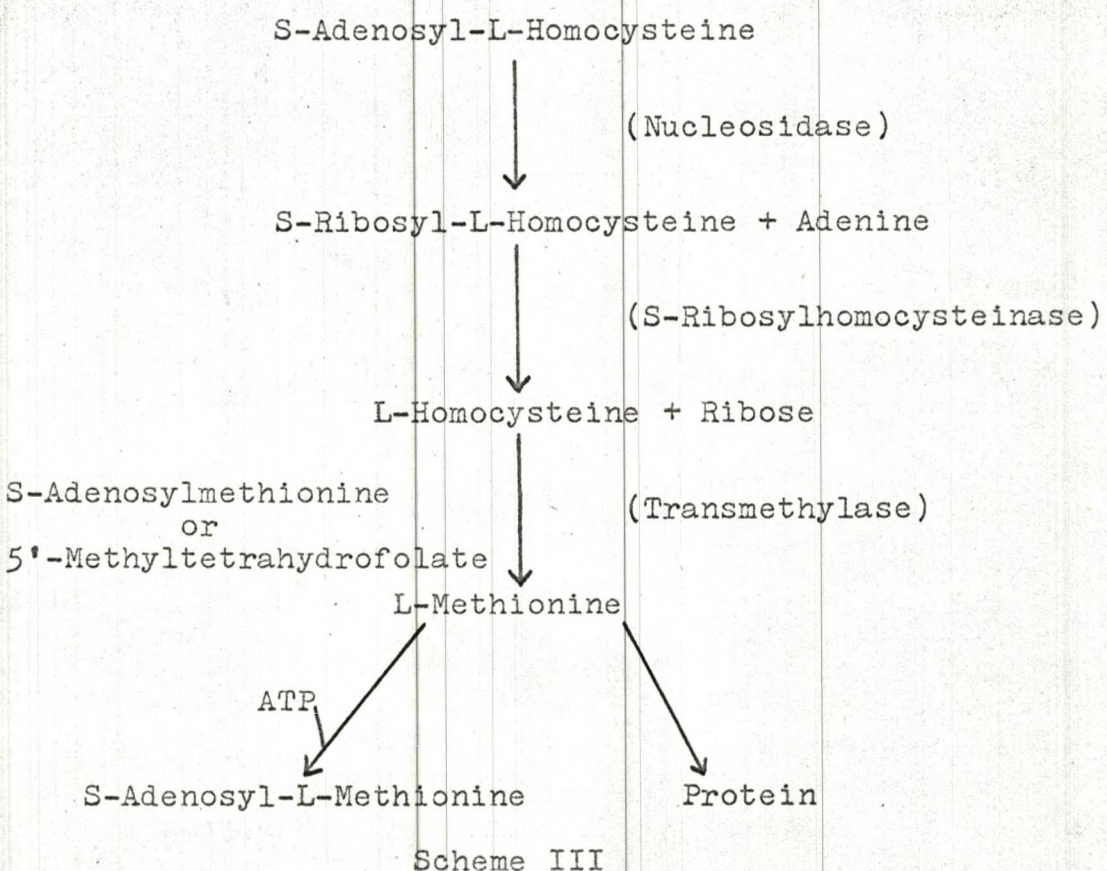
Shapiro (30) proposed that in reaction 4 (Scheme II) 5'-methylthioadenosine in some way enters the methionine pathway at some point where it can serve as the source of sulfur of homocysteine. This proposal was based on results obtained by growing A. aerogenes mutants with supplements of S^{35}-adenosylmethionine with and without homocysteine and then determining the amount of S^{35} incorporated into protein methionine (30). Mutant 68 grew on S-adenosylmethionine with over 80% incorporation of S^{35} into protein methionine in the absence of homocysteine. He assumed that homocysteine-S^{35} was derived from S^{35}-adenosylmethionine via 5'-methylthioadenosine.

Reaction 5 (Scheme II) was also proposed by Duerre and Bowden (32) in 1964 as a result of investigations of

the utilization of radioactive S-ribosylhomocysteine by growing cultures of various bacteria, yeasts, and molds. Substantial amounts of tritium were incorporated by cells of E. coli W and B. They investigated the distribution of tritium from S-ribosylhomocysteine within various fractions of E. coli W cells. When the cells were cultured in the presence of S-ribosyl-H^3-homocysteine the bulk of the isotope was found in the nucleic acid extracts. When the cells were cultured in the presence of S-ribosyl-homocysteine-H^3 the bulk of the isotope was found associated with the protein fraction. These findings indicated that the ribose moiety was metabolized independent of the homocysteine moiety. The results of Duerre and Bowden (32) would suggest that homocysteine could be formed from S-ribosylhomocysteine and then converted to methionine via transmethylation or direct methylation. This would be in agreement with the pathway of methionine biosynthesis proposed by Shapiro (30) (Scheme II).

Preliminary investigations of the metabolism of S-ribosylhomocysteine by Duerre and Miller (33) indicated that the thioether linkage of S-ribosylhomocysteine was cleaved enzymatically by cell-free extracts of E. coli W. They tentatively identified one of the reaction products as homocysteine by thin layer chromatography of the N-ethylmaleimide derivative. The other reaction product was tentatively identified as a pentose

by column chromatography. Free sulfhydryl groups were also detected using S-adenosylhomocysteine as substrate; however, enzymatic cleavage of the glycosyl bond of this compound appeared to be required prior to cleavage of the thioether linkage. Since they demonstrated that homocysteine formed from either substrate could be readily converted to methionine provided the necessary cofactors were present, they tentatively proposed a reaction sequence leading to the incorporation of the homocysteine moiety of S-adenosylhomocysteine into protein methionine of E. coli (Scheme III).



Scheme III

MATERIALS AND METHODS

Organism

Strain W of Escherichia coli, ATCC 9637, was obtained from American Type Culture Collection, Rockville, Maryland, and was maintained at 4°C on Difco Stock Culture Agar (Appendix I). The organism was transferred monthly to assure viable cells for experiments and checked periodically by the Gram stain and reaction on eosin-methylene blue agar to determine culture purity.

Reagents

L-Homocysteine thiolactone, L-homocysteine (free base), and N-ethylmaleimide were obtained from Nutritional Biochemicals Corporation, Cleveland, Ohio. Deoxyribonuclease II was secured from Worthington Biochemical Corporation, Freehold, N.J., and ribonuclease was obtained from Boehringer Mannhein Corporation, New York, N.Y. Sephadex G-25 and G-150 were obtained from Pharmacia Fine Chemicals Inc., New York, N.Y.; Amberlite CG-120 from Mallinckrodt Chemical Works, St. Louis, Mo.; Dowex resins from J.T. Baker Chemical Company, Phillipsburg, N.J. Orcinol was acquired from Mann Research Laboratories Inc., New York, N.Y. and ninhydrin from Pierce Chemical Company, Rockford, Ill. L-Homocys-

teine thiolactone was submitted to California Biochemical Corporation, Los Angeles, Calif., for tritiation (Wilzbach process). All other chemicals were obtained from Fischer Scientific Company, Minneapolis, Minn.

Preparation of Substrates

S-Adenosyl-L-Homocysteine

S-Adenosyl-L-Homocysteine was prepared enzymatically (Reaction 2, "Historical Review") by the procedure of Duerre (20). This consisted of diluting 1.5 mmoles of adenosine, 3.0 mmoles of DL-homocysteine (free base), and 1.0 mmoles phosphate buffer (pH 6.5) to a volume of 100 ml with water. After flushing the system with nitrogen, 5.0 ml of crude rat liver homogenate was added, and the reaction mixture was incubated at 37°C for 3 hours. After incubation, 0.025 ml of thiodiglycol was added to maintain S-adenosylhomocysteine in the reduced state. The reaction mixture was deproteinized by heating in a boiling water bath for 5 minutes, cooled on ice, and centrifuged at 9,000 X g for 20 minutes to remove the precipitate. S-Adenosylhomocysteine was purified from the crude reaction mixture by column chromatography on Dowex 50 W-X8 resin (H^+ form). A column 15 sq cm in cross-sectional area with a resin bed 7-10 cm deep was sufficient to purify 100 ml of the reaction mixture.

From 3-4 liters of 3.0 N H_2SO_4 were required to remove the undesired compounds. Elution was followed by ultraviolet absorption at 260 mμ, and when the column was free of impurities, S-adenosylhomocysteine was eluted with 3-4 liters of 6.0 N acid. S-Adenosylhomocysteine was precipitated from the acid eluate by the addition of 20 ml of a 20% phosphotungstic acid solution for each 100 μmoles of compound. After standing overnight the supernatant fluid was removed by decantation, and the precipitate was washed twice with 10 volumes of cold demineralized water. The water was removed by centrifugation at 500 X g for 5 minutes. The precipitate was dissolved in 6 volumes of a 1:1 mixture of acetone and water, and the phosphotungstate was decomposed by the method of Schlenk and DePalma (11). The solution was extracted 4 times with 4 volumes of a 1:1 mixture of isoamyl alcohol and ether. S-Adenosylhomocysteine remained in the aqueous phase from which residual isoamyl alcohol and acetone were removed by 4 extractions with 3 volumes of ether. Nitrogen was bubbled through the solution to remove residual ether, and the aqueous solution was adjusted to pH 6.5 with $BaCO_3$. The resulting precipitate was removed by filtration and washed with a small amount of water which was combined with the original filtrate. The filtrate was lyophilized, dissolved in water (45-50 μmoles

per ml), frozen, and stored at 4°C for 4 days to complete crystallization. Crystals were then collected by filtration, washed with a small amount of cold water, and stored under vacuum until used. Recovery, based on ultraviolet absorption at 260 mμ, amounted to 35-45% of the S-adenosylhomocysteine present in the 6.0 N acid eluate. Samples of the purified S-adenosylhomocysteine were subjected to chromatography on Whatman No. 1 paper to check for ultraviolet absorbing and ninhydrin-positive impurities and were found to be pure.

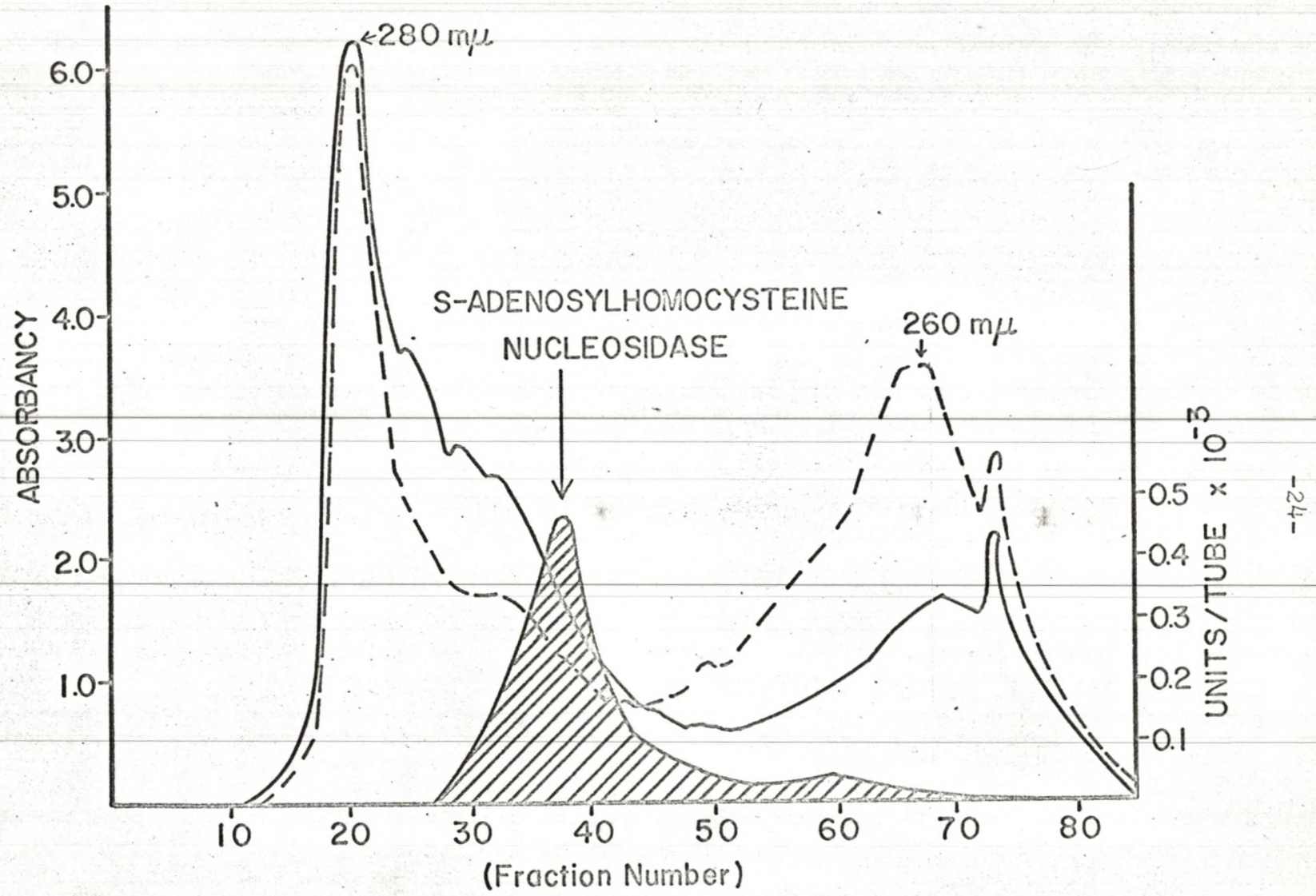
The same method was used for the preparation of S-adenosylhomocysteine labelled with tritium in the homocysteine moiety. Tritiated homocysteine (Wilzbach process) was used in the initial enzymatic reaction.

S-Ribosyl-L-Homocysteine

S-Ribosylhomocysteine was prepared enzymatically by the method of Duerre (28). The catalytic reaction that occurs is described in Reaction 4 under "Historical Review". To obtain the enzyme, S-adenosylhomocysteine nucleosidase, cell-free extracts from E. coli were incubated with deoxyribonuclease II and ribonuclease (2.0 mg each per 100 ml extract) for 1 hour at 37°C. The extract was then fractionated with ammonium sulfate saturated at 4°C (described later in this section under enzyme preparation). The precipitate obtained between

the range 40-60% saturation was dissolved in 0.1 M phosphate buffer (pH 7.8) containing 3 X 10^{-3} M mercaptoethanol and 1 X 10^{-3} M EDTA. This preparation was further fractionated by gel filtration on Sephadex G-150 (4 X 42 cm, flow rate 1.6 ml per minute) using the phosphate buffer as the eluting solvent. The elution pattern (Figure 1) shows that most of the enzyme appeared in fractions 32-42.

S-Adenosylhomocysteine was incubated with this partially purified nucleosidase (1 unit per µmole substrate) for 2 hours in the presence of 0.1 M phosphate buffer (pH 6.5). After deproteinization by heating in a boiling water bath for 5 minutes and centrifugation at 9,000 X g for 20 minutes, the clear supernatant fluid was placed on Dowex 50 W-X8 resin (H^+ form, 2 X 15 cm) previously washed with 6.0 N HCl and equilibrated with 0.1 N HCl. Development with approximately 50 ml of water and 50 ml of 0.1 N HCl removed the phosphate and mercaptoethanol. S-Ribosylhomocysteine was eluted with 1.0 N HCl and the elution pattern was followed by the orcinol reaction. The eluate was neutralized to pH 6.5 by addition of Dowex 2 resin (OH^- form) and the resin removed by filtration and washed with water until free of orcinol-positive material. The wash was combined with the resin filtrate and the solution evaporated to dryness by lyophilization. The dry S-ribosylhomocysteine


←280 mμ
6.0
5.0
4.0
3.0
2.0
1.0
ABSORBANCY
S-ADENOSYLHOMOCYSTEINE
NUCLEOSIDASE
260 mμ
0.5
0.4
0.3
0.2
0.1
UNITS/TUBE x 10-3
10
20
30
40
50
60
70
80
(Fraction Number)

was dissolved in water to a desired concentration as determined by the orcinol reaction. Chromatography of the compound on Whatman No. 1 paper was used to determine the presence of any possible ninhydrin or orcinol-positive impurities. Traces of S-ribosylhomocysteine sulfoxide were detected.

To prepare S-ribosyl-L-homocysteine-H^3, S-adenosyl-L-homocysteine-H^3 was used as substrate in the nucleosidase reaction mixture and the procedures carried out as described above.

Analytical Methods

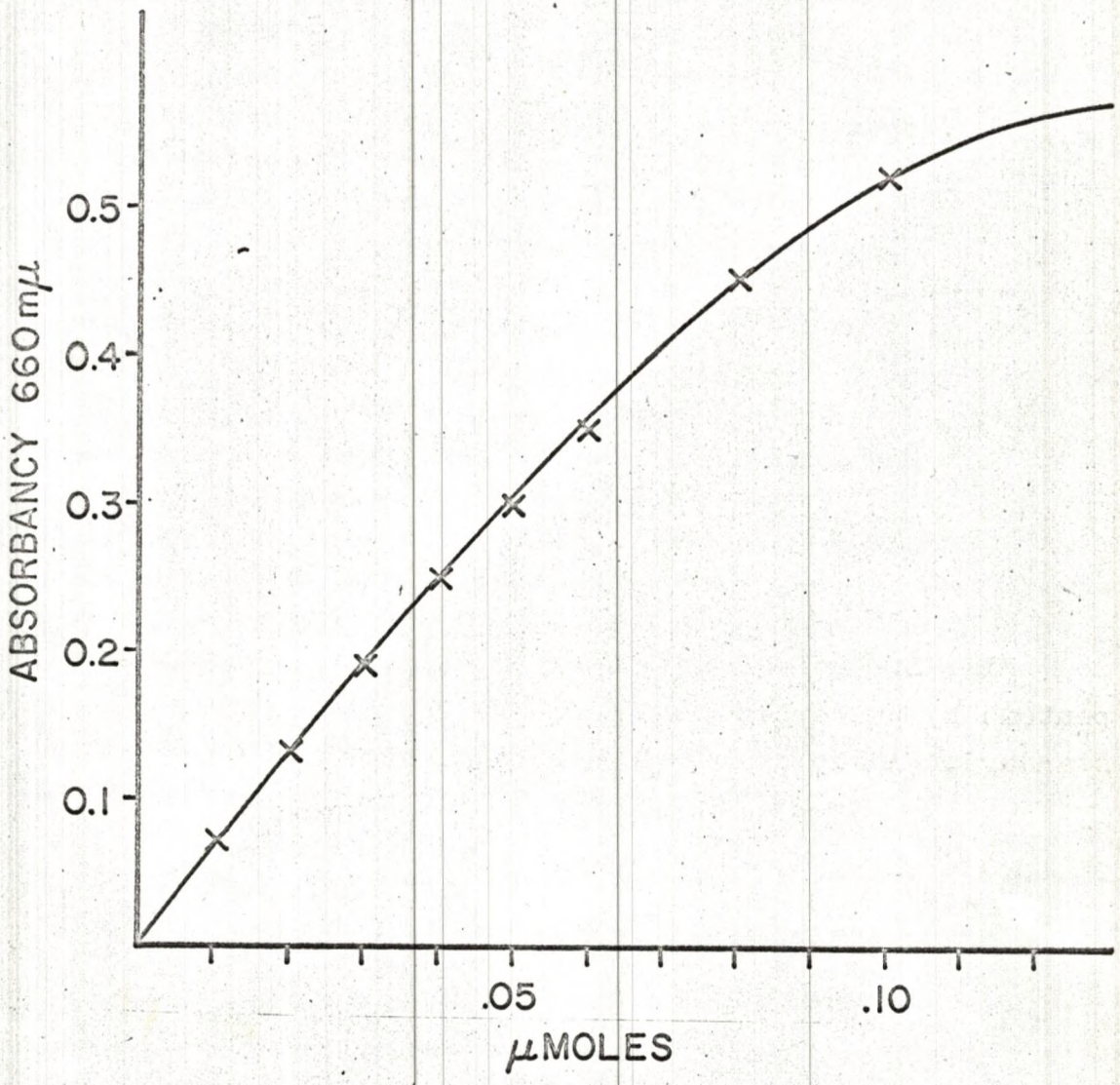
Orcinol Determination of Pentose

Pentose was measured quantitatively by the orcinol reaction described by Brown (34). Samples containing 8-20 µg of pentose were diluted to a volume of 1.0 ml with water. Two ml of Bial's reagent (Appendix I) were added and the samples placed in a boiling water bath for 20 minutes. After cooling, the samples were read in a Coleman Jr. spectrophotometer at 660 mµ. Reagent blanks were prepared by substituting water for the pentose source. Ribose added to the reaction mixture gave a linear response over a range of 2-20 µg (Figure 2).

Determination of Amino Nitrogen

Amino nitrogen was measured quantitatively by the

Fig. 2.--Reference curve for the determination of pentose by the orcinol method. Ribose was used as the standard. Additional information is given in the text.


ABSORBANCY 660 mμ
0.5
0.4
0.3
0.2
0.1
.05
.10
μMOLES

ninhydrin method of Yemm and Cocking (35). One ml samples containing 0.05-0.2 μmoles amino nitrogen were mixed with 1.0 ml of 0.2 M citrate buffer, pH 5.0 . To this mixture was added 1.2 ml of the potassium cyanide-methyl Cellosolve-ninhydrin solution (Appendix I). The solution was heated for 5 minutes and read in a Coleman Jr. spectrophotometer at 570 mμ. The reaction mixture in which the amino nitrogen source was replaced by water served as the reagent blank. Homocysteine was used as the standard for the determination of the reference curve (Figure 3).

Determination of Protein

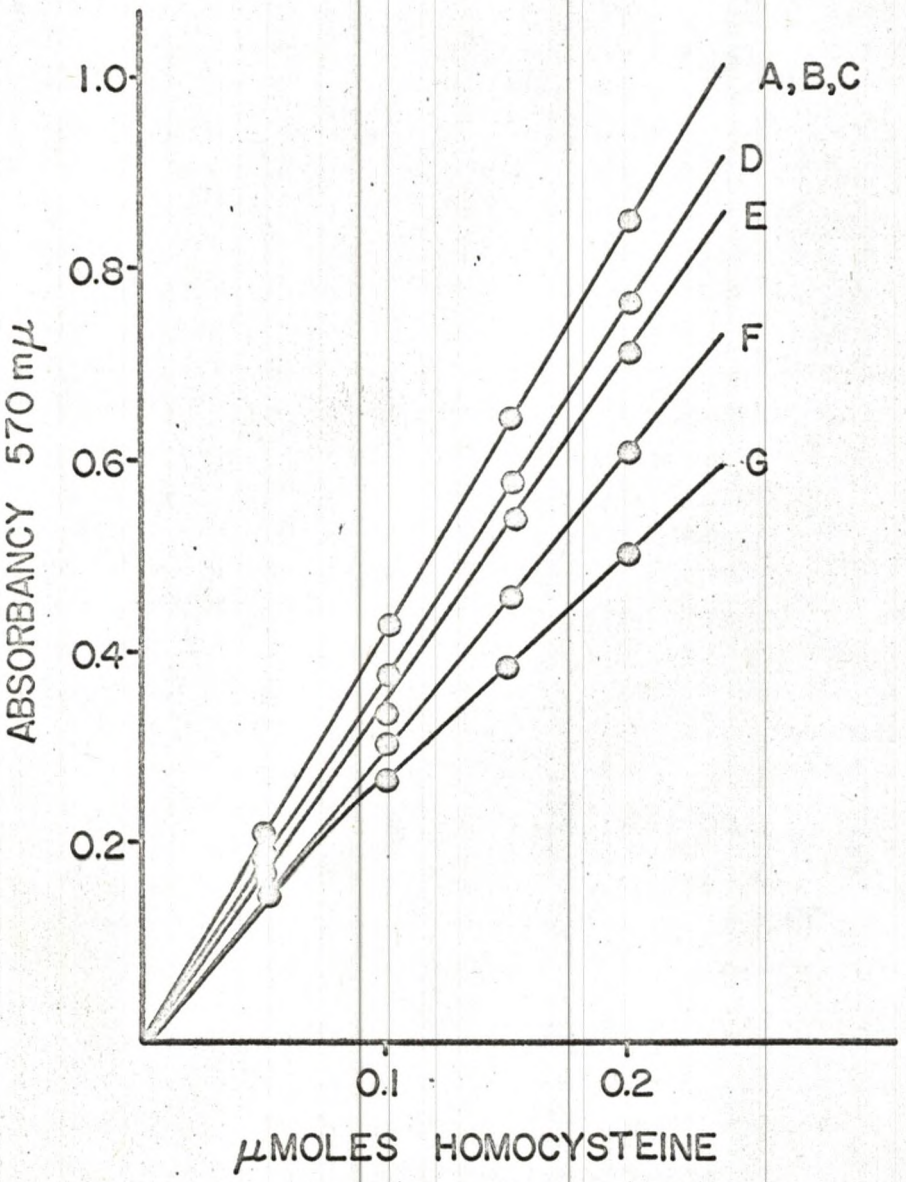
On all samples, except crude extract, protein concentration was determined by the spectrophotometric method of Warburg and Christian (36). Aliquots of samples were diluted with water until an absorbancy capable of being recorded at 260 and 280 mμ was obtained. A Beckman DU spectrophotometer was used for these determinations with water as the blank. Calculation of mg protein per ml was made using the following equation:

$$(1.55 \times O.D._{280}) - (0.76 \times O.D._{260}) \times \text{Dilution} = \text{mg per ml}$$

The high nucleic acid content of the crude extract interferred with measurement of the protein concentration by

Fig. 3.--Reference curves for the determination of free amino nitrogen by the method of Yemm and Cocking. Homocysteine, used as the standard, was prepared by incubating 0.1 mmoles of homocysteine thiolactone with 0.3 ml alkali at varying normalities for 5 minutes; neutralizing with an equivalent amount of KH_2PO_4; diluting to a known concentration.

A = Homocysteine (free base)
B = 7.0 N NaOH
C = 5.0 N NaOH
D = 3.0 N NaOH
E = 2.0 N NaOH
F = 1.0 N NaOH
G = 1.5 N NaOH

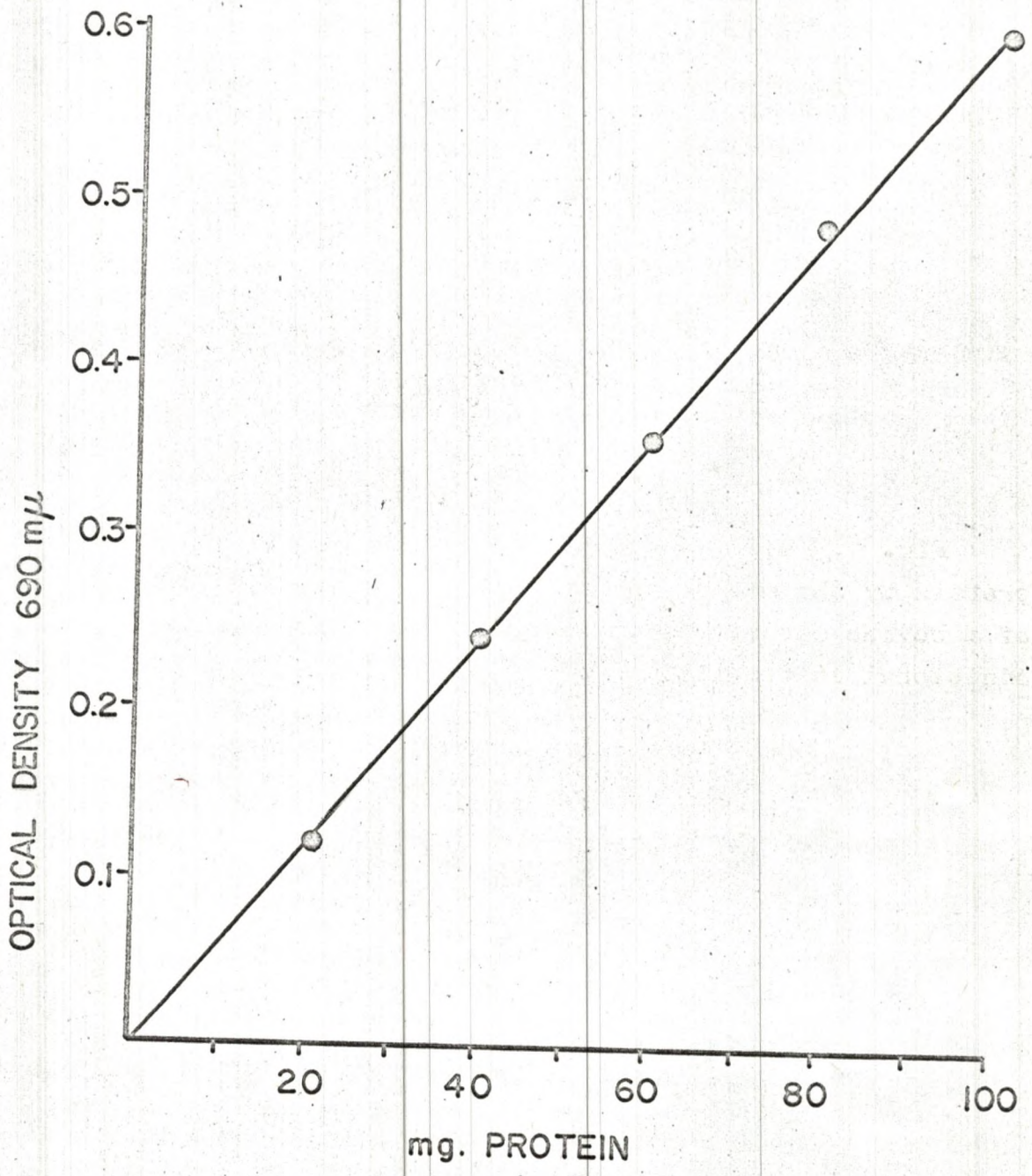

1.0
0.8
0.6
0.4
0.2
ABSORBANCY 570 mμ
A, B, C
D
E
F
G
0.1
0.2
μMOLES HOMOCYSTEINE

the method of Warburg and Christian. Therefore, the method of Lowry was used (37). Samples of the crude extract were diluted to a volume of 0.6 ml with water. Three ml of reagent D (Appendix I) were added, and the solution was mixed vigorously and allowed to stand for 10 minutes at room temperature. The reagent blank contained water instead of a protein solution. For color development 0.3 ml of Folin-Ciocalteau Phenol reagent (1.0 N with respect to sulfuric acid) was added with rapid and immediate mixing. After 30 minutes the samples were read in a Coleman Jr. spectrophotometer at 690 mμ. From the standard curve (Figure 4) it was found that linearity was maintained between 10 and 100 μg protein per ml.

S-Adenosylhomocysteine Nucleosidase Assay

Reaction mixtures containing 3.0 μmoles substrate, 100 μmoles phosphate buffer (pH 6.5), and enzyme in a total volume of 1.0 ml were incubated at 37°C for 30 minutes. To stop the reaction the tubes were placed in a boiling water bath for 5 minutes, cooled, and the precipitate removed by centrifugation at 15,000 X g for 10 minutes. The method of Benedict (38) was used to determine the amount of reducing compound (S-ribosylhomocysteine) present. Samples of the mixture were diluted to 1.0 ml with water and 1.0 ml of copper reagent, containing bisulfite, (Appendix I) was added. After mixing,

Fig. 4.--Reference curve for the determination of protein by the method of Lowry (37). Known concentrations of a bovine albumin solution was used as the standard. Additional information is given in the text.

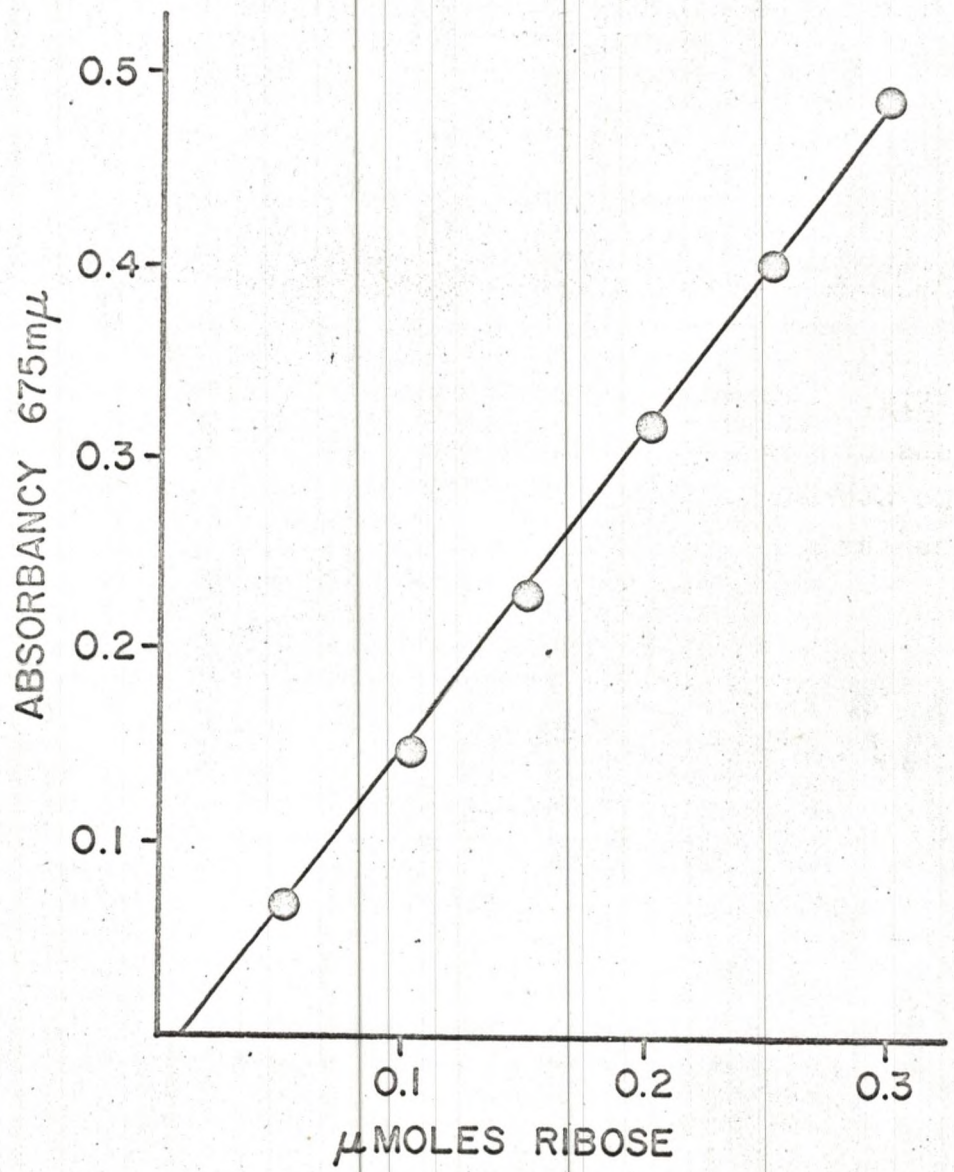

0.6
0.5
0.4
0.3
0.2
0.1
OPTICAL DENSITY 690 mμ
20
40
60
80
100
mg. PROTEIN

the tubes were placed in a boiling water bath for 6 minutes and cooled on ice. To each sample was added 1.0 ml color reagent (Appendix I). The samples were diluted to a total volume of 10 ml and allowed to stand for 10 minutes at room temperature. Absorbancy was read by a Coleman Jr. spectrophotometer at 675 mμ and compared to that obtained with a known amount of free ribose. Free ribose gave a linear response in the range 0.05-0.3 μmoles (Figure 5). One unit of activity was defined as that amount catalyzing the liberation of 1.0 μmole of reducing compound per hour under the conditions described (28).

S-Ribosylhomocysteinase Assay

The method of Duerre and Miller (33) was used for the determination of free homocysteine. Reaction mixtures containing 2.0 μmoles S-ribosylhomocysteine, 100 μmoles Tris buffer (pH 8.0), and *E. coli* extract in a total volume of 1.0 ml were incubated under an atmosphere of nitrogen for 1 hour at 37°C. The reaction was stopped by placing the tubes in a boiling water bath for 3 minutes, cooling on ice, and centrifuging at 15,000 X g for 10 minutes. To 0.3 ml of the deproteinized reaction mixture was added an equal volume of 0.375 M N-ethylmaleimide. After the tubes were allowed to stand for 10 minutes at room temperature, 0.22 ml of 1.0 N NaOH was added. The N-ethylmaleimide

Fig. 5.--Reference curve for the determination of pentose by the method of Benedict (38). Ribose was used as the standard. Additional information is given in the text.


0.5
0.4
0.3
0.2
0.1
ABSORBANCY 675mμ
0.1
0.2
0.3
μMOLES RIBOSE

derivative of homocysteine gave a pink color after the addition of alkali whereas S-ribosylhomocysteine did not. Since this color tends to fade, all tubes were read at 30 seconds after the addition of alkali in a microcuvette at 520 mμ using a Beckman DU spectrophotometer.

For all reaction tubes zero time controls were included in the assays. These controls, containing substrate, buffer, and water, were placed in a boiling water bath for 45 seconds. The prescribed amount of enzyme preparation was added, and after 3 minutes the tubes were removed, cooled on ice, and centrifuged to remove precipitated protein. The controls were then assayed in the same manner as were the reaction mixtures.

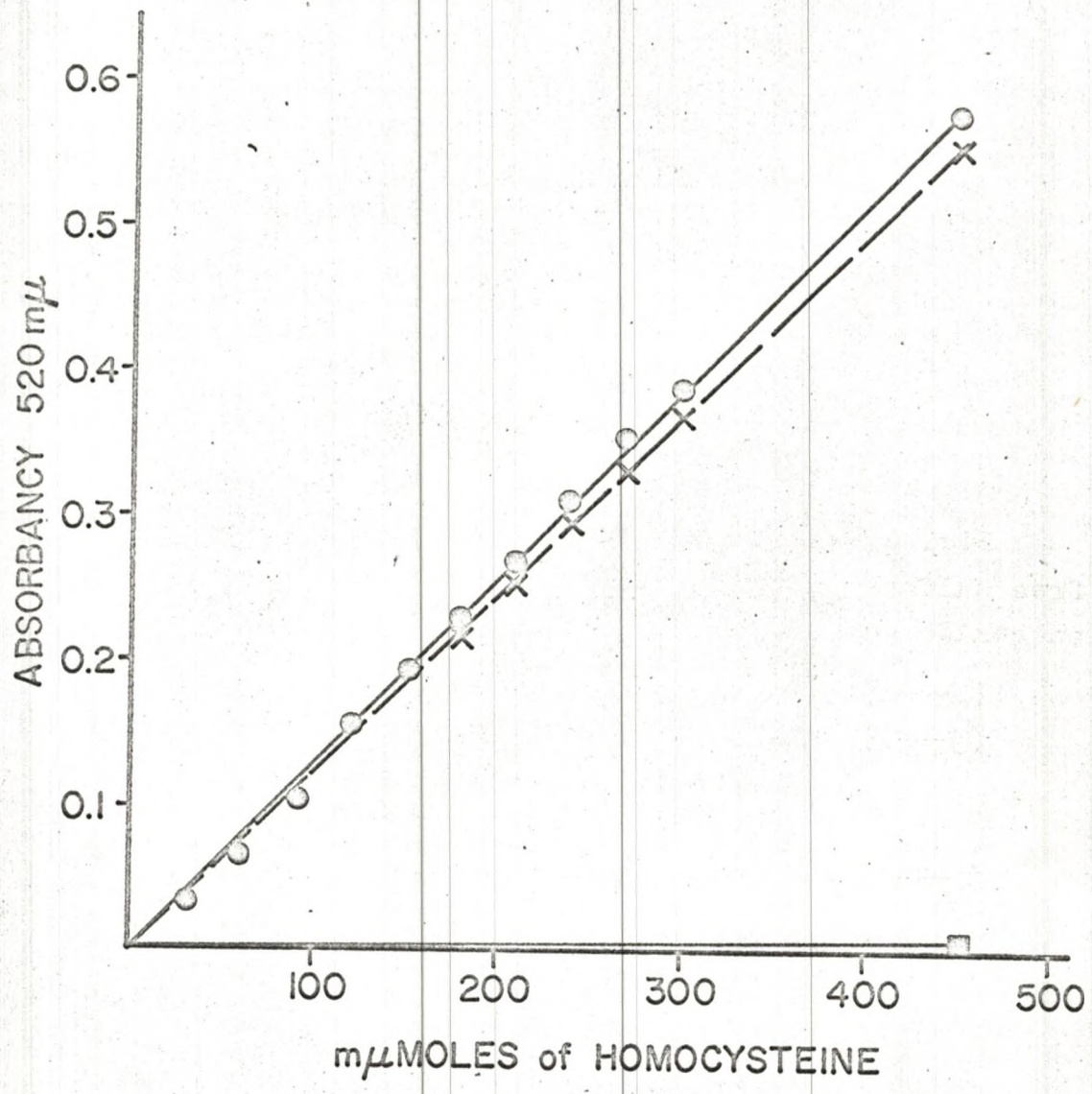
Free L-homocysteine added to the reaction mixture gave a linear response over a range of 30-450 mμmoles (Figure 6). L-Homocysteine free base was prepared by incubating 0.1 mmole of L-homocysteine thiolactone with 0.3 ml of 5.0 N NaOH for 5 minutes. The solution was then immediately neutralized with 1.5 ml of 1.0 M KH_2PO_4 and diluted to a volume of 20 ml with water giving a final concentration of 10 μmoles per ml. The linearity of the assay was not affected when homocysteine had been incubated with E. coli extract (2.0 mg) for 4 hours, nor did any measurable amount of

Fig. 6.--Reference curve for the determination of free sulfhydryl groups of homocysteine by the N-ethylmaleimide method.

(X— —X) = known quantities of L-homocysteine.

(●——●) = L-homocysteine incubated with E. coli extract (2.0 mg) for 4 hours at 37°C under an atmosphere of nitrogen.

(■——■) = E. coli extract alone.


ABSORBANCY 520 mμ
0.6
0.5
0.4
0.3
0.2
0.1
100
200
300
400
500
mμMOLES of HOMOCYSTEINE

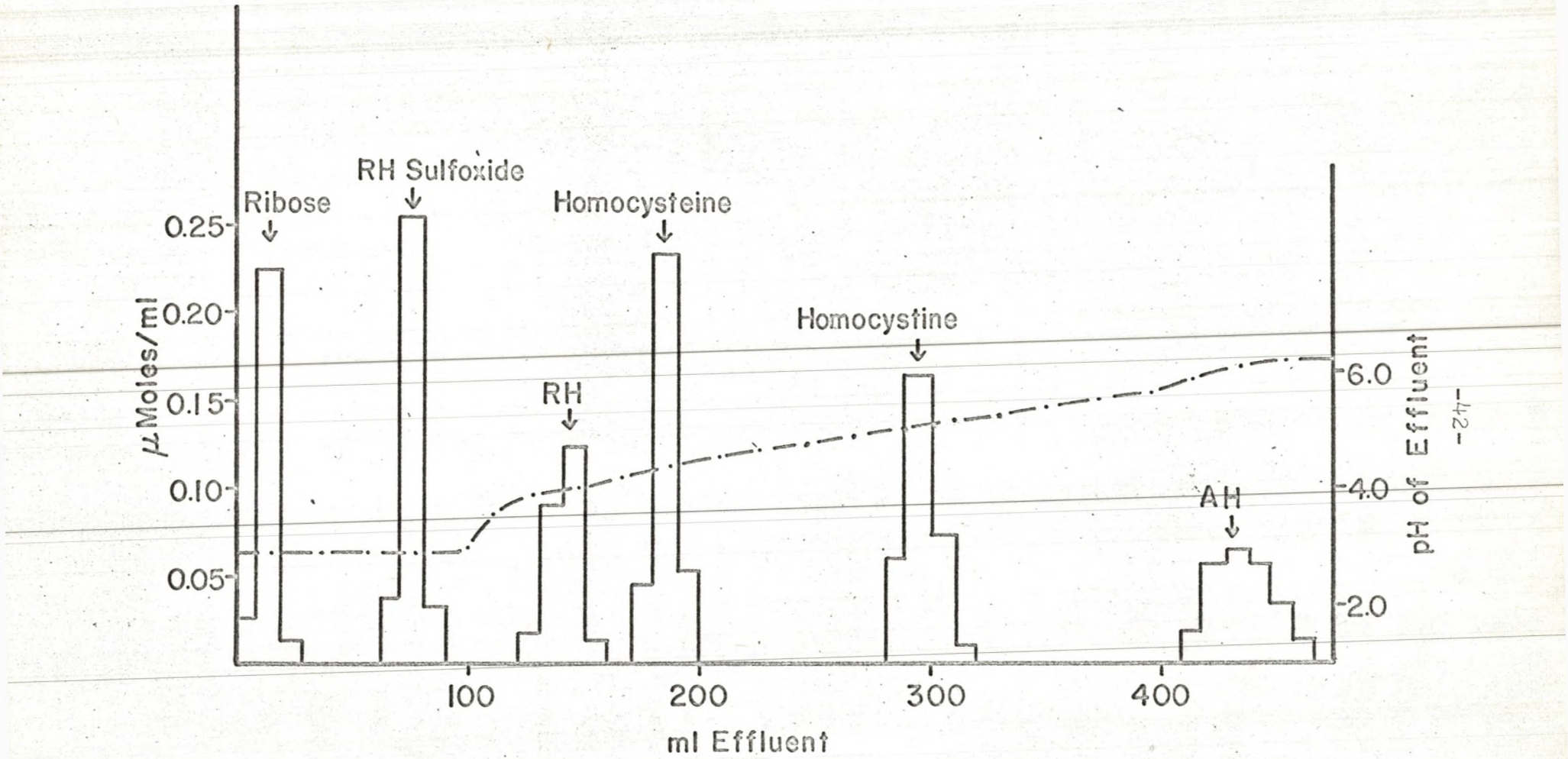
color result from deproteinized solutions without homocysteine. Neither homocysteine thiolactone, S-ribosylhomocysteine, nor S-ribosylhomocysteine sulfoxide gave positive reactions. One unit of enzyme was defined as that amount which catalyzed the generation of 1.0 mμmole of free sulfhydryl compound in 1 hour under the conditions described above.

Chromatography of Reaction Mixtures

Reaction mixtures were chromatogramed on a column (1 X 20 cm) of Amberlite CG-120, 200-400 mesh, resin. The column was previously washed with 200 ml of 1.0 N NaOH and equilibrated with 0.2 N citrate buffer, pH 2.9 . Elution was by a pH gradient obtained by allowing 250 ml of 0.2 N sodium citrate solution to flow into a mixing chamber containing an equal volume of 0.2 N citrate buffer, pH 2.9 . Both solutions contained 2.0 ml of thiodiglycol and 1.0 g Brij 35 per liter. The flow rate was 0.6 ml per minute, and the fractions were collected in 10-ml volumes. The elution patterns (Figure 7) and chemical analyses of known compounds were compared with those of products obtained from the reaction mixtures for identification purposes.

Fig. 7.-- Chromatographic separation of ribose, S-ribosylhomocysteine sulfoxide, S-ribosylhomocysteine, homocysteine, homocystine, and S-adenosylhomocysteine. A column of Amberlite CG-120 resin, 200-400 mesh, 1 X 20 cm, with a flow rate of 0.6 ml per minute was used. The column was washed with 200 ml 1.0 N NaOH, equilibrated with 0.2 N citrate buffer (pH 2.9),and eluted by a pH gradient formed by allowing 250 ml sodium citrate solution to flow into a mixing chamber containing an equal volume 0.2 N citrate buffer, pH 2.9. Both solvents contained 2.0 ml thiodiglycol and 1.0 g Brij-35 per liter. Reducing compounds were measured by the orcinol method and amino nitrogen was measured by the quantitative ninhydrin test.

RH = S-Ribosylhomocysteine.
AH = S-Adenosylhomocysteine.
(-.-) = pH gradient.


Ribose
RH Sulfoxide
Homocysteine
Homocystine
RH
AH
0.25
0.20
0.15
0.10
0.05
µMoles/ml
6.0
4.0
2.0
pH of Effluent
100
200
300
400
ml Effluent

Enzyme Preparation

Crude Extract

E. coli was cultured in 20-liter carboys containing 15 liters of Anderson's M-9 medium (Appendix I) (39). After the addition of an inoculum of 1.0% by volume of a 12-hour culture grown on the same medium, the cultures were incubated at 37°C for 12 hours. Vigorous aeration was maintained by forcing sterile air through the medium. After incubation the cells were harvested with a Servall continuous-flow centrifuge at 35,000 X g with a flow rate of approximately 35 ml per minute. The cells were washed twice with 0.1 M Tris buffer (pH 7.6) and resuspended in the same buffer at a concentration of about 400 mg per ml. The cells were disrupted in a nitrogen cooled pressure cell at 15-20,000 psi and remaining whole cells and cellular debris were removed by centrifugation at 15,000 X g for 20 minutes. The cell-free extract was then incubated for 1 hour at 37°C with deoxyribonuclease II and ribonuclease (2.0 mg each per 100 ml extract) and centrifuged at 15,000 X g for 20 minutes. This procedure yields a crude cell-free extract of approximately 20-30 mg protein per ml.

Sephadex G-25 Extract

The crude extract was passed through a column of

Sephadex G-25 (4 X 45 cm) previously equilibrated with 1 liter of 0.1 M Tris buffer, pH 7.6 . The hold-up volume of the column was 210 ml and the flow rate was 10 ml per minute. Elution was carried out with the same buffer used for equilibration. This procedure was not used as a method of protein fractionation but rather to remove small molecular weight materials from the enzyme preparation.

Ammonium Sulfate Fractionation

Ammonium sulfate, saturated at 4°C and adjusted to pH 7.0 , was added dropwise to Sephadex G-25 treated extract to 40% saturation. After mechanical stirring for 15 minutes the precipitate was removed by centrifugation at 15,000 X g for 20 minutes and discarded. The supernatant fluid was brought to 60% saturation and stirred for 15 minutes. The precipitate was again collected by centrifugation and resuspended in 0.1 M phosphate buffer, pH 7.8 . This fraction contained the S-adenosylhomocysteine nucleosidase used for the preparation of S-ribosylhomocysteine described earlier. The supernatant fluid from the 40-60% saturated fraction was then raised to 81% with solid ammonium sulfate. This precipitate, containing most of the S-ribosylhomocysteinase, was removed by centrifugation at 15,000 X g for 30 minutes and dissolved in 0.1 M Tris buffer (pH 7.6) at a concentration of 30-40 mg protein

per ml.

The method used for determining the amount of saturated ammonium sulfate necessary to attain a given saturation was as follows:

(ml Protein Solution + X)·Percent Saturation Needed = Percent Saturation of $(NH_4)_2SO_4$ · X

X = ml of Saturated $(NH_4)_2SO_4$ Needed

Example: (0-40% Saturation Needed)
(Volume of Extract = 192 ml)
(100% Saturated $(NH_4)_2SO_4$)

$$(192 + X).40 = 100.X$$
$$7680 + 40X = 100X$$
$$7680 = 60X$$
$$128 = X$$

The amount of solid ammonium sulfate required to pass from any percentage saturation to any other can be determined by the method of Dixon (40).

Sephadex G-150 Fractionation

From 400-500 mg protein of the 61-81% ammonium sulfate fraction was placed on a column of Sephadex G-150 (4 X 42cm) previously washed with 2 liters of water and equilibrated with 2 liters of 0.1 M Tris buffer (pH 7.6) containing 1 X 10^{-3} M EDTA: the same

buffer, without EDTA, was used as the eluting solvent. The column exhibited a hold-up volume of 180 ml and a flow rate of 1.4 ml per minute. The elution pattern was determined by ultraviolet absorption of each 10-ml fraction at 260 and 280 mμ using a Beckman DU spectrophotometer. The fractions were assayed for S-ribosylhomocysteinase activity, and those exhibiting the highest amount of enzymatic activity (fractions 42-51) were pooled and concentrated, by removal of water under reverse dialysis, to a volume of about 30 ml.

All procedures discussed above for the preparation of S-ribosylhomocysteinase were carried out at temperatures between 0-4°C unless otherwise stated.

RESULTS

Identification of Reaction Products Produced from Crude E. coli Extracts

A reaction mixture containing 6.0 µmoles of S-adenosylhomocysteine-H^3, 300 µmoles Tris buffer (pH 8.0), and 14 mg of Sephadex G-25 treated E. coli extract in a total volume of 3.0 ml was incubated for 2 hours at 37°C under an atmosphere of nitrogen. The mixture was then deproteinized and chromatogramed on a column of Amberlite CG-120 as described under"Materials and Methods". The elution pattern of the reaction mixture (Figure 8-A) was compared with that of the zero time control (Figure 8-B). The formation of two ninhydrin-positive, orcinol-positive, radioactively labelled compounds, one in the 190-210 ml fraction and the other in the 290-310 ml fraction, was noted in the reaction mixture. These two peaks corresponded to those obtained with homocysteine and homocystine respectively. The elution of two labelled, orcinol-positive, ninhydrin-positive compounds, one in the 130-170 ml fraction and the other in the 410-440 ml fraction, corresponded to the elution patterns obtained with S-ribosylhomocysteine and S-adenosylhomocysteine respectively. Carbohydrate passed directly through the column without retention and was recovered in the 10-20 ml fraction.

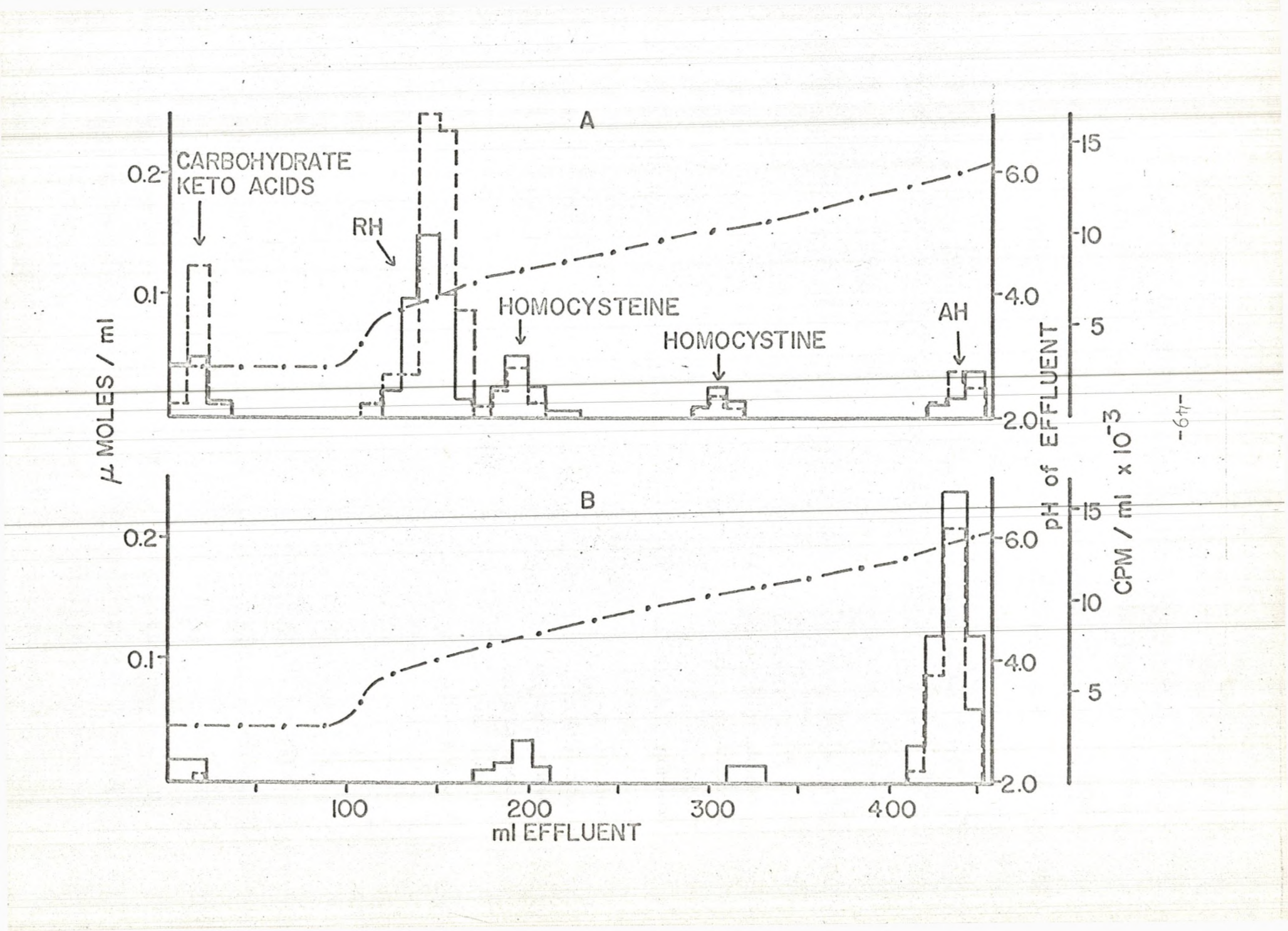
Fig. 8.--Chromatographic separation of reaction products produced from crude E. coli extracts. The chromatographic procedures used with Amberlite CG-120 resin, 200-400 mesh, are described in Figure 7. The two-hour reaction mixture contained 300 μmoles Tris buffer (pH 8.0), crude E. coli extract passed through Sephadex G-25 (4.8 mg protein per ml), and 6.0 μmoles S-adenosylhomocysteine-H^3 in a total volume of 3.0 ml. Amounts and specific activities of the compounds recovered are given in Table I.

A = reaction mixture.

B = zero time control.

(---) = radioactivity.

(-·-) = pH values.


A
CARBOHYDRATE
KETO ACIDS
RH
HOMOCYSTEINE
HOMOCYSTINE
AH
0.2
0.1
μ MOLES / ml
6.0
4.0
2.0
pH of EFFLUENT
15
10
5
B
0.2
0.1
6.0
4.0
2.0
15
10
5
CPM / ml x 10⁻³
100
200
300
400
ml EFFLUENT

A ninhydrin-negative material high in radioactivity appeared associated with the carbohydrate peak. Homocysteine oxidized with H_2O_2 and homocysteine thiolactone also passed through the column without retention under these conditions, but both were ninhydrin-positive. Since the substrate, S-adenosylhomocysteine-H^3, was labelled only in the homocysteine moiety and since the zero time control (Figure-8B) did not demonstrate a labelled compound in this region, it was indicated that the radioactivity in the 20 ml fraction from the reaction mixture was not attributable to the carbohydrate. Further analysis of this fraction revealed the presence of a significant amount of keto acid which suggested the formation of some deamination product of homocysteine.

The specific activity (cpm per μmole) of S-ribosylhomocysteine was 80% that of the substrate. This indicated that the bulk of this material was derived from S-adenosylhomocysteine (Table I). The specific activities of homocysteine, homocystine, and keto acids were 30%, 26%, and 38%, respectively, of that of the substrate indicating that a portion of these compounds was derived from S-adenosylhomocysteine. When endogenous amounts of ninhydrin-positive materials found in the zero time control were subtracted from the corresponding compounds in the reaction mixture, the specific activities of these three compounds were nearly the same as that of S-ribosyl-

TABLE I

PRODUCTS RECOVERED FROM S-ADENOSYLHOMOCYSTEINE-H^3

COMPOUND	REACTION		CONTROL	
	Amount Recovered μmoles	Specific Activity cpm/μmole	Amount Recovered μmoles	Specific Activity cpm/μmole
S-Adenosylhomocysteine-	0.63	43,500	5.70	43,500
S-Ribosylhomocysteine	3.70	35,000	--	--
Homocysteine	1.05	13,000	0.62	--
Homocystine	0.48	11,000	0.23	--
Keto Acids	1.10	16,400	0.42	300
Carbohydrate	1.00	--	0.30	--

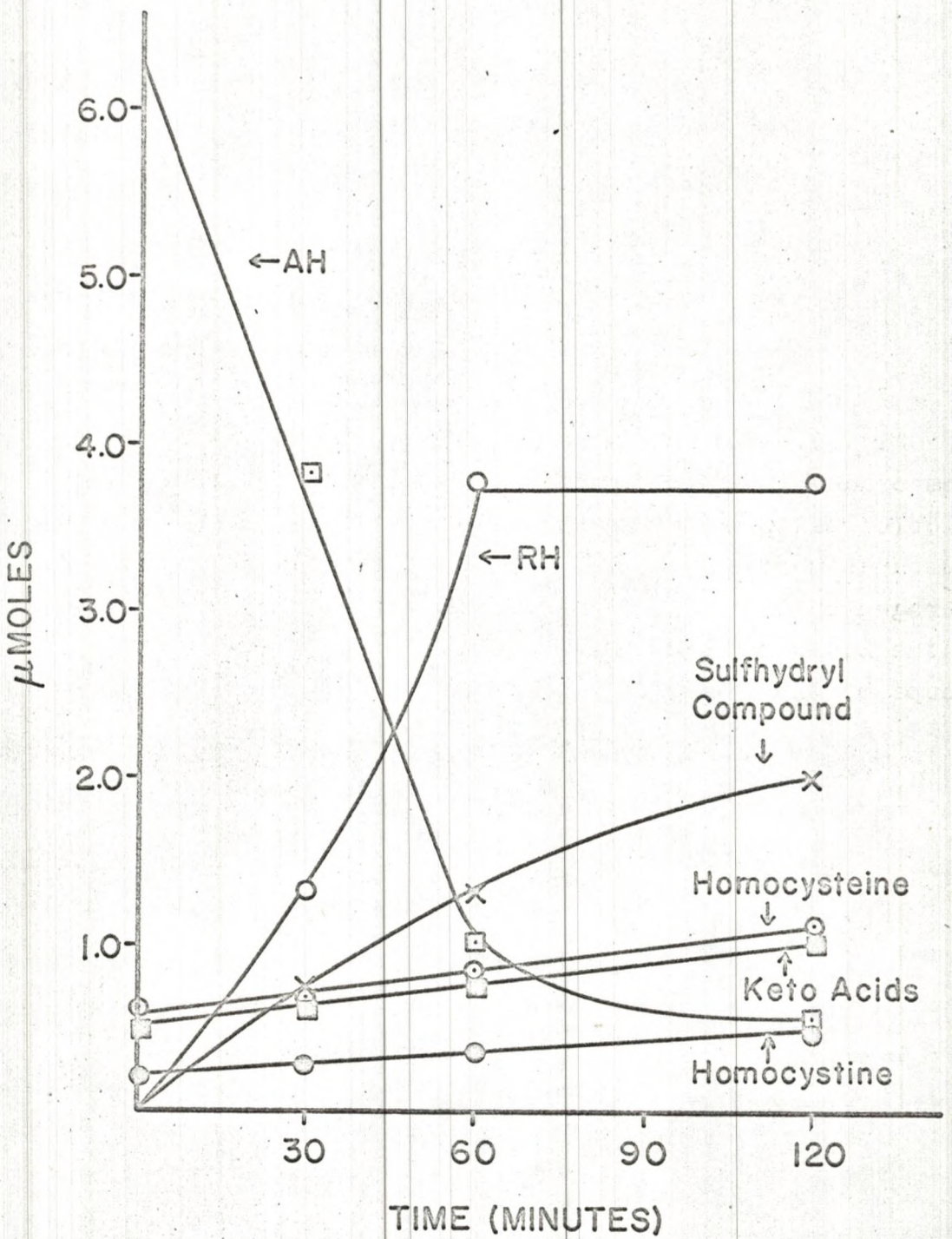
The two-hour reaction mixture contained 6.0 μmoles S-adenosylhomocysteine-H^3, 300 μmoles Tris buffer (pH 8.0), and 14 mg Sephadex G-25 treated extract in a total volume of 3.0 ml. Reducing compounds were measured by the orcinol method; amino nitrogen by the quantitative ninhydrin test; keto acids by the 2,4-dinitrophenyl hydrazine method.

homocysteine.

The amount of S-adenosylhomocysteine-H^3 degraded during the two-hour incubation was 5.1 μmoles of which 3.7 μmoles were recovered as S-ribosylhomocysteine (Table I). If homocystine and keto acids were formed from the oxidation and deamination of homocysteine, respectively, then the total amount of homocysteine formed during incubation would have been 1.6 μmoles. The sum of the amounts of S-ribosylhomocysteine and homocysteine (5.3 μmoles) would then be approximately the same as the amount of S-adenosylhomocysteine-H^3 utilized. The amount of carbohydrate formed, less the endogenous, was not equivalent to the amount homocysteine plus products derived from homocysteine.

Figure 9 shows the formation and breakdown of various compounds concerned in the reaction, previously described, as a function of time. S-Adenosylhomocysteine-H^3 was degraded by S-adenosylhomocysteine nucleosidase, present in the Sephadex G-25 extract, yielding S-ribosylhomocysteine. As the bulk of the S-adenosylhomocysteine-H^3 was utilized the formation of S-ribosylhomocysteine decreased. The thioether linkage of S-ribosylhomocysteine appeared to undergo cleavage generating free homocysteine, part of which was oxidized to homocystine and part of which was deaminated to keto acids.

Fig. 9.--Formation of reaction products produced from crude E. coli extracts as a function of time. The reaction mixture is described in Figure 8. Reducing compounds were measured by the orcinol method; amino nitrogen by the quantitative ninhydrin test; keto acids by the 2,4-dinitrophenyl hydrazine test; free sulfhydryl groups by the N-ethylmaleimide method.


6.0
5.0
4.0
3.0
2.0
1.0
μMOLES
←AH
←RH
Sulfhydryl
Compound
Homocysteine
Keto Acids
Homocystine
30
60
90
120
TIME (MINUTES)

Since the bulk of the free sulfhydryl compounds could be titrated with N-ethylmaleimide (Figure 9), oxidation of homocysteine apparently took place during chromatography of samples of the reaction mixture. Also, when samples containing free homocysteine were allowed to stand in air oxidation of homocysteine to homocystine increased.

Partial Purification

Attempts to purify the enzyme which cleaved S-ribosylhomocysteine, tentatively designated S-ribosylhomocysteinase, from E. coli cell-free extracts have produced purifications ranging from 2 to 12 fold. The procedure which yielded a partially purified extract demonstrating the greatest stability with respect to S-ribosylhomocysteinase is shown in Table II. The exact procedures for each step in the purification are described under "Materials and Methods".

The crude extract was first passed through Sephadex G-25 to remove small molecular weight materials and was then subjected to ammonium sulfate fractionation. The fraction which precipitated between 40-60% saturation contained 70% of the original S-adenosylhomocysteine nucleosidase activity. Only 16% of the original S-ribosylhomocysteinase activity was found in this fraction

TABLE II

PURIFICATION OF S-RIBOSYLHOMOCYSTEINASE FROM E. COLI STRAIN W AND ITS SEPARATION FROM S-ADENOSYLHOMOCYSTEINE NUCLEOSIDASE

PROCEDURE	VOLUME	PROTEIN		NUCLEOSIDASE		S-RIBOSYLHOMOCYSTEINASE						
	ml	mg/ml	Total mg	Units/mg AH*	Total Units $x10^{-6}$ AH	Units/mg RH**	AH	Units/ml $x10^{-3}$ RH	AH	Total Units $x10^{-5}$ RH	Purification	Yield %
Crude	234	28	6552	2560	16.9	147	53	4.1	1.5	9.6	1	100
Sephadex G-25	240	24	4600	3840	16.6	244	126	4.4	2.3	9.5	1.7	99
40-60% $(NH_4)_2SO_4$	50	39	1950	5900	12.5	85	60	3.3	2.3	1.7	--	--
61-81% $(NH_4)_2SO_4$	21	36	750	260	0.2	392	30	13.6	1.1	2.9	2.7	29
Sephadex G-150	100	0.5	52	1080	0.05	710	25	0.36	0.01	0.37	5.0	4

* S-Adenosylhomocysteine
** S-Ribosylhomocysteine

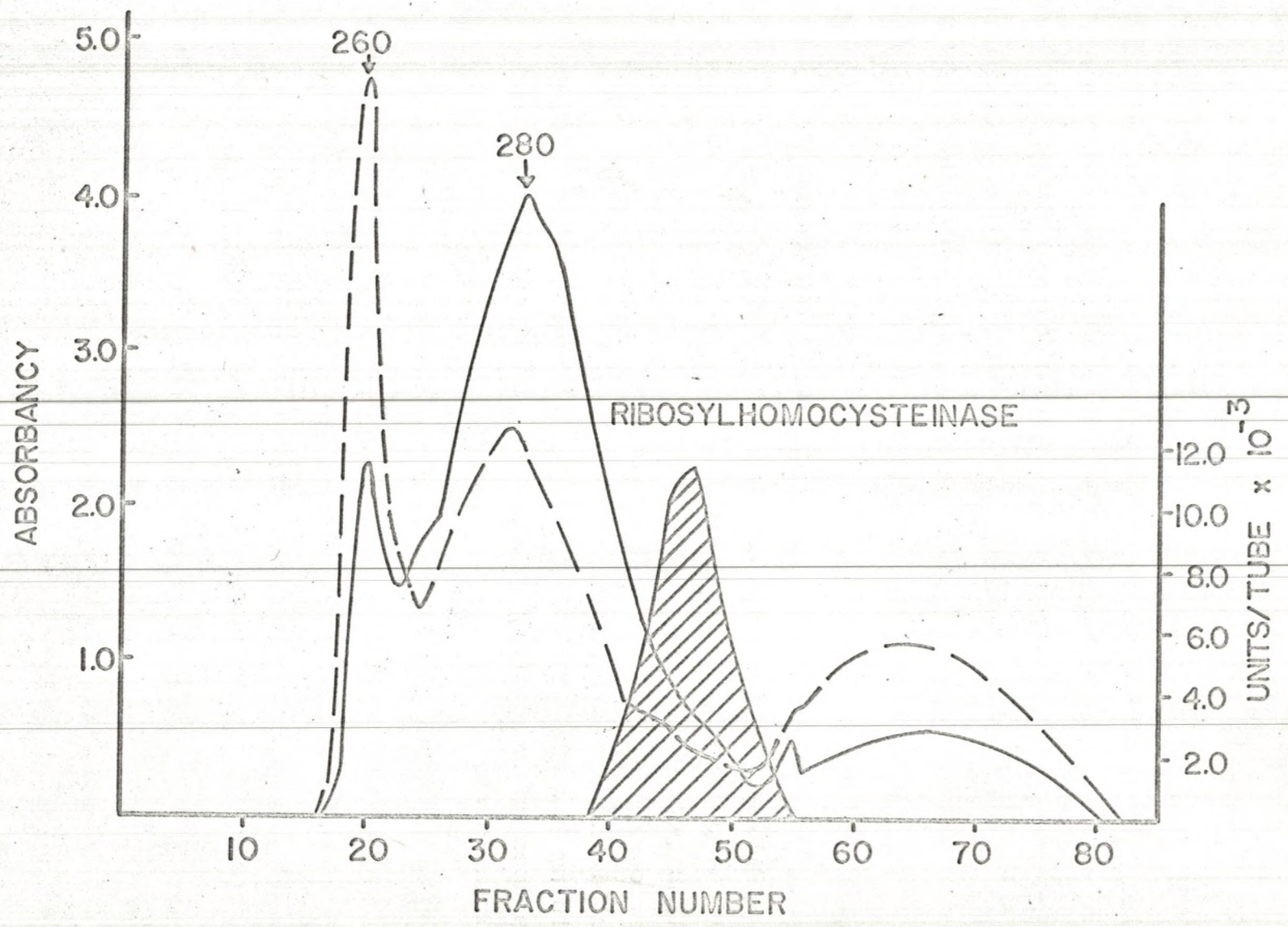
when S-ribosylhomocysteine was used as substrate. The supernatant fluid from the 40-60% fraction was raised to 81% saturation. The precipitate from this fraction contained 30% of the original S-ribosylhomocysteinase activity and yielded a 2.7-fold purification. Only 1.2% of the original S-adenosylhomocysteine nucleosidase activity was present in this fraction. No measurable S-ribosylhomocysteinase activity was detected in the fraction precipitating between 82-90% of ammonium sulfate saturation. The 61-81% ammonium sulfate fraction was chromatogramed on a column of Sephadex G-150. The elution pattern of the S-ribosylhomocysteinase is shown in Figure 10. Pooled fractions 42-52 contained 4% of the original S-ribosylhomocysteinase activity and yielded a 5-fold purification and only 0.3% of the original S-adenosylhomocysteine nucleosidase activity. This fraction contained 0.8% of the total protein initially present in the crude extract.

After passage of the extract through Sephadex G-25, an increase in activity (150%) of S-adenosylhomocysteine nucleosidase was noted. A concomitant increase (230%) in S-ribosylhomocysteinase activity with S-adenosylhomocysteine as substrate was also noted; with S-ribosylhomocysteine an increase of 165% in activity was observed. The apparent decrease in the ratio of specific activities (RH/AH)[2] might be attributed to the

---

[2] RH = S-Ribosylhomocysteine
AH = S-Adenosylhomocysteine

Fig. 10.--Gel filtration of the 61-81% ammonium sulfate-precipitated fraction on Sephadex G-150. The column (4 x 42 cm, flow rate 1.4 ml per minute), was previously washed with 2 liters of water and equilibrated with 2 liters of 0.1 M Tris buffer containing 1 x 10^{-3} M EDTA: the same buffer, without EDTA, was used as the eluting agent. The S-ribosylhomocysteinase assay is described under "Materials and Methods".


260
280
5.0
4.0
3.0
2.0
1.0
ABSORBANCY
RIBOSYLHOMOCYSTEINASE
12.0
10.0
8.0
6.0
4.0
2.0
UNITS/TUBE x 10⁻³
10
20
30
40
50
60
70
80
FRACTION NUMBER

necessity of cleavage of the glycosyl bond of S-adenosylhomocysteine prior to cleavage of the thioether linkage. This observation was further established by the finding that the ratio in the 61-81% ammonium sulfate fraction increased from 2.8 in the crude to 13.1 in this fraction. Further increase in this ratio (28.4) was noted after passage of the 61-81% ammonium sulfate fraction through Sephadex G-150. These increases were apparently due to the removal of S-adenosylhomocysteine nucleosidase during purification.

The 61-81% saturated ammonium sulfate fraction after passage through Sephadex G-25 failed to yield an increase in specific activity when adsorbed onto Cγ alumina or calcium phosphate gels at various buffer concentrations. Passage of the same extract through a column of Sephadex G-100 using Tris buffer (0.1 M, pH 7.6) as the eluting solvent resulted in no increase in specific activity of the enzyme. Gel filtration of the 61-81% ammonium sulfate fraction on a column of Sephadex G-200 using the same buffer as the eluting agent gave only a slight increase in specific activity and all measurable S-ribosylhomocysteinase activity was lost within 24 hours. The enzyme activity could not be recovered after Sephadex G-150 treated E. coli extract was passed through a column of DEAE-cellulose using a salt concentration gradient (0.1 M Tris buffer, pH 7.6 and 1.0 M NaCl in the same buffer) as the eluting agent.

Ethyl alcohol fractionation (65-80% saturation) of the Sephadex G-150 treated *E. coli* extract gave a final 12-fold purification of S-ribosylhomocysteinase, but all measurable activity was lost within 24 hours at 4°C.

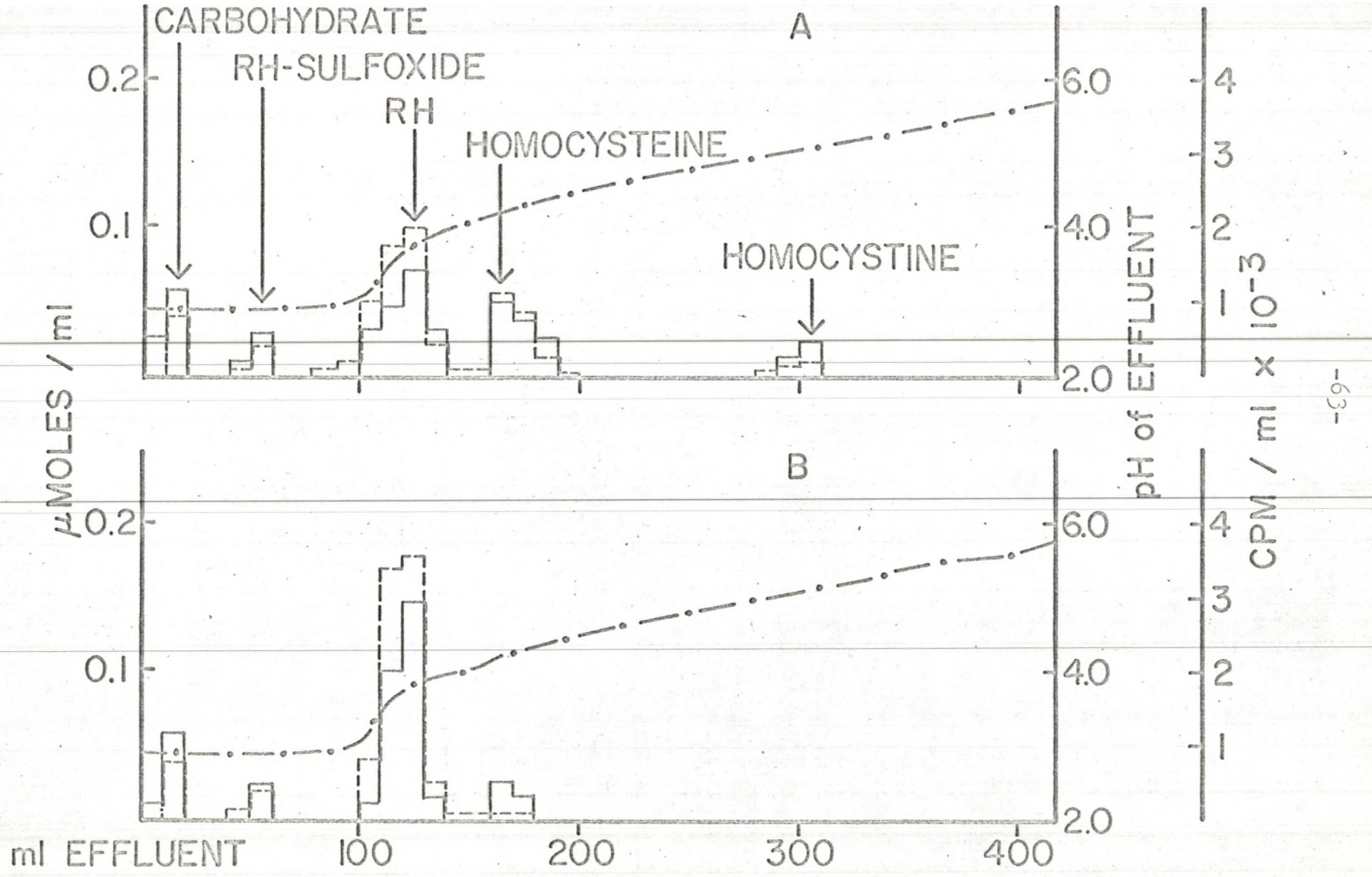
Identification of Reaction Products Produced from Partially Purified E. coli Extracts

Identification of reaction products was determined from reaction mixtures containing the partially purified (Sephadex G-150) extract. The reaction mixture containing 3.0 µmoles of S-ribosylhomocysteine-H^3, 300 µmoles Tris buffer (pH 8.0), and 1.3 mg Sephadex G-150 extract in a total volume of 3.0 ml was incubated for 2 hours at 37°C under an atmosphere of nitrogen. The mixture was deproteinized, assayed for free sulfhydryl compounds, and chromatogramed on a column of Amberlite CG-120 as described under "Materials and Methods". The elution patterns of the reaction mixture and of the zero time control are shown in Figure 11-A and 11-B respectively.

A ninhydrin-positive orcinol-negative compound in the 160-180 ml fraction was observed. This compound corresponded to free homocysteine and had a specific activity of 23,000 cpm per µmole as compared to 26,000 cpm per µmole for the substrate. Another ninhydrin-positive, orcinol-negative, radioactive compound was

Fig. 11.--Chromatographic separation of reaction products produced from partially purified (Sephadex G-150 treated) E. coli extract. The chromatographic procedures are described in Figure 7. The two-hour reaction mixture contained 3.0 μmoles S-ribosylhomocysteine-H^3, 300 μmoles Tris buffer (pH 8.0), and 1.3 mg of Sephadex G-150 treated E. coli extract in a total volume of 3.0ml.

A = reaction mixture.
B = zero time control.
(---) = radioactivity.
(-·-) = pH values.


CARBOHYDRATE
RH-SULFOXIDE
RH
HOMOCYSTEINE
HOMOCYSTINE
A
B
0.2
0.1
6.0
4.0
2.0
4
3
2
1
μMOLES / ml
pH of EFFLUENT
CPM / ml x 10⁻³
ml EFFLUENT
100
200
300
400

found in the 290-300 ml fraction and exhibited an elution pattern corresponding to that of homocystine. The orcinol-positive, ninhydrin-positive, labelled compound in the 100-140 ml fraction gave an elution pattern similar to that of S-ribosylhomocysteine, and the orcinol-positive, ninhydrin-positive, labelled material in the 50-60 ml fraction gave an elution pattern similar to that of S-ribosylhomocysteine sulfoxide. In the 20 ml fraction an orcinol-positive ninhydrin-negative material was obtained giving the same elution pattern as that of ribose.

A radioactive material was observed in the 20 ml fraction; however, this was also present in the zero time control. Since S-ribosylhomocysteine was prepared enzymatically using partially purified S-adenosylhomocysteine nucleosidase containing small amounts of S-ribosylhomocysteinase, traces of the S-ribosylhomocysteine formed may have been cleaved generating homocysteine. Thus, contaminating amounts of homocysteine persent in the zero time control may have been deaminated to keto acids. Therefore, keto acids found in the 20 ml fraction could account for this labelled compound observed in the zero time control. The amount of S-ribosylhomocysteine-H^3 recovered in the zero time control (Figure 11-B) was 2.8 µmoles and in the reaction mixture (Figure 11-A) 1.7 µmoles; thus, 1.1 µmoles were utilized

in the two-hour reaction. The amount of homocysteine recovered was 0.7 μmoles and of homocystine 0.3 μmoles. The specific activity of the carbohydrate peak in the reaction mixture was essentially the same as that of the corresponding peak in the zero time control. This indicated that the homocysteine generated in the reaction mixture was not deaminated to keto acids as was suggested to occur with the crude extract. Oxidation of homocysteine to homocystine was still observed and accounted for 0.6 μmoles of homocysteine. The total amount of homocysteine generated in the reaction mixture was, therefore, 1.3 μmoles which approximately agrees with the amount of S-ribosylhomocysteine utilized. The amount of carbohydrate recovered form the reaction mixture, less the endogenous, was not equivalent to the amount of S-ribosylhomocysteine utilized.

Properties of the Enzyme

Stability

The stability of the 61-81% ammonium sulfate fraction was studied using phosphate and Tris buffers at various concentrations and pH values. Aliquots of the washed precipitate dissolved in phosphate buffer (0.1 M and 0.05 M, pH 7.6) exhibited a marked reduction in S-ribosylhomocysteinase activity. Samples were also

dissolved in 0.05 M and 0.1 M Tris buffer ranging in pH from 6.8 to 8.7 . Those in 0.05 M and 0.1 M (pH 8.2 and 8.7) lost 80% of the S-ribosylhomocysteinase activity after 7 days at 4°C, and those in 0.1 M (pH 6.8) demonstrated no initial activity. Tris buffer, 0.1 M (pH 7.6), provided the best conditions for stability of S-ribosylhomocysteinase. Figure 12 compares the stability of the crude extract, the ammonium sulfate fraction (61-81%), and the Sephadex G-150 treated preparation. Each was prepared in 0.1 M Tris buffer (pH 7.6) and divided into several aliquots which were immediately frozen. All samples were thawed only once before being assayed for S-ribosylhomocysteinase activity. The crude extract (20 mg protein per ml) retained 82% of its original activity after 32 days, and the 61-81% ammonium sulfate fraction (36 mg protein per ml) retained 93% of its original activity after 14 days. The Sephadex G-150 treated preparation (3 mg protein per ml) lost all measurable activity after 7 days.

pH Optimum

The optimum pH for cleavage of the thioether linkage of S-ribosylhomocysteine by the partially purified enzyme (Sephadex G-150) was between 8.0 and 8.1 . The dependency of the enzymatic reaction upon pH in shown in Figure 13. The two-hour reaction mixture contained

Fig. 12.--Effect of storage at -15°C on various fractions of S-ribosylhomocysteinase. All samples were thawed only once before assay of enzyme activity by the N-ethylmaleimide test described under "Materials and Methods".

(●-.-●) = crude extract.

(●---●) = 61-81% ammonium sulfate-precipitated extract.

(●——●) = Sephadex G-150 treated extract.

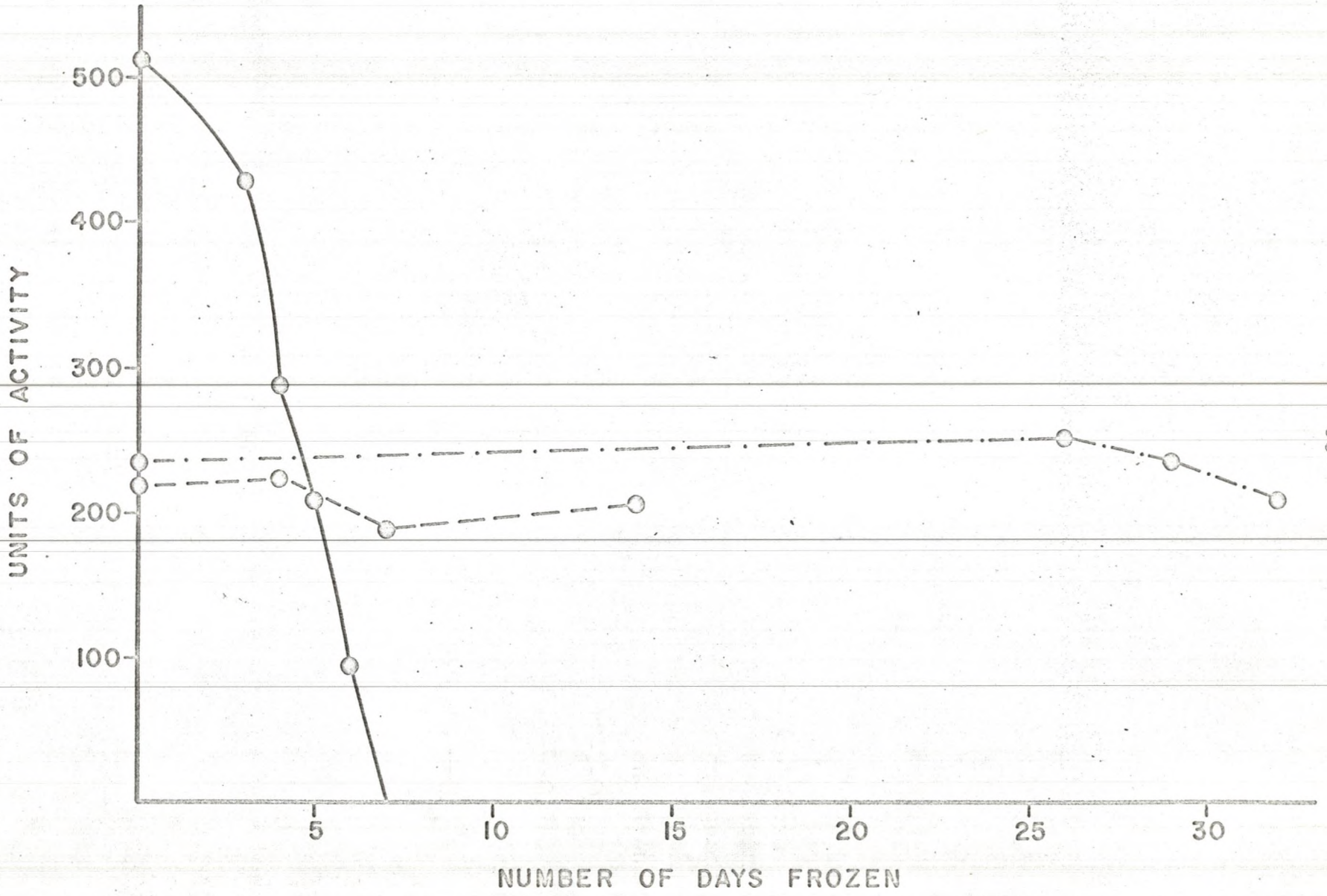
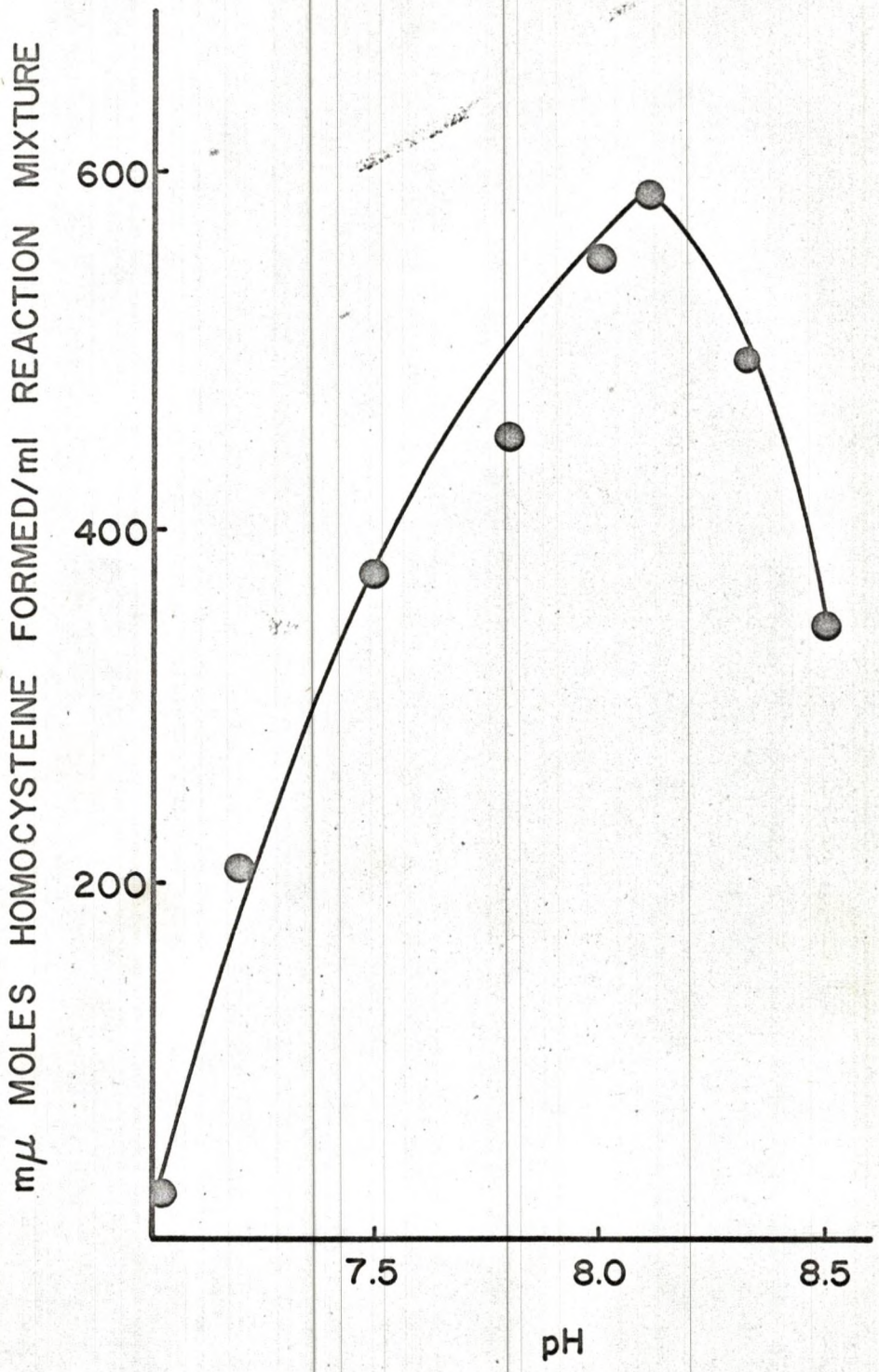

500
400
300
200
100
UNITS OF ACTIVITY
5
10
15
20
25
30
NUMBER OF DAYS FROZEN

Fig. 13.--Dependency of the enzymatic reaction on pH. Each tube contained 2.0 µmoles of S-ribosylhomocysteine, 0.7 mg of Sephadex G-150 treated extract, and 100 µmoles Tris (at varying pH values) in a total volume of 1.0 ml. The pH values which appear in the figure were measured at the end of the one-hour incubation. Activity was measured by the N-ethylmaleimide test described under "Materials and Methods".


mμ MOLES HOMOCYSTEINE FORMED/ml REACTION MIXTURE
600
400
200
7.5
8.0
8.5
pH

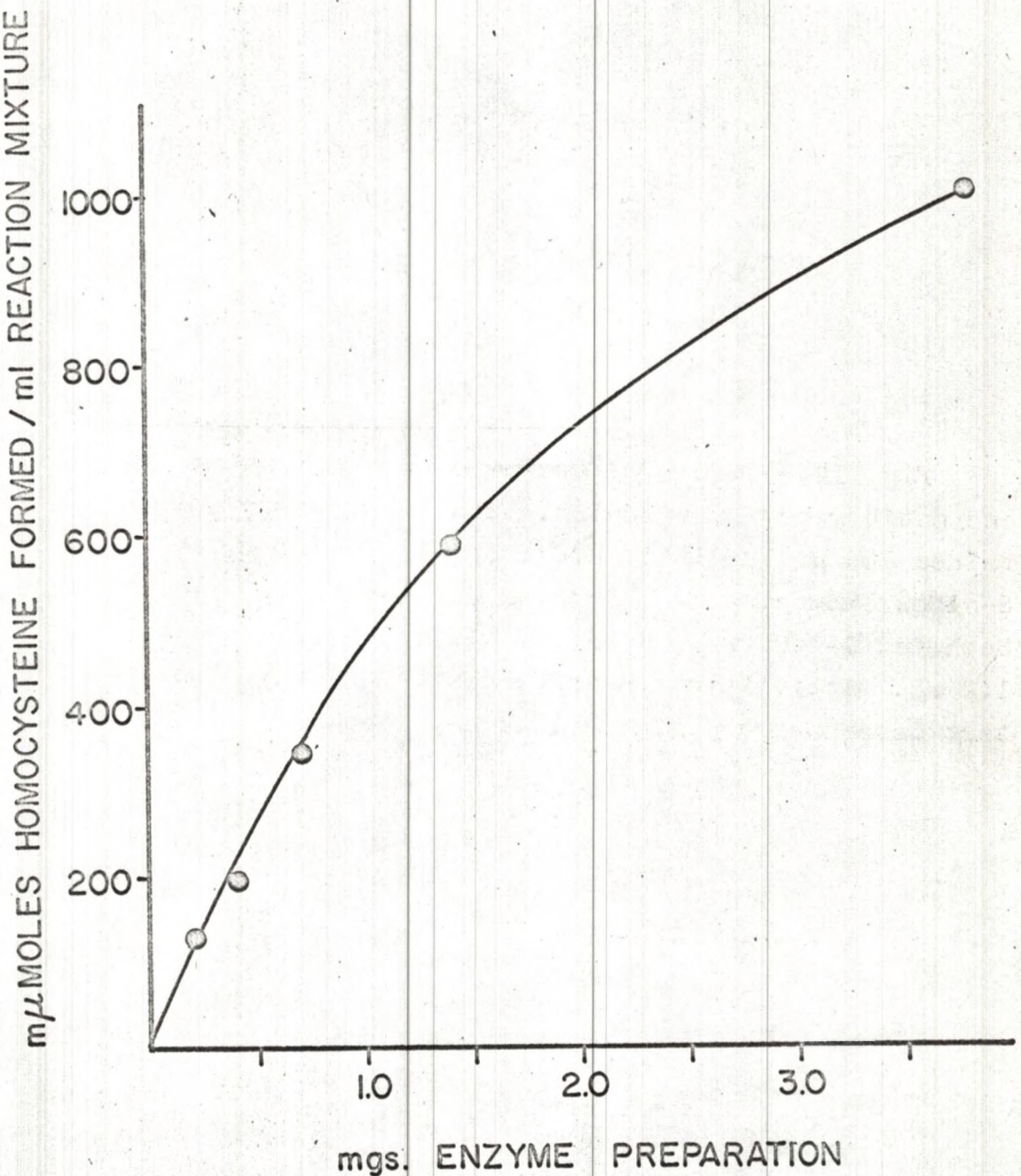
2.0 μmoles of S-ribosylhomocysteine, 0.7 mg of Sephadex G-150 treated extract, and 100 μmoles of Tris buffer in a total volume of 1.0 ml. The pH values of the reaction mixtures were determined at the end of incubation. No measurable activity resulted below pH 7.0 . Unpredictable results were obtained when reaction mixtures exhibiting a final pH above 8.6 were assayed. This did not occur in reaction mixtures exhibiting a final pH below 8.6.

Effect of Protein Concentration on Catalytic Reaction

The partially purified Sephadex G-150 treated extract was used to study the relationship between the rate of formation of free homocysteine and enzyme concentration. The reaction mixtures containing varying amounts of extract were incubated for 1 hour at 37°C, deproteinized , and assayed for the presence of free sulfhydryl groups. Figure 14 shows that enzyme concentration up to 1.0 mg per ml reaction mixture gave a linear response of S-ribosylhomocysteinase activity. As the enzyme concentration increased above 1.0 mg per ml reaction mixture, the activity correspondingly decreased, apparently due to depletion of substrate or to the presence of an inhibitor in the enzyme preparation.

Figure 15 compares S-ribosylhomocysteinase activity

Fig. 14.--Effect of enzyme concentration on the enzymatic reaction velocity. Reaction mixtures contained 100 µmoles Tris buffer (pH 8.0), 2.0 µmoles S-ribosylhomocysteine, and varying amounts of Sephadex G-150 treated extract in a total volume of 1.0 ml. Activity was measured by the N-ethylmaleimide test described under "Materials and Methods".


mμMOLES HOMOCYSTEINE FORMED / ml REACTION MIXTURE
1000
800
600
400
200
1.0
2.0
3.0
mgs. ENZYME PREPARATION

at two different concentrations of enzyme (Sephadex G-150) to time of incubation. The reaction mixture containing 0.35 mg protein per ml gave a linear response of S-ribosylhomocysteinase activity for approximately 1 hour at 37°C, and the mixture containing 2.1 mg protein per ml gave a linear response of enzyme activity for approximately 45 minutes. The falling off of enzyme activity with continued incubation with both concentrations was possibly due to inhibition by products of the reaction, decrease in saturation of the enzyme with substrate, or inactivation of the enzyme.

Determination of Km

Reaction mixtures were prepared containing varying amounts of S-ribosylhomocysteine ranging from 5.0×10^{-4} to 4.0×10^{-3} M (0.5 to 4.0 μmoles per ml reaction mixture). The mixtures were incubated for 1 hour, deproteinized, and assayed for free sulfhydryl groups. For determination of Km the Lineweaver-Burk plot (42) was used comparing the reciprocals of substrate concentration to the reciprocals of S-ribosylhomocysteinase activity (Figure 16). When 1/v was plotted against 1/S, where (v) equals reaction velocity and (S) equals substrate concentration, a straight line was obtained which intercepted the base line at a point giving -1/Km and which intercepted the vertical axis at a point giving

Fig. 15.--Comparison of reaction velocity as a function of time with two protein concentrations. Reaction mixtures contained 100 μmoles Tris buffer (pH 8.0), 2.0 μmoles S-ribosylhomocysteine, and Sephadex G-150 treated E. coli extract in a total volume of 1.0 ml. Activity was measured by the N-ethylmaleimide test described under "Materials and Methods".

●——● = 2.1 mg protein per ml.

●---● = 0.35 mg protein per ml.

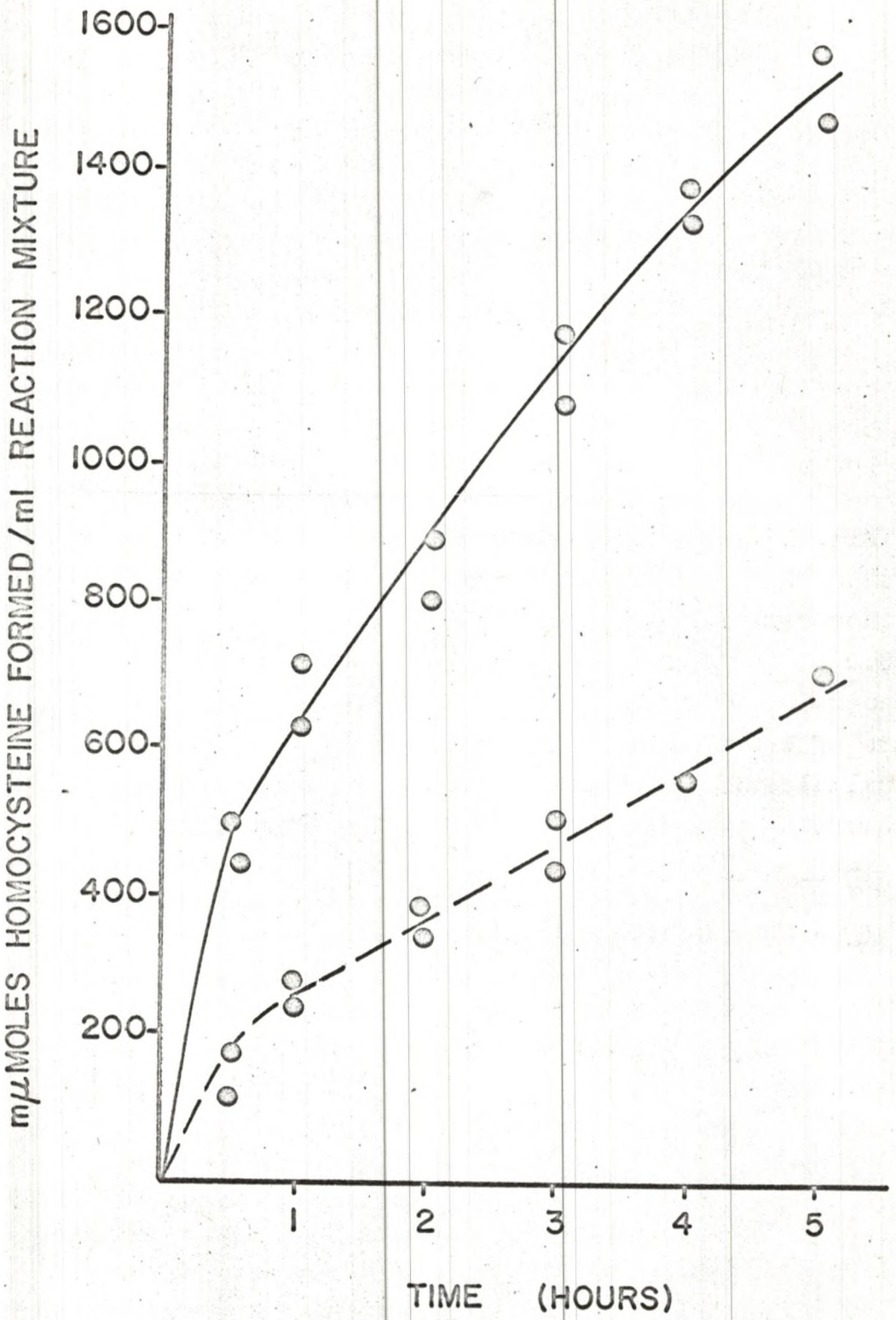
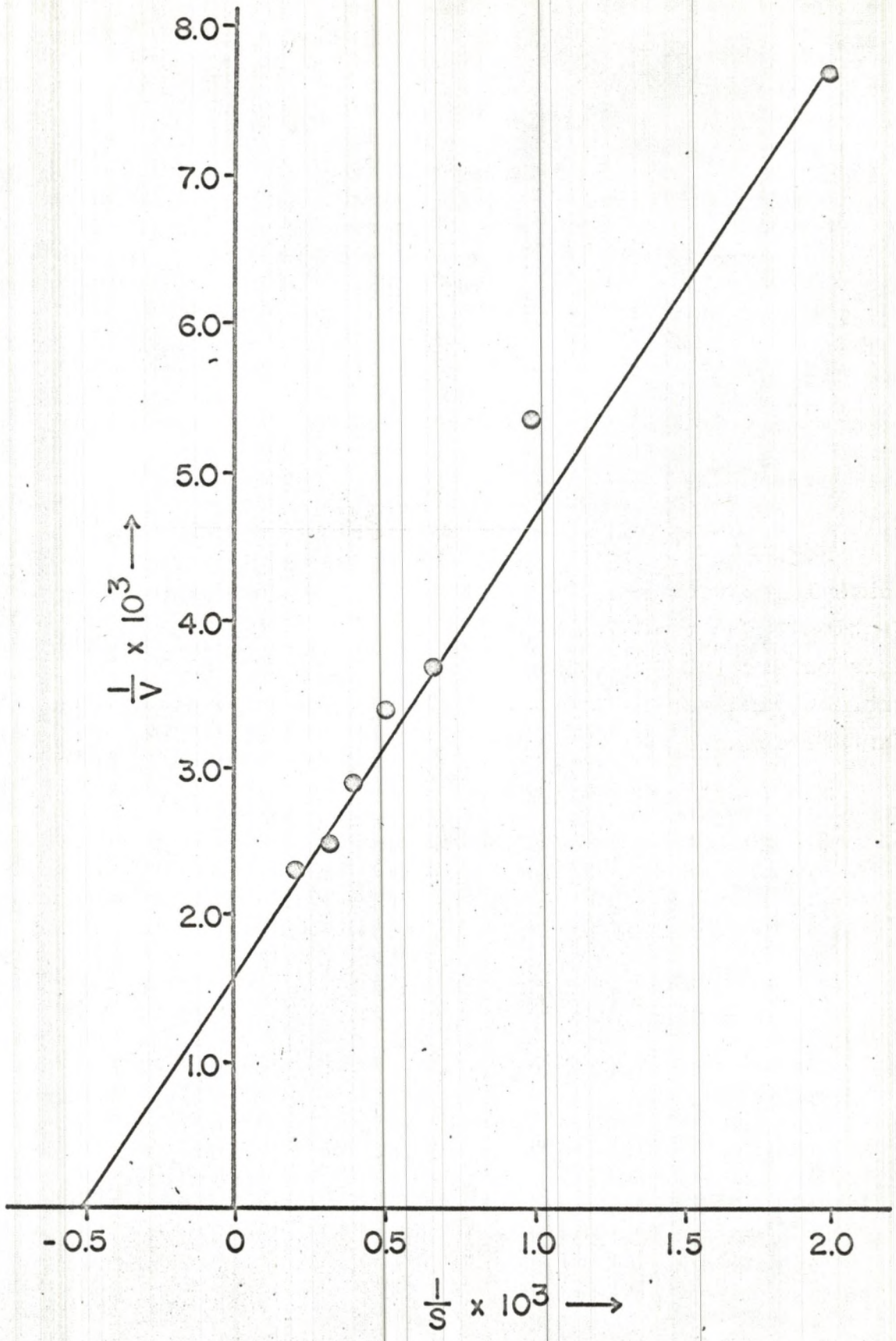

1600
1400
1200
1000
800
600
400
200
mμMOLES HOMOCYSTEINE FORMED/ml REACTION MIXTURE
1
2
3
4
5
TIME (HOURS)

Fig. 16.--Lineweaver-Burk reciprocal plot of initial reaction velocity versus substrate concentration. The reaction mixtures contained 100 μmoles Tris buffer (pH 8.0), 1.0 mg Sephadex G-150 treated extract, and varying amounts of S-ribosylhomocysteine. Further details are given in the text.

v = reaction velocity.

S = substrate concentration (molarity).


8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
$\frac{1}{V} \times 10^3 \longrightarrow$
-0.5
0
0.5
1.0
1.5
2.0
$\frac{1}{S} \times 10^3 \longrightarrow$

1/Vm (Km is the Michaelis constant and Vm is the maximal velocity).

The Km value for S-ribosylhomocysteinase under these conditions was found to be 1.94×10^{-3} M. The value for Vm was found to be 625 mμmoles of homocysteine formed per hour per ml reaction mixture.

DISCUSSION

The results of preliminary investigations indicated that the thioether linkage of S-ribosylhomocysteine was cleaved by cell-free extracts of Escherichia coli. One of the reaction products was tentatively identified as homocysteine by paper chromatography of the N-ethylmaleimide derivative.

In the present study using partially purified enzyme, homocysteine has definitely been established as one of the reaction products by ion exchange chromatography. However, the amount of free homocysteine recovered in the free sulfhydryl form was not equivalent to the amount of substrate used. When S-ribosylhomocysteine-H^3 was used as substrate, labelled homocystine, an oxidation product of homocysteine, was found. When the amount of this oxidation product recovered was added to the amount of the free sulfhydryl form found, recovery of homocysteine was essentially quantitative.

The amount of carbohydrate, less the endogenous, was not equivalent to the amount of homocysteine plus products derived from homocysteine in either the reaction mixtures of crude extracts or those of partially purified extracts. This suggests that ribose *per se* was not formed or that some ribose trapping agent present

in the extracts was interfering with its recovery. Identification of reaction products using S-ribosylhomocysteine labelled in the ribose moiety as substrate, will be used to clarify this point.

Duerre and Miller (33) suggested that cleavage of the glycosidic bond of S-adenosylhomocysteine by S-adenosylhomocysteine nucleosidase was required prior to cleavage of the thioether linkage by S-ribosylhomocysteinase. To further elucidate the cleavage of this compound, attempts were made during the purification of S-ribosylhomocysteinase to remove as much of the S-adenosylhomocysteine nucleosidase as possible. Separation of these two enzymes was achieved during the ammonium sulfate fractionation. Most of the S-adenosylhomocysteine nucleosidase was obtained with 40-60% saturation and the S-ribosylhomocysteinase with 61-81% saturation. Only a small amount (0.3%) of the S-adenosylhomocysteine nucleosidase remained after further fractionation of the S-ribosylhomocysteinase on Sephadex G-150. The ratio of the specific activity obtained with S-ribosylhomocysteine to that obtained with S-adenosylhomocysteine as substrates demonstrated the necessity of cleavage of the glycosidic bond of S-adenosylhomocysteine prior to cleavage of the thioether linkage. If S-ribosylhomocysteinase had cleaved the thioether linkage of S-adenosylhomocysteine without prior degradation by the

nucleosidase, the ratio of specific activities involving both substrates would have been the same throughout purification.

Lability of S-ribosylhomocysteinase was noted after several of the attempted fractionation procedures. Thus, the possibility exists that the enzyme is protected by as yet unknown factors in the extract. Metal ion inhibition or removal of a required cofactor may explain these reductions or loses in activity under various conditions. However, cofactor requirements or the effect of possible inhibitors could not be demonstrated during the course of this study.

During the determination of the pH optimum for cleavage of the thioether linkage of S-ribosylhomocysteine, unpredictable results were obtained from those reaction mixtures exhibiting a final pH above 8.6 when assayed for free sulfhydryl groups by the N-ethylmaleimide method. It was observed that significant amounts of protein remained in these reaction mixtures after heating for 3 minutes in a boiling water bath as compared to those exhibiting a final pH below 8.6 . Thus, free sulfhydryl groups in the unprecipitated protein may have interfered with the assay.

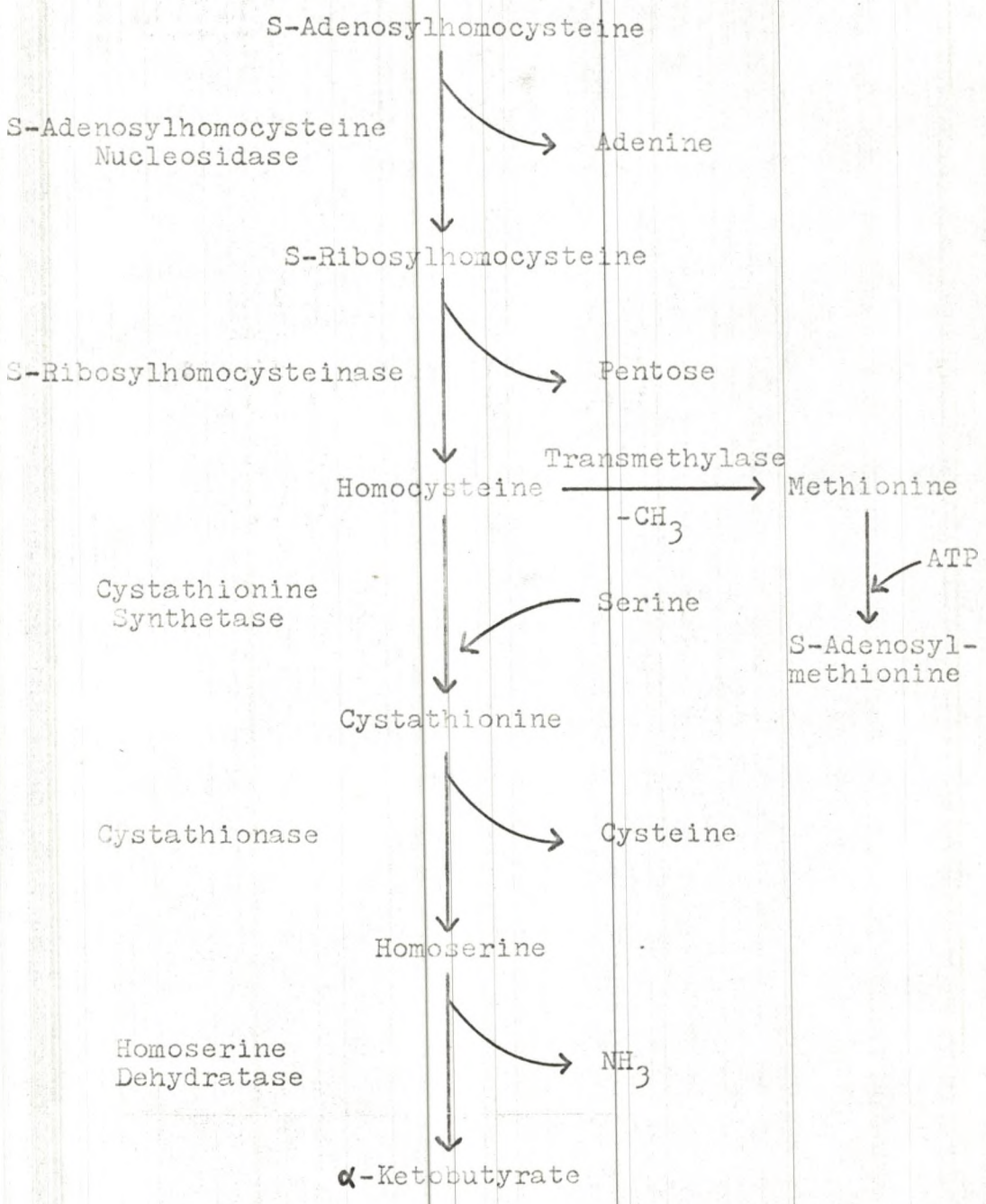
When S-adenosylhomocysteine-H^3 was utilized as substrate with crude E. coli extracts labelled homocysteine and homocystine were recovered by chromatography on

Amberlite CG-120. However, the sum of the amounts of these compounds recovered was not equivalent to the amount of substrate utilized. A labelled ninhydrin-negative material, passing directly through the CG-120 column, gave a positive reaction for keto acids with 2,4-dinitrophenyl hydrazine. It is postulated that enzymes present in the crude extract catalyzed reactions utilizing the generated homocysteine as the initial substrate culminating in the formation of a keto acid as an end product (Scheme IV). A mechanism as such for the formation of ∝-ketobutyrate from homocysteine and serine has been reported (41,42).

In the reaction mixture containing crude enzyme the amount of free sulfhydryl compounds present plus the amount of S-ribosylhomocysteine present at the end of the two-hour incubation was approximately the same as the amount of S-adenosylhomocysteine-H^3 utilized. However, the amount of homocysteine recovered in both the free and oxidized forms was less than the amount of sulfhydryl compounds detected. This suggests the possible transfer of the sulfhydryl group of homocysteine to other compounds, perhaps cysteine, during formation of ∝-ketobutyrate.

Since keto acids were not detected in the reaction mixture of the partially purified (Sephadex G-150) extract, one or more of these enzymes was apparently removed during the purification process resulting in

an extract not capable of forming keto acids from homocysteine.



Scheme IV

SUMMARY

Cell-free extracts of Escherichia coli strain W were shown to contain an enzyme, tentatively designated S-ribosylhomocysteinase, which cleaved the thioether linkage of S-ribosylhomocysteine. The reaction appeared to be of the hydrolytic type as it resulted in the generation of free homocysteine and possibly ribose. Homocysteine was quantitatively recovered from reaction mixtures by ion exchange chromatography as free homocysteine and homocystine. Crude extracts further degraded homocysteine (enzymatically) to α-ketobutyrate. Recovery of homocysteine from reaction mixtures containing the partially purified extract suggested the removal of these degradative enzymes, but oxidation of the homocysteine to homocystine still occurred. Ribose was detected in the reaction mixtures of both crude and partially purified extracts, but the yield was not quantitative.

The purification procedure outlined demonstrated sufficient stability of the enzyme necessary for further study. Comparison of the specific activity of S-ribosylhomocysteinase in various purified states using S-ribosylhomocysteine and S-adenosylhomocysteine as substrates revealed that cleavage of the glycosidic

bond of S-adenosylhomocysteine by S-adenosylhomocysteine nucleosidase was required prior to cleavage of the thioether linkage by S-ribosylhomocysteinase.

Various properties of the enzyme, such as the effects of pH and enzyme concentration on cleavage of the thioether linkage of S-ribosylhomocysteine, optimal pH for stability, and Km were determined.

APPENDIX I

PREPARATION OF REAGENT SOLUTIONS

(3) Orcinol Determination of Pentose

Bial's reagent

Orcinol	0.2	g
HCl (conc.)	60	ml
$FeCl_3$	0.2	ml

(5) Determination of Pentose (Benedict)

Copper reagent

A. Na_2CO_3 (anhydrous)	15	g
Alanine	3	g
Rochelle salts	2	g
Water	250	ml
B. $CuSO_4 \cdot 5H_2O$	3	g
Water	100	ml

C. Mix A and B, dilute to 500 ml with water, store at 4°C

Bisulfite solution

1% $NaSO_3$ in water

Copper reagent containing bisulfite

Bisulfite solution	1	ml
Copper reagent	20	ml

Do not use after second day

Color reagent

Molybdic acid	150	ml

Na_2CO_3 (anhydrous) ..... 75 ml

Water ..... 500 ml

Heat to boiling; filter; wash residue from filter until filtrate plus washings equal 600 ml.

H_3PO_4 ..... 300 ml

Dilute with water to 1 liter.

Ninhydrin Determination of Amino Nitrogen

Potassium cyanide-methyl Cellosolve solution

KCN (0.01 M) ..... 5 ml

Methyl Cellosolve ..... 245 ml

Methyl Cellosolve-ninhydrin solution

5% w/v solution of ninhydrin in methyl Cellosolve.

Potassium cyanide-methyl Cellosolve ninhydrin solution

Methyl Cellosolve-ninhydrin solution ..... 50 ml

Potassium cyanide-methyl Cellosolve solution ..... 250 ml

Store overnight before use.

(9) Determination of Protein (Lowry)

Reagent A

2.0% Na_2CO_3 in o.1 N NaOH

Reagent B

0.5% $CuSO_4 \cdot 5H_2O$ in water

Reagent C

1.0% Na or K tartrate in water

Reagent D

Reagent A	50	ml
Reagent B	0.5	ml
Reagent C	0.5	ml

(1) M-9 Medium (Anderson)

Compound	Amount per liter	
KH_2PO_4	3	g
K_2HPO_4	6	g
NaCl	3	g
$(NH_4)_2SO_4$	2	g
$MgSO_4$	0.1	g
Glucose (autoclaved separately)	5	g

(2) Salt Solution Added to M-9 Medium

Compound	Amount per liter	
$CaCl_2$	0.25	g
$ZnSO_4$	1	mg
$MnCl_2$	1	mg
$FeCl_3$	0.5	mg

$CuSO_4$	0.1	mg
KI	0.1	mg

Add 1.0 ml of this solution to every 15 liters of M-9 medium.

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ABSTRACT

DETERMINATION OF THE PRESENCE AND PROPERTIES OF S-RIBOSYLHOMOCYSTEINASE FROM *ESCHERICHIA COLI*

Chris H. Miller, Master of Science

The thesis here abstracted was written under the direction of Dr. John A. Duerre and approved by Dr. John W. Vennes and Dr. Robert C. Nordlie as members of the examining committee, of which Dr. Duerre was Chairman.

An investigation was undertaken to purify and characterize an enzyme found in *Escherichia coli* that cleaves the thioether linkage of S-ribosylhomocysteine. A 12-fold purification of the enzyme has been achieved with the following fractionation procedures: ammonium sulfate, Sephadex G-150, and ethyl alcohol. Homocysteine has been quantitatively recovered from reaction mixtures by chromatography on Amberlite CG-120. The sulfhydryl group of homocysteine was also quantitatively measured in reaction mixtures with N-ethylmaleimide. In addition, ribose was detected in the reaction mixtures; however, the yield was not quantitative. The ethyl alcohol fraction was very labile losing 95% of the enzymatic activity after 24 hours at 4°C. The optimal pH for stability of the enzyme was 7.6 with 0.1 M Tris-HCl buffer. The

optimal pH for enzymatic activity was 8.0, and the Km value for the enzyme was 1.94×10^{-3} M. S-Adenosylhomocysteine did not serve as a substrate; therefore, cleavage of the glycosidic bond of S-adenosylhomocysteine by S-adenosylhomocysteine nucleosidase was required prior to cleavage of the thioether linkage by S-ribosylhomocysteinase. When crude E. coli extracts were used it was noted that a portion of the homocysteine generated was enzymatically degraded to α-ketobutyrate.

This abstract of a thesis submitted by Chris H. Miller in partial fulfillment of the requirements for the Degree of Master of Science in the University of North Dakota is hereby approved by the Committee under whom the work has been done.

Chairman

Dean of the Graduate School