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THE IN VIVO AND IN VITRO METABOLISM OF S-ADENOSYL-L-HOMOCysteine By THE RAT

by

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A Dissertation
Submitted to the Faculty
of the
University of North Dakota
in partial fulfillment of the requirements
for the degree of
Doctor of Philosophy

Grand Forks, North Dakota
June
1969
DEDICATION

To my wife, Sharon, and children Matthew and Kristine
This dissertation submitted by Chris H. Miller in partial fulfillment of the requirements for the Degree of Doctor of Philosophy from the University of North Dakota is hereby approved by the Faculty Advisory Committee under whom the work has been done.

(Chairman)

Dean of the Graduate School
Permission

Title: The In Vivo and In Vitro Metabolism of S-Adenosyl-L-homocysteine by the Rat

Department: Microbiology

Degree: Doctor of Philosophy

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ABSTRACT

Radioactively labeled S-adenosyl-L-homocysteine was administered intravenously to rats to determine the fate of the homocysteine moiety and to isolate and identify any excretory products that arose from the metabolism of this compound in vivo. After intravenous injection of S-adenosyl-L-homocysteine-\(^3\)H (homocysteine labeled) into rats, less than 15% of the radioisotope was incorporated into protein methionine or excreted as \(\alpha\)-ketobutyrate. The remaining tritium was associated with a previously unidentified keto acid which was excreted in the urine. Intravenous injection of S-adenosyl-\(^3\)H-L-homocysteine (adenosine labeled) or S-adenosyl-L-homocysteine-\(^35\)S revealed that both the purine moiety and the sulfur atom remained associated with this keto acid excretory product. The compound was isolated from the urine by ion-exchange chromatography, purified and crystallized. Based on chemical, elemental, and ultraviolet and infrared spectral analyses as well as the information obtained from the radioisotope tracer studies, the chemical structure of the compound was proposed as S-adenosyl-\(\gamma\)-thio-\(\alpha\)-ketobutyrate.

-x-
To determine the type of reaction responsible for the formation of S-adenosyl-γ-thio-α-ketobutyrate, S-adenosyl-3H-L-homocysteine was incubated with various rat tissue extracts under conditions which would favor oxidative deamination or transamination. Radioactive S-adenosyl-γ-thio-α-ketobutyrate was isolated by chromatography from reaction mixtures in which kidney and liver extracts were used. The reaction was found to be catalyzed by the general L-amino acid oxidase, EC 1.4.3.2. Incubation of the partially purified oxidase with S-adenosyl-L-homocysteine resulted in the oxidative deamination of the substrate to S-adenosyl-γ-thio-α-ketobutyrate with the utilization of 0.48 μmoles of oxygen per μmole of ammonia and keto acid formed in the presence of catalase. Without catalase oxygen consumption was doubled. The pH optimum for S-adenosyl-γ-thio-α-ketobutyrate formation ranged from 8.8 to 9.2 and the Km value for S-adenosyl-L-homocysteine was 2.5 x 10^{-2} M as compared to those of 1.3 x 10^{-2} M and 1.9 x 10^{-2} M for L-leucine and L-methionine, respectively.

Cell-free extracts of the major rat tissues were surveyed for the presence of S-adenosyl-L-homocysteine hydrolase, and only liver extracts exhibited activity. When isolated livers were perfused with buffer containing erythrocytes, serum albumin and tritiated S-adenosyl-L-homocysteine, neither hydrolysis to adenosine and L-homocysteine -xi-
nor deamination to S-adenosyl-$\gamma$-thio-$\alpha$-ketobutyrate occurred. The apparent impermeability of the liver cells to S-adenosyl-L-homocysteine would explain why this compound was not readily hydrolyzed during the in vivo experiments. However, formation of S-adenosyl-L-homocysteine intracellularly in the liver as a result of transmethylation reactions, could be subsequently followed by hydrolysis to adenosine and L-homocysteine. S-Adenosyl-L-homocysteine formed in other organs would not be hydrolytically cleaved but could be oxidatively deaminated by the kidney L-amino acid oxidase and eliminated in the urine as S-adenosyl-$\gamma$-thio-$\alpha$-ketobutyrate.
INTRODUCTION

Experimental evidence from various laboratories have established that S-adenosyl-L-homocysteine is a direct product of transmethylation reactions involving S-adenosyl-L-methionine. Demonstration of a large number of transmethylation reactions and their ubiquitous occurrence has stimulated interest in the metabolic fate of S-adenosyl-L-homocysteine. The formation of free L-homocysteine from S-adenosyl-L-homocysteine has been demonstrated in cell-free extracts from rat liver, yeasts and gram negative bacteria.

In vitro studies with Escherichia coli and Aerobacter aerogenes indicated that two separate enzymes acting in sequence catalyze the generation of free L-homocysteine from S-adenosyl-L-homocysteine. The glycosyl bond of the compound is first hydrolyzed by S-adenosyl-L-homocysteine nucleosidase resulting in the formation of adenine and S-ribosyl-L-homocysteine. The S-ribosyl-L-homocysteine cleavage enzyme then cleaves the thioether linkage of the latter compound yielding free L-homocysteine which can be methylated to methionine. In vivo studies with these bacteria indicated an impermeability to S-adenosyl-L-homocysteine but not to S-ribosyl-L-homocysteine. Neither
of these bacterial enzymes has been demonstrated in yeasts or mammals.

In vivo and in vitro investigations have shown that the generation of L-homocysteine from S-adenosyl-L-homocysteine in yeasts results from a reversible hydrolysis catalyzed by S-adenosyl-L-homocysteine hydrolase. The products of this reaction, adenosine and L-homocysteine, can be converted to ATP and methionine and utilized in the formation of S-adenosyl-L-methionine. Cell-free extracts prepared from rat liver also contain S-adenosyl-L-homocysteine hydrolase, but the actual in vivo metabolism of S-adenosyl-L-homocysteine in the rat has not been investigated.

In the studies presented here, radioactively labeled S-adenosyl-L-homocysteine was administered intravenously to rats to determine the fate of the homocysteine moiety, and to isolate and identify any excretory products that arose from metabolism of this compound in vivo. Metabolism of the compound by cell-free rat tissue extracts and by perfused livers was also investigated.
Metabolism of S-Adenosyl-L-homocysteine in Mammals

Early considerations upon the mechanism of biological transmethyllations indicated that the methyl group of methionine was transferred to suitable acceptors in the presence of ATP (9). Cantoni (15) demonstrated that enzymatic activation was a prerequisite to the utilization of methionine in this capacity. Incubation of L-methionine and ATP with a partially purified enzyme (ATP:methionine S-adenosyltransferase, EC 2.4.2.13) prepared from rabbit liver resulted in the formation of a sulfonium compound which served as a methyl donor in transmethylation reactions. This compound was subsequently identified as S-adenosyl-L-methionine (Formula I) (1, 2). S-Adenosyl-L-methionine has been shown to be involved in the enzymatic methylation of several compounds including nucleic acids, nicotinamide, histamine, guanidinoacetate and a variety of other compounds (47, 59). The demethylated product of transmethylation reactions involving S-adenosyl-L-methionine is S-adenosyl-L-homocysteine. This product was first characterized by Cantoni and Scarano (16) in 1954. S-Adenosyl-L-
methionine-$^{35}$S and guanidinoacetate were incubated with liver guanidinoacetate methyltransferase (S-adenosylmethionine:guanidinoacetate N-methyltransferase, EC 2.1.1.2). The unreacted S-adenosyl-L-methionine-$^{35}$S was precipitated with ammonium reineckate and the supernatant fluid was acidified and applied to a column of Norite A. After washing with water, aqueous pyridine was used to elute the radioactive material. Paper chromatography of the eluate separated the radioactive compound from contaminating creatine, guanidinoacetate and pyridine. The purified compound was ninhydrin-positive, reacted with the nitroprusside reagent, and had an ultraviolet absorption spectrum characteristic of adenine nucleosides with a maximum absorption at 260 m\(\mu\). The structure of S-adenosyl-L-homocysteine (Formula II) was confirmed by chemical synthesis (3).

The only information concerning the metabolic fate of S-adenosyl-L-homocysteine in mammalian systems was provided by De La Haba and Cantoni (19) in 1959. They reported that cell-free extracts prepared from rat liver contained an enzyme (S-adenosyl-L-homocysteine hydrolase, EC 3.3.1.1) which catalyzed the condensation of adenosine and L-homocysteine to S-adenosyl-L-homocysteine (Reaction A).

\[
\text{Adenosine} + \text{L-Homocysteine} \leftrightarrow S\text{-Adenosyl-L-homocysteine} + \text{H}_2\text{O}
\] (A)
S-ADENOSYLMETHIONINE

Formula I

S-ADENOSYLMETHIONINE

Formula II
S-Adenosyl-L-homocysteine was identified as the condensation product of this reaction by its chemical conversion to S-adenosyl-L-methionine, and by comparison of the elemental analysis of the crystalline product to the theoretical values for S-adenosyl-L-homocysteine. With the partially purified enzyme it was observed that the extent of hydrolysis of the thioether linkage of S-adenosyl-L-homocysteine was very small. However, hydrolysis could be accelerated by enzymatically removing either one of the reaction products with adenosine aminohydrolase, EC 3.5.4.4 or dimethylthetin:L-homocysteine S-methyltransferase, EC 2.1.1.3.

**Metabolism of S-Adenosyl-L-homocysteine in Bacteria**

The bacterial metabolism of S-adenosyl-L-homocysteine was first studied in detail by Duepre (20) in 1962. He reported that cell-free extracts prepared from *Escherichia coli*, *Aerobacter aerogenes* and *Salmonella typhimurium* contained an enzyme, S-adenosyl-L-homocysteine nucleosidase, which hydrolytically cleaved the glycosyl bond of S-adenosyl-L-homocysteine yielding adenine and S-ribosyl-L-homocysteine. The products of the reaction were shown to be identical to those which had been obtained from acid hydrolysis of S-adenosyl-L-homocysteine (21). The enzyme purified 160-fold from *E. coli* also was shown to hydrolytically cleave 5'-methylthioadenosine to
adenine and 5-methylthioribose but was not active with the common
purine or pyrimidine nucleosides or related sulfonium compounds.

Further studies revealed that crude cell-free extracts prepared
from E. coli also catalyzed the formation of a free sulfhydryl compound
from both S-ribosyl-L-homocysteine and S-adenosyl-L-homocysteine
(25). The S-ribosyl-L-homocysteine cleavage enzyme was partially
purified and used in studies which confirmed the identification of L-
homocysteine as a product of the enzymatic cleavage (46). Attempts
were made during the purification to separate the S-ribosyl-L-
homocysteine cleavage enzyme from the S-adenosyl-L-homocysteine
nucleosidase. Good separation was achieved. The ratio of specific
activities obtained with S-ribosyl-L-homocysteine and S-adenosyl-
L-homocysteine as substrates during the purification demonstrated the
necessity for cleavage of the glycosyl bond of S-adenosyl-L-homocysteine
prior to cleavage of the thioether linkage. These studies indicated that
two separate enzymatic reactions were responsible for the generation
of homocysteine from S-adenosyl-L-homocysteine in E. coli, and that
the sequence of reactions occurred through the formation of a single
intermediate S-ribosyl-L-homocysteine.

The in vivo metabolic fate of S-adenosyl-L-homocysteine and
S-ribosyl-L-homocysteine was investigated by studying the utilization
of the tritiated compounds by growing cultures of various bacteria (23).
Escherichia coli, in particular, incorporated substantial amounts of S-ribosyl-L-homocysteine but was impermeable to S-adenosyl-L-homocysteine. Since the bulk of the radioisotope from S-ribosyl-L-homocysteine-³H (homocysteine labeled) was recovered in the protein residue of the fractionated cells, it appeared that this compound was cleaved and the homocysteine moiety was incorporated into protein methionine after methylation. The metabolism of S-adenosyl-L-homocysteine is illustrated in Scheme I.

Metabolism of S-Adenosyl-L-homocysteine in Yeasts

The enzymatic formation of S-adenosyl-L-homocysteine in yeasts results from transmethylation reactions involving S-adenosyl-L-methionine (47, 59) and from the condensation of adenosine and L-homocysteine (22, 27). When growing cultures of Saccharomyces cerevisiae or Candida utilis were supplemented with L-homocysteine, an increase in the production of S-adenosyl-L-homocysteine was observed (27). Sufficient amounts of adenosine in the metabolic pool precluded the necessity for adenosine supplementation. Cell-free extracts prepared from these yeasts also catalyzed the formation of S-adenosyl-L-homocysteine from adenosine and L-homocysteine. Supplementation of the growing cultures with radioactive S-adenosyl-8-¹⁴C-L-homocysteine resulted in the incorporation of the radioisotope into S-adenosyl-L-methionine which had a specific activity of about 80%
S-ADENOSYL-L-HOMOCYSTEINE

Nucleosidase

S-RIBOSYL-L-HOMOCYSTEINE + ADENINE

Cleavage Enzyme

L-HOMOCYSTEINE + C5

Transmethylase

L-METHIONINE

Transmethylase

PROTEIN

Methionine Adenosyltransferase

ATP

S-ADENOSYL-L-METHIONINE

Scheme I
that of the substrate. Since only a small amount of the radioactivity was incorporated into S-adenosyl-L-methionine formed from non-labeled S-adenosyl-L-homocysteine and labeled adenosine, it was proposed that S-adenosyl-L-homocysteine was directly methylated to S-adenosyl-L-methionine (27). However, this methylation reaction could not be demonstrated when S-adenosyl-L-homocysteine and various one-carbon donors were incubated with cell-free extracts of the yeasts.

Recent investigations by Duerre (22), again employing radioisotope tracer techniques in vivo with S. cerevisiae, indicated that S-adenosyl-L-homocysteine was cleaved and that the resultant products were incorporated into methionine, S-adenosyl-L-methionine, cysteine, proteins and nucleic acids. Examination of cell-free extracts of S. cerevisiae confirmed the existence of S-adenosyl-L-homocysteine hydrolase and revealed the presence of an enzymatic system which catalyzed the methylation of L-homocysteine per se and L-homocysteine derived from S-adenosyl-L-homocysteine to methionine. The methyl generating system was analogous to that involved in the methylation of L-homocysteine by E. coli and mammalian liver (63, 70, 74). The results presented by Duerre (22) indicated that the direct methylation of S-adenosyl-L-homocysteine need not occur to account for the in vivo incorporation of radioisotopes from S-adenosyl-L-homocysteine into
The first detailed study concerning the enzymatic oxidation of L-amino acids was initiated in 1935. At that time, Krebs (39) reported the oxidation of L- and D-amino acids by homogenates of rat liver and kidney. This work stimulated a general study of the enzymes involved in the oxidative deamination of amino acids which resulted in the characterization of L-amino acid oxidase (L-amino acid:oxygen oxidoreductase (deaminating), EC 1.4.3.2). This enzyme which oxidatively deaminates several L-amino acids has been observed in a variety of organisms including mammals (5, 6), birds (10, 61), snakes (76, 77), invertebrates (8, 52), fungi (14, 38, 64), and bacteria (62). The enzyme species studied from the various sources differ with respect to specificity. The L-amino acid oxidase from Neurospora crassa, Mytilus edulis, turkey liver and chicken liver readily oxidize the basic amino acids such as L-lysine and L-ornithine while the mammalian, ophidian and bacterial oxidases are not active towards

S-adenosyl-L-methionine. S-Adenosyl-L-homocysteine could be hydrolytically cleaved and the homocysteine methylated to methionine. In the presence of ATP the resultant methionine could be converted to S-adenosyl-L-methionine by methionine adenosyltransferase. The proposed metabolism of S-adenosyl-L-homocysteine in S. cerevisiae is shown in Scheme II (22).

L-Amino Acid Oxidase

The L-amino acid oxidase was initiated in 1935. At that time, Krebs (39) reported the oxidation of L- and D-amino acids by homogenates of rat liver and kidney. This work stimulated a general study of the enzymes involved in the oxidative deamination of amino acids which resulted in the characterization of L-amino acid oxidase (L-amino acid:oxygen oxidoreductase (deaminating), EC 1.4.3.2). This enzyme which oxidatively deaminates several L-amino acids has been observed in a variety of organisms including mammals (5, 6), birds (10, 61), snakes (76, 77), invertebrates (8, 52), fungi (14, 38, 64), and bacteria (62). The enzyme species studied from the various sources differ with respect to specificity. The L-amino acid oxidase from Neurospora crassa, Mytilus edulis, turkey liver and chicken liver readily oxidize the basic amino acids such as L-lysine and L-ornithine while the mammalian, ophidian and bacterial oxidases are not active towards
S-ADENOSYL-L-HOMOCYSTEINE

ADENOSINE

L-HOMOCYSTEINE

S-Adenosyl-L-methionine or Methyltetrahydrofolate

ATP

L-METHIONINE

PROTEIN

S-ADENOSYL-L-METHIONINE

-CH₃

Scheme II
these amino acids. A particular L-amino acid may be oxidized by
the L-amino acid oxidase from each of the known sources, but the
rates of oxidation may differ (44).

The mammalian L-amino acid oxidase was first isolated and
described by Blanchard et al. (5) in 1944 and more recently has been
studied by Nakano and co-workers (48, 49), Paik and Kim (50) and
Salvatore and his associates (55). The presence of the enzyme has
been examined in tissues from rat, sheep, ox, pig, dog, rabbit,
guinea pig, cat, mouse and hamster, but only rat kidney, and to a
lesser extent rat liver, have provided a satisfactory source of active
enzyme (5, 55). The rat kidney enzyme has been shown to oxidize
practically all of the monoaminomonocarboxylic acids of the L-series.
In descending rate-order these include leucine, aminocaproic acid,
methionine, proline, aminovaleric acid, phenylalanine, tryptophan,
tyrosine, isoleucine, valine, cystine and alanine (5, 48). Activity with
glycine, L-threonine, L-serine, L-glutamic and L-aspartic acids,
L-lysine, L-ornithine or L-arginine has not been demonstrated (51).
The N-monomethyl derivatives of L-leucine, L-methionine and L-
homocysteine (5) as well as several α-hydroxy acids of the L-series such
as isovaleric, butyric, isocaproic and caproic acids, phenylglycolic,
phenyllactic and lactic acids (7) are oxidized quite rapidly by the kidney
L-amino acid oxidase.
The enzyme purified from rat kidney has been shown to have a pH optimum in the alkaline range (5, 50), a molecular weight of about 88,900 (48) and to contain two molecules of riboflavinphosphate per molecule of enzyme (6, 48). In contrast, the L-amino acid oxidase crystallized from the venom of Crotalus adamanteus (eastern diamondback rattlesnake) has a pH optimum near 7.5, a molecular weight of about 130,000 and contains two molecules of flavin adenine dinucleotide per molecule of enzyme (71). The molecular activity of the kidney L-amino acid oxidase with L-leucine as substrate has been shown to be 6.0 moles of keto acid formed per min per mole of enzyme (6, 48). This value is low compared to those of other flavo-proteins (2000 for mammalian kidney D-amino acid oxidase and 7000 for snake L-amino acid oxidase) (44).

The L-amino acid oxidase catalyzes the oxidative deamination of L-amino acids as described in Reaction B. In the presence of catalase the hydrogen peroxide produced is decomposed to water and molecular oxygen and, in the overall reaction 0.5 mole of oxygen is utilized for each mole of amino acid oxidized (Reactions C and D). In the absence
H₂O → H₂O + 1/2 O₂  

RCH(NH₂)COOH + 1/2 O₂ → RCOOCH + NH₃  

of catalase the hydrogen peroxide produced causes the decarboxylation of the newly formed keto acid (Reaction E) and, in the overall reaction (F) one mole of oxygen is utilized for each mole of amino acid oxidized.

RCOOCOH + H₂O₂ → RCOOH + CO₂ + H₂O  

RCH(NH₂)COOH + O₂ → RCOOCH + CO₂ + NH₃ (B + E)  

The reaction mechanism is thought to involve the oxidation of the amino acid to the imino acid with reduction of the flavin prosthetic groups, hydrolysis of the imino acid to the corresponding keto acid and ammonia, and reoxidation of the reduced flavin by molecular oxygen (45, 72).

Thus, Reaction B would be the sum of the following reactions (E is enzyme):

RCH(NH₂)COOH + E-FAD → E-FADH₂ + RC(NH)COOH  

RC(NH)COOH + H₂O → RCOOCH + NH₃  

E-FADH₂ + O₂ → E-FAD + H₂O₂
The actual involvement of the two flavin prosthetic groups are not fully understood. It has been suggested that the enzyme contains two active centers each of which is associated with one molecule of flavin. During reduction of the enzyme-flavin complex by the amino acid a semiquinone intermediate apparently is formed by transfer of one electron from the substrate to the flavin. This half-reduced enzyme then would be further reduced by an additional electron transfer or be directly reoxidized by molecular oxygen (41).
EXPERIMENTAL PROCEDURE

Materials

Adenosine and L-homocysteine thiolactone, uniformly labeled with tritium by the Wilzbach process (73) were obtained from New England Nuclear Corporation, Boston, Massachusetts. L-Homocysteine-$^{35}$S thiolactone was obtained from Volk Radiochemical Company, Skokie, Illinois. Amino acids, pyridoxal phosphate, NAD, NADP, purines and α-keto acids were purchased from Calbiochem, Los Angeles, California, or from Nutritional Biochemicals Corporation, Cleveland, Ohio. The α-keto acids which were not available commercially were prepared by the complete oxidation of the corresponding amino acid by crystalline L-amino acid oxidase from Crotalus terr. terr. venom in the presence of excess beef liver catalase. L-Amino acid oxidase and catalase were purchased from Boehringer Mannheim Corporation, New York, New York. The Dowex, Amberlite and ion retardation resins were obtained from the J. T. Baker Chemical Company, Phillipsburg, New Jersey; Mallinckrodt Chemical Works, St. Louis, Missouri; and Bio Rad Laboratories, Richmond, California, respectively.
Orcinol, N-ethylmaleimide and 1,2,3-triketohydrindene were purchased from Eastman Organic Chemicals, Rochester, New York, and sodium citrate, citric acid and 2,4-dinitrophenylhydrazine from Matheson Coleman and Bell, East Rutherford, New Jersey. General chemicals were purchased from Fisher Scientific, Minneapolis, Minnesota, and Mallinckrodt Chemical Works. Most of the rat kidneys used in this investigation were purchased from Pel-Freeze Biologicals, Rogers, Arkansas. Additional supplies were obtained from Dr. R. Shipley, Eli Lilly and Company, Indianapolis, Indiana, and from rats raised at the University of North Dakota Medical School. All the kidneys came from Sprague-Dawley rats.

**Preparation of Substrates**

**Preparation of S-Adenosyl-L-homocysteine**

S-Adenosyl-L-homocysteine was enzymatically synthesized from adenosine and L-homocysteine by a modification of the procedures outlined by Duerre (21). Rat livers were homogenized in 3 volumes of 0.01 M acetic acid for 30 sec in a Waring blender at maximum speed. Cellular debris was removed by centrifugation at 18,000 x g for 20 min. Solid ammonium sulfate was added with constant stirring to the supernatant fluid to 40% saturation and the resultant precipitate was removed by centrifugation at 15,000 x g for 10 min. The supernatant fluid was raised to 50% saturation with solid ammonium sulfate and mechanically
stirred for 15 min. The precipitate was collected as before by centrifugation and dissolved in 0.05 M potassium phosphate buffer, pH 7.0. This fraction served as the source of S-adenosyl-L-homocysteine hydrolase and, the specific activity was usually about 0.3 μmoles of homocysteine utilized per min per mg of protein.

For each mmole of adenosine, 3.0 mmoles of DL-homocysteine and 18.0 units of the partially purified enzyme were incubated in 0.1 M phosphate buffer, pH 6.5, at 37° under an atmosphere of nitrogen. After an incubation period of 90 min, thiodiglycol (0.025 ml per 100 ml reaction mixture) was added to maintain S-adenosyl-L-homocysteine in the reduced state. The reaction mixture was deproteinized by heating in a boiling water bath for 5 min, cooling on ice and centrifuging for 10 min at 10,000 x g. S-Adenosyl-L-homocysteine was then isolated from the supernatant fluid by column chromatography. Amberlite CG-120 resin, 200 to 400 mesh, was charged with 6.0 N H₂SO₄ and equilibrated with 1.0 N H₂SO₄. A column 15 cm² in cross-sectional area with a resin bed of 10 cm was sufficient for purification of 4 mmoles of S-adenosyl-L-homocysteine. After application of the sample, 1.5 liters of 3.0 N H₂SO₄ was required to remove the undesired compounds. Elution was followed by ultraviolet absorption at 260 mμ, and when the column was free of impurities, S-adenosyl-L-homocysteine was eluted with 2.5 to 3.0 liters of 6.0 N H₂SO₄. S-Adenosyl-L-
homocysteine was precipitated from solution by the addition of phosphotungstic acid. The water was removed by centrifugation at 500 x g for 5 min and, the precipitate was washed twice with 0.05 N H2SO4 and dissolved in 6 volumes of a 1:1 mixture of acetone and water.

The phosphotungstate complex was dissociated by the method of Schlenk and DePalma (57). The solution was extracted 4 times with 4 volumes of a 1:1 mixture of isoamyl alcohol and ether. S-Adenosyl-L-homocysteine 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 4.0 with freshly prepared BaCO3. 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/ml), frozen, and stored at 4° for 4 days to effect crystallization. Crystals were collected by filtration, washed with a small amount of cold water, and stored under vacuum until used. The total yield of S-adenosyl-L-homocysteine calculated from the amount of adenosine in the initial reaction mixture ranged from 40-50%. Samples of the purified compound were chromatographed on Whatman No. 1 paper to check for ultraviolet and ninhydrin-positive impurities and were found to be pure.
The same procedure was used for the preparation of S-adenosyl-$^3$H-L-homocysteine, S-adenosyl-L-homocysteine-$^3$H and S-adenosyl-L-homocysteine-$^{35}$S. Adenosine-$^3$H, homocysteine-$^3$H or homocysteine-$^{35}$S was used in the initial reaction mixture (26).

Preparation of S-Adenosyl-L-methionine

The method of Schlenk et al. (56) utilizes the synthetic capabilities of yeasts to form S-adenosyl-L-methionine from exogenously supplied L-methionine and endogenously formed ATP. Saccharomyces cerevisiae, NRL strain, was cultured in Snell’s medium (Appendix) supplemented with L-methionine (0.75 g per liter). After incubation of the culture for two days at 30° with moderate aeration, the cells were harvested by continuous-flow centrifugation and washed twice with demineralized water. The cells were extracted with 4 volumes of 1.5 N perchloric acid for 1 hour at room temperature with constant stirring. Cellular debris was removed by centrifugation at 10,000 x g for 10 min and discarded. The S-adenosyl-L-methionine was precipitated from the supernatant fluid by the addition of 1/2 volume of a cold freshly prepared saturated solution of ammonium reineckate. After storage at 4° for 8 hours, the precipitate was collected by suction filtration, washed with a small amount of cold water and dissolved in 10–20 volumes of a 1:1 mixture of acetone and 1N H$_2$SO$_4$. The S-adenosyl-L-methionine was applied to a column of Dowex 50 W–X8 resin and eluted by the same procedures used for the preparation of S-adenosyl-L-
homocysteine. The compound was precipitated with phosphotungstic acid and separated from the phosphotungstate with organic solvents by those procedures described for the purification of S-adenosyl-L-homocysteine. The lyophilized compound was diluted with water to a desired concentration and checked for purity by chromatography on Whatman No. 1 paper. The preparations were found to contain trace amounts of an ultraviolet light absorbing impurity.

**Preparation of Homocysteine from Homocysteine Thiolactone**

The method described by Duerre and Miller (24) was used for cleavage of the thiolactone ring of either DL- or L-homocysteine thiolactone. The free sulfhydryl form was prepared by incubating 0.1 mmole of the thiolactone with 0.3 ml of 3.0 N NaOH for 5 min at room temperature. The solution was immediately neutralized with 0.9 ml of 1.0 M KH₂PO₄ and diluted with water to yield the desired concentration of homocysteine.

**Analytical Methods**

**Determination of α-Keto Acids**

The 2,4-dinitrophenylhydrazine method of Friedemann and Haugen (31) was used to measure α-keto acids. Samples containing 0.05 to 0.30 μmoles of keto acid were diluted to 1.0 ml with water and 0.05 ml of 100% trichloroacetic acid was added. After mixing, 0.25 ml of a 0.1% solution of 2,4-dinitrophenylhydrazine in 20% HCl was added
and the tubes incubated at room temperature for 5 min. Two ml of 4.0 N NaOH was added with immediate mixing. After the tubes were incubated at room temperature for 10 min, the absorbancy of the samples was determined at 515 m\(\mu\) with a Coleman Jr. spectrophotometer. Figure 1 shows the relationship obtained with this procedure between absorbancy and \(\mu\)moles of \(\alpha\)-ketobutyric, \(\alpha\)-keto-\(\gamma\)-methiolethylbutyric and \(\alpha\)-ketoisocapric acids.

**Determination of Pentoses**

Pentose was measured quantitatively by reaction with Bial's reagent as described by Brown (13). Samples containing pentose were diluted with water to 1.0 ml. Two ml of Bial's reagent (Appendix) were added and the samples placed in a boiling water bath for 20 min and cooled on ice. The samples were read in a Coleman Jr. spectrophotometer at 660 m\(\mu\). Reaction mixtures containing known quantities of ribose, adenosine, inosine, xanthosine or S-adenosyl-L-homocysteine reacted identically and gave a linear response over a range of 0.02 to 0.08 \(\mu\)moles (Figure 2).

**Determination of Amino Nitrogen**

Amino nitrogen was measured quantitatively by the ninhydrin method of Yemm and Cocking (75). One ml samples were mixed with 1.0 ml of 0.2 M citrate buffer, pH 5.0. To this mixture was added 1.2 ml of potassium cyanide-methyl cellosolve-ninhydrin solution (Appendix).
Fig. 1. — Reference curve for the determination of α-ketobutyric acid (○○), α-ketoisocaproic acid (●●) and α-keto-γ-methiolbutyric acid (□□) by the 2,4-dinitrophenylhydrazine method. α-Ketobutyrate and α-ketoisocaproic acid were obtained commercially. α-Keto-γ-methiolbutyrate was prepared by complete oxidation of known amounts of L-methionine by crystalline L-amino acid oxidase in the presence of excess catalase.
Fig. 2. -- Reference curve for the determination of ribose, adenosine, inosine, xanthosine and S-adenosyl-L-homocysteine by reaction with Bial's reagent. All of the pentose containing compounds gave the same response as did ribose.
ABSORBANCY $A_{660\mu m}$ vs. $\mu$ MOLES PENTOSE
The solution was heated in a boiling water bath for 15 min, cooled in ice water and read in a Coleman Jr. spectrophotometer at 570 μm. L-Homocysteine was used as the standard for determination of the reference curve and gave a linear response of 0.05 to 0.25 μmoles (Figure 3).

**Determination of Free Sulfhydryl Compounds**

The N-ethylmaleimide method described by Duerre and Miller (25) was used to measure free sulfhydryl compounds. To 0.3 ml of sample was added an equal volume of 0.375 M N-ethylmaleimide. After incubation at room temperature for 10 min, 0.175 ml of 1.0 N NaOH was added. Since the pink color which forms tends to fade, all samples were read 30 sec after the addition of alkali in a microcuvette at 520 μm using a Beckman DU spectrophotometer. Known quantities of L-homocysteine added to the reaction mixtures gave a linear response over a range of 0.05 to 0.4 μmoles (Figure 4).

**Determination of Protein**

Protein concentration was determined spectrophotometrically by the method of Warburg and Christian (69). Aliquots of the samples were diluted with water until an absorbancy capable of being recorded at 260 and 280 μm was obtained. A Beckman DU or DB spectrophotometer was used for this determination with water as the blank. Protein concentration in mg per ml was calculated from the following relationship:
Fig. 3. — Reference curve for the determination of amino nitrogen by the ninhydrin method. L-Homocysteine prepared as described in the text was used as the standard.
ABSORBANCY 570nm

μMOLES L-HOMOCYSTEINE
Fig. 4. — Reference curve for the determination of free sulphydryl compounds by the N-ethylmaleimide method. L-Homocysteine prepared as described in the text was used as the standard.
Determination of Radioactive Isotopes

Tritium-labeled compounds were measured with a Packard Tri-Carb liquid scintillation spectrometer at a counting efficiency of 24%.

The solvent system consisted of toluene and ethanol (4:1) with 6.0 g of 2,5-diphenyloxazole (PPO) and 100 mg of 1,4-bis[2-(5-phenyloxazoly)] benzene (POPOP) per liter. All counting vials contained 0.25 ml sample and 19 ml of the organic solvent. $^{35}$S-labeled compounds were measured with a Tracerlab Omni/Guard low beta planchet counter.

Thin Layer Chromatography of $\alpha$-Keto Acids

The technique used for the identification of $\alpha$-keto acids and their 2,4-dinitrophenylhydrazine derivatives was essentially that of Dancis, Hutzler and Levitz (18). The hydrazine derivatives of the keto acids were prepared by the addition of a 20% molar excess of 0.01 M 2,4-dinitrophenylhydrazine in 2.0 N HCl to a solution of the keto acids. After approximately 5 hours, the crystals were collected by centrifugation, washed 3 times with cold water and dissolved in ethanol. Aliquots were applied to thin layer silica gel plates which had been activated at 110° for 20 min. The solvent system was isoamyl alcohol-0.25 N ammonium hydroxide (20:1).

\[
(1.55 \times \text{O.D.}_{280}) - (0.76 \times \text{O.D.}_{260}) = \text{mg per ml}
\]
Amberlite CG-120 Chromatography

Ion-exchange chromatography with Amberlite CG-120 resin was used to isolate and identify various compounds. Prior to application of the sample, the resin was washed with 1.0 N NaOH and equilibrated with 0.067 sodium citrate buffer, pH 2.9. Elution was by a 0.067 M sodium citrate gradient with a pH range of 2.9 to 6.1. The elution patterns of several known compounds are shown in Figure 5.

Enzyme Assays

L-Amino Acid Oxidase Assay

L-Amino acid oxidase activity was routinely measured by determining the rate of oxygen consumption with the Gilson oxygraph model KM equipped with the YSI Clark oxygen electrode. The electrode chamber with a reaction mixture volume of 1.5 ml contained enzyme, 25 μmoles of substrate, 150 μmoles of Tris-HCl buffer, pH 9.0, and 45 units of catalase. The temperature of the chamber was kept at 37°. The percent oxygen utilized per min was calculated from the slope of the recorded line obtained with the oxygraph at a given chart speed. This value was multiplied by the number of μmoles of dissolved oxygen present in 1.5 ml of water at 37° (0.314) and divided by the total mg of protein in the reaction mixture. The resulting value represented the specific activity of the enzyme or μmoles of oxygen utilized per min
Fig. 5. — Chromatographic separation of various compounds.

A column of Amberlite CG-120 resin, 200 to 400 mesh, 0.7 x 20 cm, with a flow rate of 0.6 ml per min, was used. Before application to the column, all samples were adjusted to pH 2.9. The column was eluted with a pH gradient formed by allowing 250 ml of 0.067 M sodium citrate, pH 8.3, to flow into a mixing chamber containing an equal volume of 0.067 M citrate buffer, pH 2.9. Elution was continued with 75 ml of 0.067 M sodium citrate, pH 8.3. Pentose and pentose containing compounds were measured by reaction with Bial’s reagent; amino nitrogen containing compounds were measured by the ninhydrin test; keto acids were determined by reaction with 2,4-dinitrophenylhydrazine; purines and purine containing compounds were measured by ultraviolet light absorption.
per mg of protein. One unit of activity was defined as that amount of enzyme which catalyzed the utilization of 1.0 μmole of oxygen per min at 37° under the conditions described.

Activity was also measured by determining oxygen consumption manometrically with the Warburg and by colorimetric detection of the amount of newly formed α-keto acid by the 2,4-dinitrophenyl-hydrazine method (31).

**S-Adenosyl-L-homocysteine Hydrolase Assay**

Reaction mixtures containing 6.0 μmoles of L-homocysteine, 6.0 μmoles of adenosine, 100 μmoles of potassium phosphate buffer, pH 6.5, and enzyme in a total volume of 1.0 ml were incubated at 37° under an atmosphere of nitrogen for 1 hour. To stop the reaction the tubes were placed in a boiling water bath for 3 min and cooled on ice; the precipitated protein was removed by centrifugation at 1500 x g for 5 min. The amount of L-homocysteine utilized for the formation of S-adenosyl-L-homocysteine was measured by determining the amount of L-homocysteine in the incubated reaction mixtures and in zero-time controls. The N-ethylmaleimide method for detection of free sulfhydryl compounds was used to measure the L-homocysteine. One unit of activity was defined as that amount of enzyme which catalyzed the utilization of 1.0 μmole of L-homocysteine per min under the conditions described.
Experiments In Vivo

From 5 to 100 μmoles of S-adenosyl-L-homocysteine dissolved in 0.9% NaCl were injected into the tail vein of adult white rats. The animals were placed in restraining cages and the urines collected and adjusted to pH 11.0 with NaOH and placed under vacuum for 6 hours to remove the ammonia. The urines were adjusted to pH 7.0 with NaOH and desalted by passage through a column of AG-11A8 ion retardation resin with water as the eluting agent (53). The column size varied with that of the sample. The pH of the urine was adjusted to 2.9 and aliquots were analyzed chromatographically on Amberlite CG-120 resin. In experiments with radioactive S-adenosyl-L-homocysteine, the rats were sacrificed at various time intervals by decapitation and the liver, kidneys, spleen, small intestine, lungs, heart, brain, muscle and testes were removed, weighed and homogenized in demineralized water to a fine cream with a Waring blender. Samples of each homogenate were extracted with equal volumes of cold 10% trichloroacetic acid and centrifuged at 10,000 x g for 10 min. The precipitate was washed by centrifugation with cold 5% trichloroacetic acid, and the wash was combined with the original extract. The radioactive isotope content of the homogenates, extracts and precipitates was determined.
Experiments In Vitro

Adult white rats were killed by decapitation and the kidneys, liver, spleen, heart, small intestine, muscle, brain and testes removed. Each tissue was homogenized at 4° for 1 min at one-half maximum speed in a Waring blender in 0.05 M potassium phosphate buffer, pH 7.5. The homogenates were centrifuged at 1000 x g for 10 min. The supernatant fluids were dialyzed at 4° against 100 volumes of 0.05 M potassium phosphate buffer, pH 7.5 for 12 hours with 3 changes of the buffer during the period. The dialyzed extracts were assayed for the presence of an enzyme which would catalyze the formation of S-adenosyl-γ-thio-α-ketobutyrate from S-adenosyl-L-homocysteine. The tissue extracts were incubated in the presence of 10mM S-adenosyl-3H-L-homocysteine (adenosine labeled) and 0.1 M Tris-HCl buffer at pH values ranging from 7.0 to 9.0. The reaction mixtures were incubated for 2 hours at 37° and deproteinized by the addition of trichloroacetic acid at a final concentration of 10%. One to three ml of the deproteinized reaction mixtures were chromatographed on Amberlite CG-120 resin and analyzed for the presence of radioactive S-adenosyl-γ-thio-α-ketobutyrate.

Enzyme Preparation

The method used for the partial purification of the enzyme which oxidatively deaminated S-adenosyl-L-homocysteine was essentially
that used for the purification of rat kidney L-amino acid oxidase by Blanchard and co-workers (6). Rat kidneys were minced in a minimum amount of demineralized water for 45 sec in a Waring blender and added slowly to 3 volumes of acetone cooled to \(-15^\circ\). The precipitate was collected by suction filtration, washed 3 times with cold acetone and broken up into a fine powder while drying in air. The powder was resuspended in acetone at room temperature, collected by filtration and again dried in air. A yield of about 25 g of dry powder per 100 g of wet kidneys was obtained. The powder was extracted with 10 volumes of demineralized water for 30 min with constant stirring and the residue was removed by centrifugation at 10,000 \(x\) g for 10 min. To the dark red supernatant fluid was added anhydrous \(\text{Na}_2\text{SO}_4\) (15 g per 100 ml of extract) and then acetic acid to pH 5.1. After stirring the suspension for 10 min, the precipitate was collected by centrifugation at 10,000 \(x\) g for 10 min, suspended in demineralized water (30 ml per 100 g of wet kidneys) and adjusted to pH 8.5 with NaOH. The suspension was heated rapidly to 55°, maintained at that temperature for 5 min and rapidly cooled in ice water. To the supernatant fluid resulting from centrifugation at 10,000 \(x\) g for 10 min, was added anhydrous \(\text{Na}_2\text{SO}_4\) (15 g per 100 ml of extract) and then acetic acid to pH 5.6. The precipitate was collected by centrifugation at 10,000 \(x\) g.
for 10 min, suspended in a small volume of water, adjusted to pH 8.5 with NaOH and clarified by centrifugation at 10,000 x g for 20 min. All procedures were carried out at 10° unless otherwise stated. The final protein concentration ranged from 30 to 60 mg per ml and the specific activity with L-leucine as substrate ranged from 0.28 to 0.36 units per mg of protein. Large quantities of rat kidneys were necessary to obtain sufficient amounts of the enzyme. The total amount of the partially purified enzyme recovered from each 100 g of kidneys would catalyze only 10 to 15 $\mu$moles of S-adenosyl-L-homocysteine per min, and this represented about 50% of the total amount in the crude acetone preparation.

**Perfusion Experiments**

Preparation of the perfusate was described by Veneziale and co-workers (68). The erythrocytes from 75 ml of heparinized rat blood were collected by centrifugation at 100 x g for 30 min at 4°. The cells were washed twice with 0.9% NaCl and resuspended in a total volume of 95 ml of Krebs-Ringer bicarbonate solution (Appendix) containing 3.0 g of bovine serum albumin and 34.1 mg of heparin. The perfusate was prepared and stored at 4° for about 18 hours prior to its use. The method of perfusion was that of Brauer et al. (12). Large male Sprague-Dawley rats, fasted for 24 hours, were anesthetized with ether and the thoracic and abdominal cavities were exposed. After cannulation of the bile duct, portal vein and inferior vena cava in
that order, the liver was excised and connected to the perfusion apparatus. The oxygenated perfusate entered through the cannulated portal vein, left through the cannulated vena cava and was then reoxygenated under 1 atm of pressure with a mixture of 95% oxygen and 5% carbon dioxide. Viability of the liver was determined by the blood flow rate and by the general appearance of the organ.
\textbf{RESULTS}

\textbf{Experiments In Vivo}

\textbf{Radioisotope Tracer Studies}

Radioactively labeled S-adenosyl-L-homocysteine was administered intravenously to rats to determine the fate of the homocysteine moiety. Adult white rats received intravenous injections of 100 \( \mu \)moles of S-adenosyl-L-homocysteine-\(^3\)H labeled in the homocysteine moiety (58,500 cpm per \( \mu \)mole). The animals were sacrificed at various time intervals and portions of the various tissues were removed and analyzed as described in "EXPERIMENTAL PROCEDURE". In the rats sacrificed 4 hours after injection the amounts of tritium found associated with the tissue proteins of liver, kidney and small intestine were 2300, 1600 and 600 cpm per g, respectively. This represented less than 1\% of the total radioisotope administered. Essentially no tritium was found associated with the proteins of spleen, muscle, lungs, heart, brain or testes. In the rats sacrificed 8 to 12 hours after injection even lesser amounts of tritium were associated with the tissue proteins. From 50 to 60\% of the radioisotope appeared in
the urine within the first 4 hours after injection, and only trace amounts were detected in urine collected 12 hours after injection.

A sample of urine collected 2 hours after injection of S-adenosyl-L-homocysteine-$^3$H was chromatographed on Amberlite CG-120 resin (Figure 6A). Approximately 5% of the tritium in the urine sample passed directly through the column without retention (Peak 1). This peak contained both ninhydrin and keto acid-positive material. The remaining tritium was associated with a ninhydrin-negative keto acid-positive compound which emerged from the column at a gradient pH of 4.6 (Peak 2). The fractions representing Peaks 1 and 2 were pooled separately and the keto acid material in each peak was precipitated by the addition of 2,4-dinitrophenylhydrazine. Essentially all of the tritium found in each peak was associated with the crystalline keto acid hydrazones. The radioactive keto acid hydrazones from Peaks 1 and 2 were co-chromatographed on thin layer silica gel with several known C₃ to C₆ keto acid hydrazones as described in "EXPERIMENTAL PROCEDURES". The $R_f$ values obtained are shown in Table I. The $R_f$ values for the two radioactive keto acids found in Peak 1 correspond to those of the isomers of the α-ketobutyric acid derivatives. The $R_f$ values of the dinitrophenylhydrazone of the radioactive keto acid found in Peak 2 did not correspond to that of any keto
Fig. 6. — Chromatographic separation of compounds present in urine after intravenous injection of radioactive S-adenosyl-L-homocysteine. The chromatographic procedures used with Amberlite CG-120 resin are described in Figure 5. A, chromatograph of 0.5 ml urine collected 2 hours after injection of 100 μmoles of S-adenosyl-L-homocysteine-H (58,500 cpm per μmole). B, chromatograph of 0.5 ml urine collected 2 1/4 hours after injection of 34 μmoles of S-adenosyl-H-L-homocysteine (67,300 cpm per μmole). C, chromatograph of urine collected 2 1/2 hours after injection of 94 μmoles of S-adenosyl-L-homocysteine-S (160,000 cpm per μmole). (————) pH gradient; (————) radioactivity; (————) amino nitrogen as measured by the ninhydrin test with L-homocysteine as the standard; (————) keto acids as measured by the 2,4-dinitrophenylhydrazine test with α-ketobutyrate as the standard.
acid derivative listed in Table I or to any of those reported by Dancis and co-workers (18).

To further investigate the metabolism of S-adenosyl-L-homocysteine in the rat, S-adenosyl-\(^{3}H\)-L-homocysteine (adenosine labeled) and S-adenosyl-L-homocysteine-\(^{35}S\) were injected intravenously and the animals were killed 4 to 5 hours after injection. Less than 1% of the sulfur-35 administered as S-adenosyl-L-homocysteine was associated with the tissue proteins of liver, kidney and small intestines with only trace amounts of the radioisotope associated with the proteins of the other tissues. Small amounts of tritium were detected in most of the tissues from rats injected with S-adenosyl-\(^{3}H\)-L-homocysteine; however, all of the radioisotope could be extracted from the tissues with cold 5% trichloroacetic acid. None of the purine moiety of S-adenosyl-L-homocysteine appeared to be incorporated into nucleic acids. The rats injected with the sulfur-and purine-labeled S-adenosyl-L-homocysteine excreted 50 to 60% of the radioisotope (sulfur-35 or tritium) within 4 to 5 hours after injection. Chromatography of the urine from these animals on Amberlite CG-120 resin demonstrated that most of the sulfur-35 or tritium was associated with the compound found in the second keto acid-positive peak (Figure 6, B and C). When 2,4-dinitrophenyl-hydrazine was added to the fractions represented by these peaks essentially all of the tritium and sulfur-35 was found associated with the precipitated keto acid hydrazone.
Table I

Thin layer chromatography of dinitro-phenylhydrazones of keto acids

<table>
<thead>
<tr>
<th>Compound</th>
<th>$R_F$</th>
<th>$R_F$^a</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\alpha$-Keto-$\beta$-methylvaleric acid</td>
<td>0.52</td>
<td>0.24</td>
</tr>
<tr>
<td>$\alpha$-Ketobutyric acid</td>
<td>0.28</td>
<td>0.10</td>
</tr>
<tr>
<td>Pyruvic acid</td>
<td>0.21</td>
<td>0.07</td>
</tr>
<tr>
<td>$\alpha$-Ketoisocapric acid</td>
<td>0.52</td>
<td>0.25</td>
</tr>
<tr>
<td>$\alpha$-Ketoisovaleric acid</td>
<td>0.40</td>
<td></td>
</tr>
<tr>
<td>$\alpha$-Ketovaleric acid</td>
<td>0.46</td>
<td>0.18</td>
</tr>
<tr>
<td>$\alpha$-Ketoglutaric acid</td>
<td>0.00</td>
<td></td>
</tr>
<tr>
<td>Peak 1</td>
<td>0.28</td>
<td>0.10</td>
</tr>
<tr>
<td>Peak 2</td>
<td>0.02</td>
<td></td>
</tr>
</tbody>
</table>

^a When two $R_F$ values are given, they refer to isomers 1 and 2, respectively.
The above findings revealed that both the homocysteine and purine moieties as well as the sulfur atom of S-adenosyl-L-homocysteine remained associated with the excreted compound. This compound appeared to be similar to S-adenosyl-L-homocysteine; however, the parent compound contained an amino group which was not detected in the excreted product. This suggested that a deamination of S-adenosyl-L-homocysteine may have occurred.

Preparation of the Major Keto Acid Excretory Product

Preparation of the excretory product in quantities sufficient for the chemical and physical characterization of the compound was undertaken. Two mmoles of S-adenosyl-L-homocysteine were dissolved in 0.9% NaCl and injected intravenously into 12 rats. The urine from these rats was collected over an 8 hour period, adjusted to pH 2.9 and directly chromatographed on Amberlite CG-120 resin, 200 to 400 mesh. A column 2 x 20 cm was used with a pH gradient formed by allowing 500 ml of 0.067 M sodium citrate, pH 8.3, to flow into a mixing chamber containing an equal volume of 0.067 M citrate buffer, pH 2.9. Fractions of 10 ml were collected at a rate of 0.6 ml per min. This system was adequate for the fractionation of 20 ml of urine without desalting. Elution of the keto acid product was followed by the 2,4-dinitrophenylhydrazine test. The compound was eluted from the column at about midpoint in the gradient (pH 4.5). The fractions containing the compound were pooled
and adjusted to pH 0.5 with $\text{H}_2\text{SO}_4$, and 30 ml of a 20% aqueous solution of phosphotungstic acid was added to the solution. The resultant precipitate was allowed to settle overnight at 4° and was collected by centrifugation at 5000 x g for 10 min. The precipitate was washed with 10 volumes of cold 0.01 N $\text{H}_2\text{SO}_4$ and dissolved in 6 volumes of a 1:1 mixture of acetone and water. The phosphotungstate complex was dissociated with organic solvents as described for the preparation of S-adenosyl-L-homocysteine in "EXPERIMENTAL PROCEDURE". The aqueous solution of the keto acid was adjusted to pH 4.0 with freshly prepared $\text{BaCO}_3$ and the resulting $\text{BaSO}_4$ precipitate was removed by filtration and discarded. The filtrate was lyophilized and dissolved in water to a concentration of about 40 μmoles per ml. This solution was passed through a Millipore filter (0.45 μm pore size) and crystallization of the keto acid was effected by the addition of ethanol. The white crystals were collected by filtration, washed with a small amount of cold ethanol and stored under vacuum over activated silica gel. Approximately 15% of the 2.0 mmoles injected into the rats was recovered as the crystalline keto acid excretory product.

**Characterization of the Purified Excretory Product**

Samples of the compound were subjected to chromatographic analysis on Whatman No. 1 paper with solvent systems of ethanol-water-acetic acid (65:34:1) and 1-propanol-water-acetic acid (65:31:4). The
$R_f$ values obtained for the compound in the above solvent systems were 0.62 and 0.55, respectively. The keto acid was found to be free of all impurities as measured by ultraviolet light absorption, iodoplatinate, ninhydrin, or ammoniacal silver chloride reagents (Appendix). The compound could be located on the chromatograms with iodoplatinate (reacts with sulfur compounds), ammoniacal silver chloride (reacts with carbohydrates) and by ultraviolet absorption but not with the ninhydrin reagent (reacts with amino groups).

The purified compound had an absorption maximum at 262 nm (Figure 7) with a molar extinction coefficient ($E_m$) of 12,300 in water as compared to an absorption maximum of 260 nm and an $E_m$ of 15,400 for S-adenosyl-L-homocysteine. The purified compound had no reactive amino group, but reacted quantitatively in the 2,4-dinitrophenylhydrazine test with $\alpha$-ketobutyrate as the standard. The compound also reacted with Bial's reagent and gave a maximum absorption at 660 nm; however, its reactivity on a molar basis was only 20% that of the ribose standard. The melting point range was 131 to 136° with decomposition.

On the basis of the previous radioisotope and chemical data, a molecular formula of $C_{14}H_{17}O_6N_5S$ and a molecular weight of 383.4 was tentatively suggested for the purified compound as compared to those of $C_{14}H_{20}O_5N_5S$ and 384.4 for S-adenosyl-L-homocysteine. The results of the elemental analysis of the pure crystalline compound were as follows:
Fig. 7. — Ultraviolet absorption spectrum of the purified excretory product at a concentration of 0.05 μmoles per ml in water at 25°. A Beckman DB recording spectrophotometer was used.
The infrared spectra of the purified compound, designated as S-adenosyl-\(\gamma\)-thio-\(\alpha\)-ketobutyrate, and of S-adenosyl-L-homocysteine are shown in Figure 8. The bands at 1600 cm\(^{-1}\), 1050 cm\(^{-1}\) and 1080 cm\(^{-1}\) might be attributed to the structural characteristics of the purine ring. The strong carbonyl band at 1720 cm\(^{-1}\) satisfies the requirement for the carboxylic acid group present in both compounds. However, this band must also accommodate the ketone group present in S-adenosyl-\(\gamma\)-thio-\(\alpha\)-ketobutyric acid. Both compounds exhibited strong bands from 2800 to 3550 cm\(^{-1}\) which indicate the presence of \(\text{NH}_2\), C-H and OH groups.

The structure proposed for the purified excretory product (Formula III) is based on the results obtained from the radioisotope experiments and on the chemical, spectrophotometric and elemental analyses of the compound.

\[\text{C}_{14}\text{H}_{17}\text{O}_{6}\text{N}_{5}\]

Calculated: C 43.85, H 4.46, O 25.04, N 18.26, S 8.36

Found\(^1\) : C 43.59, H 4.41, O 24.51, N 17.30, S 7.85

\(^1\) Analyzed by Alfred Bernhardt Mikroanalyischers Laboratorium in Max-Planck-Institute fur Kohlenforschung, 433 Mulheim Ruhr, West Germany.
Fig. 8. — Infrared spectra of S-adenosyl-L-homocysteine and S-adenosyl-γ-thio-α-ketobutyrate in Nujol preparations. A Beckman IR-12 dual beam spectrophotometer was used at a recording speed of 80 cm\(^{-1}\) per min. Nujol absorbs strongly in the regions from 1400 to 1500 cm\(^{-1}\) and 2800 to 3000 cm\(^{-1}\).
S-ADENOSYL-γ-THIO-α-KETOBUTYRIC ACID

Formula III
Tissue Survey

S-Adenosyl-$\gamma$-thio-$\alpha$-ketobutyrate is a resultant product of the in vivo metabolism of S-adenosyl-L-homocysteine and, its enzymatic formation could result in a variety of tissues from a deamination process such as transamination or oxidative deamination with oxygen, NAD or NADP as the acceptor. To investigate the in vitro formation of S-adenosyl-$\gamma$-thio-$\alpha$-ketobutyrate from S-adenosyl-L-homocysteine, dialyzed extracts prepared from rat kidney, liver, spleen, heart, small intestine, muscle, brain and testes were incubated with S-adenosyl-3H-L-homocysteine (adenosine labeled) under the conditions described in "EXPERIMENTAL PROCEDURE". Chromatographic analysis of the various assay mixtures on Amberlite CG-120 resin resulted in the isolation of radioactive S-adenosyl-$\gamma$-thio-$\alpha$-ketobutyrate only from those reaction mixtures in which kidney and liver extracts were used (Figure 9). The compound was eluted from the columns at pH 4.5 and it exhibited all the chemical, physical and chromatographic properties of the S-adenosyl-$\gamma$-thio-$\alpha$-ketobutyrate previously identified during the in vivo experiments. In addition to the S-adenosyl-$\gamma$-thio-$\alpha$-ketobutyrate, radioactive purines and purine nucleosides were isolated from assay mixtures in which dialyzed liver extracts were used (Figure 9A). No purines or purine nucleosides were isolated from reaction mixtures
Fig. 9. — Chromatographic separation of reaction products produced by crude tissue extracts. Crude dialyzed tissue extracts were incubated in 10 mM S-adenosyl-[3H]-L-homocysteine and 0.1 M Tris-HCl buffer, pH 8.5, for 2 hours at 37°. After deproteinization 1.0 ml samples were applied to columns of Amberlite CG-120 resin (0.7 x 20 cm) and eluted as described in Figure 5. A, chromatograph of a reaction mixture in which liver extract was used. B, chromatograph of a reaction mixture in which kidney extract was used. (— — — ) pH gradient; (•——•) radioactivity; (———) reducing compounds as measured by the orcinol test with ribose as the standard; (— — — ) keto acids as measured by the 2,4-dinitrophenylhydrazine test with α-ketobutyrate as the standard. The compounds were identified by comparison of their chemical and chromatographic properties to those of the authentic compounds demonstrated in Figure 5. The purine containing compounds could be quantitated by their molar extinction coefficients. ATKB, S-adenosyl-γ-thio-α-ketobutyrate; AH, S-adenosyl-L-homocysteine.
CITRATE pH GRADIENT

SODIUM CITRATE

A

HYPOXANTHINE

ATKB

INOSINE

ADENINE

ADENOSINE

SULFOXIDE

B

ATKB

SULFOXIDE

AH

AH

pH of EFFLUENT

μMOLES/ml

ml EFFLUENT

cpm/ml x 10^4
in which extracts from other tissues were used. Chromatographic analysis of corresponding zero-time control mixtures resulted in the recovery of only radioactive S-adenosyl-L-homocysteine and its sulfoxide derivative.

The addition of pyridoxal phosphate and either α-ketobutyrate, α-ketoglutarate or pyruvate (final concentrations were 10 mM) to reaction mixtures containing S-adenosyl-\(^3\)H-L-homocysteine and liver or kidney extracts failed to enhance the formation of S-adenosyl-\(\gamma\)-thio-α-ketobutyrate above the level observed in non-supplemented mixtures. The formation of the corresponding amino acid of α-ketobutyrate, α-ketoglutarate or pyruvate also was not detected when the reaction mixtures were analyzed by paper chromatography (33). The presence of NAD or NADP had no effect on the formation of S-adenosyl-\(\gamma\)-thio-α-ketobutyrate as determined by chromatographic analysis on Amberlite CG-120 resin of supplemented and non-supplemented reaction mixtures. Neither transamination nor oxidative deamination with NAD or NADP as the acceptor appeared to be involved in the formation of S-adenosyl-\(\gamma\)-thio-α-ketobutyrate.

When reaction mixtures were incubated under an atmosphere of nitrogen, S-adenosyl-\(\gamma\)-thio-α-ketobutyrate formation was not observed. Manometric measurements in the presence of air by the Warburg apparatus revealed that oxygen consumption was coincident
with keto acid formation. Thus, the deamination of S-adenosyl-L-homocysteine to S-adenosyl-\( \gamma \)-thio-\( \alpha \)-ketobutyrate appeared to be similar to the oxidative deamination reaction catalyzed by the kidney and liver L-amino acid oxidase. Partial purification of L-amino acid oxidase from rat kidneys was performed as described in "EXPERIMENTAL PROCEDURE". The specific activity of the enzyme at various stages of purity was determined with L-leucine, L-methionine and S-adenosyl-L-homocysteine as substrates. Oxygen consumption was measured with the Gilson oxygraph in reaction mixtures containing enzyme, 16.6 mM substrate, 0.1 M Tris-HCl buffer, pH 9.0, and excess catalase. The ratios of specific activities of S-adenosyl-L-homocysteine to L-leucine, S-adenosyl-L-homocysteine to L-methionine and L-methionine to L-leucine remained essentially constant throughout purification (Table II).

Identification of Products from the Oxidative Deamination of S-Adenosyl-L-homocysteine

Warburg vessels containing partially purified L-amino acid oxidase, 25 \( \mu \)moles of S-adenosyl-\(^3\)H-L-homocysteine, 300 \( \mu \)moles of Tris-HCl buffer, pH 9.0 and 60 units of catalase in a total volume of 3.0 ml were incubated at 37\(^\circ\) for 1 hour. During this time oxygen consumption was measured manometrically. In one of the vessels the reaction was stopped by mixing the enzyme solution with 1.0 ml of
Table II

Specific activities during purification of the rat kidney L-amino acid oxidase

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Specific activities&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Ratio of specific activities</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>L-Leucine</td>
<td>L-Methionine</td>
</tr>
<tr>
<td>Crude</td>
<td>0.019</td>
<td>0.014</td>
</tr>
<tr>
<td>First Na&lt;sub&gt;2&lt;/sub&gt;SO&lt;sub&gt;4&lt;/sub&gt; precipitation</td>
<td>0.037</td>
<td>0.028</td>
</tr>
<tr>
<td>Heat treatment</td>
<td>0.121</td>
<td>0.089</td>
</tr>
<tr>
<td>Second Na&lt;sub&gt;2&lt;/sub&gt;SO&lt;sub&gt;4&lt;/sub&gt; precipitation</td>
<td>0.358</td>
<td>0.252</td>
</tr>
</tbody>
</table>

<sup>a</sup> Units per mg protein

<sup>b</sup> S-Adenosyl-L-homocysteine

<sup>c</sup> Below sensitivity of the assay
saturated \( \text{K}_2\text{CO}_3 \) contained in a side arm of the vessel. The liberated ammonia was absorbed by the \( \text{H}_2\text{SO}_4 \) in the center well, and samples of this acid were assayed for ammonia by reaction with Nessler's reagent (11). The other reaction mixtures were deproteinized by heating in a boiling water bath for 3 min and centrifuging at 5000 x g for 5 min. The resulting supernatant fluid was chromatographed on Amberlite CG-120 resin. An elution pattern of a reaction mixture is given in Figure 10A and is compared to that of a zero-time control (Figure 10B). The radioactive keto acid which was eluted from the column at a gradient pH of 4.5 (Figure 10A) was not present in the zero-time control (Figure 10B). This compound exhibited all the properties previously described for S-adenosyl-\( \gamma \)-thio-\( \alpha \)-ketobutyrate. S-Adenosyl-\( ^3 \text{H} \)-L-homocysteine emerged from the columns at its characteristic gradient pH value of 5.7. Table III lists the quantitative relationships observed between oxygen uptake and ammonia and keto acid production. The amount of S-adenosyl-L-homocysteine utilized during the reaction was equivalent to the amount of S-adenosyl-\( \gamma \)-thio-\( \alpha \)-ketobutyrate recovered, and the specific activity (cpm per \( \mu \)mole) of the deaminated product was 92% that of the substrate. The oxygen consumption data indicated that 0.48 \( \mu \)mole of oxygen was consumed per \( \mu \)mole of S-adenosyl-\( \gamma \)-thio-\( \alpha \)-ketobutyrate formed in the presence of catalase. The amount of ammonia liberated was 0.93 \( \mu \)mole per \( \mu \)mole of keto acid formed.
Fig. 10. — A, chromatographic separation of reaction products produced by partially purified rat kidney L-amino acid oxidase. The reaction mixture, containing 25 μmoles of S-adenosyl$^3$H-L-homocysteine, 300 μmoles of Tris-HCl buffer, pH 9.0, and 60 units of catalase in a total volume of 3.0 ml, was incubated for 1 hour at 37°. After deproteination, the mixture was placed on Amberlite CG-120 resin (0.7 x 20 cm) and eluted as described in Figure 5. B, elution pattern of an identical mixture deproteinized at zero time. (-----) pH gradient; (-----) reducing compounds as measured by the orcinol test with ribose as the standard; (-----) keto acids as measured by the 2,4-dinitrophenylhydrazine test with α-ketobutyrate as the standard; (-----) radioactivity. ATKB, S-adenosyl-γ-thio-α-ketobutyrate; AH, S-adenosyl-L-homocysteine.
Table III

Summary of products recovered from S-adenosyl-$^3$H-L-homocysteine

<table>
<thead>
<tr>
<th>Compound</th>
<th>Reaction mixture$^a$</th>
<th>Zero-time control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Amount recovered</td>
<td>Specific activity</td>
</tr>
<tr>
<td></td>
<td>µmoles</td>
<td>cpm/µmole</td>
</tr>
<tr>
<td>S-Adenosyl-L-homocysteine</td>
<td>23.22</td>
<td>204,000</td>
</tr>
<tr>
<td>S-Adenosyl-$\gamma$-thio-α-ketobutyrate</td>
<td>2.10</td>
<td>188,000</td>
</tr>
<tr>
<td>Oxygen$^b$</td>
<td>1.00</td>
<td>---</td>
</tr>
<tr>
<td>Ammonia$^b$</td>
<td>1.95</td>
<td>---</td>
</tr>
</tbody>
</table>

$^a$ Reaction mixture described in Figure 10A

$^b$ Endogenous values subtracted
Substrate Specificity

In addition to the several natural L-amino acids found to be
deaminated by the kidney L-amino acid oxidase (5), S-adenosyl-L-
homocysteine, L-homocysteine and S-adenosyl-L-methionine were
found to serve as substrates for this enzyme. The enzyme velocity
with various substrates was determined in the presence and absence
of catalase. The reaction mixtures contained 15.0 \( \mu \)moles of sub-
strate, 150 \( \mu \)moles of Tris-HCl buffer, pH 9.0, 30 units of catalase
(when added) and enzyme in a total volume of 1.5 ml. Oxygen uptake
was measured with the Gilson oxygraph and the results are shown in
Table IV. S-Adenosyl-L-homocysteine was 30% as reactive as L-
leucine, 41% as reactive as L-methionine and twice as active as S-
adenosyl-L-methionine. With all substrates tested oxygen consumption
was approximately doubled when catalase was omitted from the reaction
mixtures. Activity with L-homocysteine could not be determined by
the Gilson oxygraph method since free sulfhydryl compounds adversely
affect the electrode system. Incubation of L-homocysteine with the
partially purified enzyme in Warburg vessels did result in oxygen con-
sumption and keto acid formation. However, a meaningful comparison
of these two parameters was difficult to obtain due to the auto-oxidation
of the L-homocysteine.
Table IV

Relative reaction velocities

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Catalase</th>
<th>Specific activity&lt;sup&gt;a&lt;/sup&gt;</th>
<th>% of L-leucine activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-Leucine</td>
<td>-</td>
<td>0.579</td>
<td>100</td>
</tr>
<tr>
<td>L-Leucine</td>
<td>+</td>
<td>0.316</td>
<td></td>
</tr>
<tr>
<td>L-Methionine</td>
<td>-</td>
<td>0.424</td>
<td>73</td>
</tr>
<tr>
<td>L-Methionine</td>
<td>+</td>
<td>0.203</td>
<td></td>
</tr>
<tr>
<td>S-Adenosyl-L-homocysteine</td>
<td>-</td>
<td>0.172</td>
<td>30</td>
</tr>
<tr>
<td>S-Adenosyl-L-homocysteine</td>
<td>+</td>
<td>0.090</td>
<td></td>
</tr>
<tr>
<td>S-Adenosyl-L-methionine</td>
<td>-</td>
<td>0.090</td>
<td>15</td>
</tr>
<tr>
<td>S-Adenosyl-L-methionine</td>
<td>+</td>
<td>0.045</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Units per mg protein
Optimal pH for Enzymatic Activity

The optimal pH range for the oxidative deamination of S-adenosyl-L-homocysteine by the partially purified kidney L-amino acid oxidase was from 8.8 to 9.2. Figure 11 shows the effect of pH on the enzyme velocity with S-adenosyl-L-homocysteine, L-methionine and L-leucine as substrates. Incubation of L-homocysteine with the partially purified enzyme in Warburg vessels revealed that maximum oxygen consumption and keto acid production occurred between pH 8.4 and 9.0. With S-adenosyl-L-methionine as substrate, oxygen consumption increased as the pH increased between 7.7 and 9.2. Substrate lability prevented reproducible measurements of activity above pH 9.2.

Km Determination

Reaction mixtures containing the partially purified L-amino acid oxidase and varying amounts of S-adenosyl-L-homocysteine ranging from 0.005 M to 0.022 M were incubated in the presence and absence of catalase and oxygen consumption was measured with the Gilson oxygraph. A Lineweaver-Burk reciprocal plot (40) of the substrate concentrations versus the velocities gave a Km value of \(2.48 \times 10^{-2}\) M for S-adenosyl-L-homocysteine and a Vmax of 0.2 \(\mu\)mole of oxygen utilized per min per mg protein in the presence of catalase (Figure 12). In the absence of catalase the value for Km remained the same and that for Vmax was exactly doubled. The Km value obtained
Fig. 11. -- Effect of pH on the rate of oxidation of S-adenosyl-L-homocysteine (○), L-methionine (□) and L-leucine (△). The reaction mixtures consisted of partially purified L-amino acid oxidase, 20 μmoles of substrate, 45 units of catalase, and 150 μmoles of Tris-HCl buffer (for the range of 7.0 to 9.0) or 150 μmoles of glycine -- NaOH buffer (for the range of 9.1 to 11.0) in a total volume of 1.5 ml. Oxygen consumption was measured with the Gilson oxygraph.
AMOLES OXYGEN UTILIZED PER MIN PER mg PROTEIN

FINAL pH

7.5 8.0 8.5 9.0 9.5 10.0 10.5
Fig. 12. — Lineweaver-Burk double reciprocal plot of initial velocity versus S-adenosyl-L-homocysteine concentration in the presence (○—○) and absence (●—●) of catalase. The Gilson oxygraph was used to measure oxygen consumption in reaction mixtures of 0.1 M Tris-HCl buffer, pH 9.0, excess partially purified enzyme and varying amounts of S-adenosyl-L-homocysteine. The final concentration of catalase (when added) was 30 units per ml.
\[
\frac{1}{V} = 10^{-2M} \times (S\text{-ADENOSYL-L-HOMOCYSTEINE})^{-1}
\]
for L-leucine with the partially purified L-amino acid oxidase was $1.25 \times 10^{-2}$ M (Figure 13) and that for L-methionine was $1.82 \times 10^{-2}$ (Figure 14). The Vmax values with these two substrates also were doubled when catalase was omitted from the reaction mixtures.

**Perfusion Experiments**

The techniques used for perfusion of rat livers are described in "EXPERIMENTAL PROCEDURE". A rat liver weighing 16.0 g was perfused for 20 min with 95 ml of Krebs-Ringer bicarbonate solution containing red blood cells and serum albumin. Six ml of a buffered solution containing 300 μmoles of S-adenosyl-$^{3}$H-L-homocysteine (204,000 cpm per μmole) was added to the perfusate and perfusion was continued at an average rate of 19 ml per min. At time intervals of 30, 90, 120 and 150 min after addition of the substrate, 5 ml samples of the perfusate were removed and deproteinized by the addition of trichloroacetic acid. The precipitate was removed by centrifugation and the supernatant fluids were adjusted to pH 2.9 with NaOH and chromatographed on Amberlite CG-120 resin. Hydrolysis of the substrate to adenosine and L-homocysteine by S-adenosyl-L-homocysteine hydrolase, previously found to be highly active in cell-free rat liver extracts (19), did not
Fig. 13. — Lineweaver-Burk double reciprocal plot of initial velocity versus L-leucine concentration in the presence (○—○) and absence (○——○) of catalase. The Gilson oxygraph was used to measure oxygen consumption in reaction mixtures of excess partially purified enzyme, 0.1 M Tris-HCl buffer, pH 9.0, and varying amounts of L-leucine. The final concentration of catalase (when added) was 30 units per ml.
Fig. 14. — Lineweaver-Burk double reciprocal plot of initial velocity versus L-methionine concentration in the presence (○—○) and absence (□—□) of catalase. The Gilson oxygraph was used to measure oxygen consumption in reaction mixtures of 0.1 M Tris-HCl buffer, pH 8.5, excess partially purified enzyme and varying amounts of L-methionine. The final concentration of catalase (when added) was 30 units per ml.
occur. Complete recovery of the S-adenosyl-L-homocysteine was obtained in all samples and no radioactive adenosine or S-adenosyl-$\gamma$-thio-$\alpha$-ketobutyrate was detected.

An identical perfusion experiment was performed with 500 $\mu$moles each of adenosine and L-homocysteine as substrates. Perfusate samples were removed at the various time intervals stated above, deproteinized and chromatographically analyzed for the presence of S-adenosyl-L-homocysteine on Amberlite CG-120 resin. A sample of the perfusate obtained before the addition of the substrates served as the chromatographic control. After 30 min of perfusion, the total perfusate contained 20 $\mu$moles of S-adenosyl-L-homocysteine, and this amount remained unchanged for the duration of the 2.5 hour perfusion experiment. The adenosine was apparently metabolized quite rapidly since only 20% of the initial amount was recovered after 30 min of perfusion and none was recovered after 90 min.
DISCUSSION

S-Adenosyl-L-methionine is a principle methyl group donor in a variety of biological systems. In the rat several tissues including adrenal gland, liver, heart, spleen, kidney, lung and brain have been shown to contain this compound in concentrations ranging from 10 to 48 µg per g (4), and many of these tissues exhibit specific trans-methylase activity with S-adenosyl-L-methionine as the methyl donor (47, 59). The enzymatic removal of the labile methyl group from this compound results in the formation of S-adenosyl-L-homocysteine. In vitro studies by De La Haba and Cantoni (19) indicated the presence of an enzyme, S-adenosyl-L-homocysteine hydrolase, in cell-free rat liver extracts which catalyzed the reversible hydrolysis of S-adenosyl-L-homocysteine to adenosine and L-homocysteine. From this study in vitro it was not possible for these investigators to state whether S-adenosyl-L-homocysteine is hydrolyzed in vivo or whether it is metabolized through a pathway not yet discovered. If the S-adenosyl-L-homocysteine hydrolase system is the mechanism by which S-adenosyl-L-homocysteine is metabolized in mammals, then the homocysteine generated from this reaction should be utilized in the
formation of methionine. The presence of transmethylase systems involving homocysteine have been established in mammals. These would include dimethylthetin:L-homocysteine S-methyltransferase, EC 2.1.1.3, (28), N₅-methyl-tetrahydrofolate:L-homocysteine methyltransferase (36, 54, 70) and betaine:L-homocysteine S-methyltransferase, EC 2.1.1.5, (37). Since mammalian systems also contain L-serine hydro-lyase (deaminating), EC 4.2.1.13, (58) and L-homoserine hydro-lyase (deaminating), EC 4.2.1.15, (42, 43) which catalyze a transsulfuration reaction through the formation cystathionine, the sulfur atom of homocysteine could ultimately appear in the urine as inorganic sulfate or taurine via cysteine, whereas the carbon skeleton could be excreted as α-ketobutyrate. L-Homocysteine hydrogensulfide lyase (deaminating), EC 4.4.1.2, (32) previously demonstrated in rat liver, kidney and pancreas could also be involved in the formation of α-ketobutyrate from the homocysteine moiety of S-adenosyl-L-homocysteine. Several investigators (27, 30, 54) have postulated mechanisms whereby S-adenosyl-L-homocysteine also served as the methyl acceptor from methyltetrahydrofolatic acid; but direct evidence for this mechanism has not been obtained (22, 29, 63). In bacteria two separate enzymes, S-adenosyl-L-homocysteine nucleosidase and S-ribosyl-L-homocysteine cleavage enzyme, catalyze the generation of free homocysteine from S-adenosyl-L-homocysteine via formation
of the intermediate S-ribosyl-L-homocysteine (25, 46); however, these enzymes have not been detected in rat tissues. The apparent metabolic fate of S-adenosyl-L-homocysteine in the mammalian system is shown in Scheme III.

When S-adenosyl-L-homocysteine tritiated in the homocysteine moiety was administered intravenously to rats most of the radioisotope was excreted in the urine while only trace amounts were incorporated into tissue proteins. It would appear that the over-all economy of the mammalian system is extremely poor with regard to the utilization of homocysteine from S-adenosyl-L-homocysteine. Chromatographic analysis of the urine from these animals demonstrated that less than 10% of the radioisotope present was associated with \( \alpha \)-ketobutyrate. The remainder of the tritium in the urine was found associated with a previously unidentified keto acid. As with the rats administered S-adenosyl-L-homocysteine-\( ^3 \)H, rats injected with S-adenosyl-\( ^3 \)H-L-homocysteine (adenosine labeled) or S-adenosyl-L-homocysteine-\( ^35 \)S excreted about 55% of the radioisotope administered (\( ^3 \)H or \( ^35 \)S) within 4 to 5 hours. Chromatographic analysis of these urines revealed that both the sulfur atom and the adenosine moiety of the parent compound remained associated with the previously unidentified keto acid excretory product. These data suggest that the thioether linkage of
S-ADENOSYLHOMOCYSTEINE

X-CH₃

S-ADENOSYLMETHIONINE

ATP

METHIONINE

C₁

H₂S

PROTEIN

NH₃

α-KETOBUTYRATE

HOMOCYSTEINE + ADENOSINE

SERINE

CYSTATHIONINE

HOMOSERINE + CYSTEINE

CYSTEINE SULFINIC ACID

SO₄²⁻ + PYRUVATE TAURINE

Scheme III
S-adenosyl-L-homocysteine was not readily hydrolyzed in vivo, but that this compound underwent deamination and was eliminated in the urine.

Purification of this excretory product by procedures similar to those used for the purification of S-adenosyl-L-homocysteine provided sufficient crystalline compound for its chemical and physical characterization. Analytical data on the purified compound including chemical, elemental and ultraviolet and infrared spectral analyses, and information obtained from the radioisotope tracer experiments indicate the chemical structure of the excretory product to be S-adenosyl-γ-thio-α-ketobutyrate, as shown in Formula III.

To investigate the in vitro formation of S-adenosyl-γ-thio-α-ketobutyrate, various rat tissues were surveyed for their ability to deaminate S-adenosyl-L-homocysteine under conditions which would facilitate transamination or oxidative deamination. The formation of the keto acid derivative of S-adenosyl-L-homocysteine was catalyzed by kidney and liver extracts, and neither transamination nor oxidative deamination with NAD or NADP as the acceptors appeared to be involved. Since the deamination of S-adenosyl-L-homocysteine was concomitant with oxygen utilization, the reaction appeared to be similar to the oxidative deamination of L-amino acids catalyzed by the kidney and liver L-amino acid oxidase. This enzyme was partially purified from rat
kidneys by the method of Blanchard et al. (6). The first four steps of this procedure yielded a 20-fold purification and resulted in 40 to 50% recovery of the enzyme initially present in the crude fraction. Further purification resulted in severe decreases in total recovery. The relative velocities obtained with S-adenosyl-L-homocysteine, L-methionine and L-leucine as substrates for the rat kidney L-amino acid oxidase at various stages of purity suggested that S-adenosyl-L-homocysteine could serve as a substrate for this enzyme. Chromatographic analysis of reaction mixtures in which S-adenosyl-L-homocysteine was incubated with the partially purified kidney enzyme resulted in the identification of S-adenosyl-γ-thio-α-ketobutyrate as a product of the reaction. The quantitative relationships observed between oxygen consumption, keto acid and ammonia production from S-adenosyl-L-homocysteine in the presence of the partially purified oxidase were identical to those previously observed for the oxidative deamination of several natural L-amino acids by this enzyme (5).

The Km value for S-adenosyl-L-homocysteine was twice that for L-leucine and 1.3 times that observed for L-methionine. The Km for L-leucine agreed with that obtained by other investigators (48), and the Km for L-methionine has not previously appeared in the literature. The Vmax for S-adenosyl-L-homocysteine as well as those for L-leucine and L-methionine were measured in the absence of catalase.
The values obtained for all three substrates were twice those obtained in the presence of catalase. Breakdown of the hydrogen peroxide formed during the oxidative deamination reaction catalyzed by the L-amino acid oxidase will replenish one-half of the oxygen utilized in the reaction (Reaction D, "HISTORY"). Therefore, measurement of oxygen consumption in the presence of catalase results in values which are one-half those obtained in the absence of catalase. Keto acid formation from S-adenosyl-L-homocysteine, L-leucine or L-methionine in the absence of catalase was not detected, since the hydrogen peroxide formed caused the decarboxylation of these keto acids to the corresponding acid with one less carbon as shown in Reaction F ("HISTORY"). The evidence presented clearly shows that L-amino acid oxidase found in rat kidney and liver oxidatively deaminates S-adenosyl-L-homocysteine as described in Reaction J,

\[
\text{S-Adenosyl-L-homocysteine} + \text{O}_2 + \text{H}_2\text{O} \rightarrow \text{S-Adenosyl-\(\gamma\)-thio-\(\delta\)-ketobutyrate} + \text{NH}_3 + \text{H}_2\text{O}_2 \quad (J)
\]

S-Adenosyl-L-methionine also appeared to serve as a substrate for the L-amino acid oxidase. The lability of this compound at the alkaline pH values necessary for enzyme action may have resulted in the formation of decomposition products which were subsequently deaminated by the enzyme. However, L-homoserine, the most likely decomposition
product which could be deaminated, did not serve as a substrate when incubated with the partially purified enzyme. Incubation of L-homocysteine with the partially purified L-amino acid oxidase resulted in oxygen utilization and keto acid formation. The high susceptibility of L-homocysteine to spontaneous chemical oxidation may have facilitated the formation of compounds which were more or less enzymatically active than L-homocysteine itself. L-Homocysteine hydrogen sulfide lyase (deaminating) which catalyzes the formation of $\alpha$-ketobutyrate from L-homocysteine was not present in the L-amino acid oxidase preparation.

In addition to the oxidative deamination of S-adenosyl-L-homocysteine, the hydrolytic cleavage of this compound to adenosine and L-homocysteine was catalyzed by the crude dialyzed liver extracts. The adenosine resulting from hydrolysis was subsequently converted to inosine, hypoxanthine and adenine by enzymes previously demonstrated in liver extracts (17, 34, 35).

Thus, two mechanisms can be demonstrated for the enzymatic breakdown of S-adenosyl-L-homocysteine by the rat in vitro. One reaction is catalyzed by the kidney and liver L-amino acid oxidase and results in the formation of S-adenosyl-$\gamma$-thio-$\alpha$-ketobutyrate. The other reaction detected in the liver is catalyzed by S-adenosyl-L-homocysteine hydrolase and results in the formation of adenosine and
L-homocysteine. The in vivo studies clearly demonstrated that S-
adenosyl-L-homocysteine administered intravenously was not readily
hydrolyzed but was deaminated and eliminated in the urine as S-
adenosyl-\( \gamma \)-thio-\( \alpha \)-ketobutyrate. The results of liver perfusion experi-
ments demonstrated that S-adenosyl-L-homocysteine was not hydrolyzed
or deaminated. However, perfusion with equimolar amounts of adeno-
sine and L-homocysteine did result in the formation of measurable
amounts of S-adenosyl-L-homocysteine even in the presence of the
highly active erythrocyte adenosine deaminase which would tend to
remove the available adenosine. The liver cells appear to be permeable
to adenosine and L-homocysteine but relatively impermeable to S-
adenosyl-L-homocysteine. This does not preclude the possibility
that S-adenosyl-L-homocysteine formed intracellularly in the liver could
be subsequently hydrolyzed. Since S-adenosyl-L-homocysteine hydrol-
ase was not detected in muscle, spleen, heart, brain, small intestine,
or kidneys, the compound formed in these tissues would not be hydro-
lytically cleaved but could be oxidatively deaminated in the kidney and
eliminated in the urine.
SUMMARY

When S-adenosyl-L-homocysteine tritiated in the homocysteine moiety was administered intravenously to rats, less than 15% of the radioisotope was incorporated into protein methionine or excreted as α-ketobutyrate. From 70 to 80% of the tritium was found associated with a previously unidentified keto acid which was excreted in the urine. The radioactively labeled moieties of intravenously administered S-adenosyl-$^3$H-L-homocysteine and S-adenosyl-L-homocysteine-$^{35}$S also remained associated with the keto acid excretory product. The unknown compound was isolated from the urine and purified. Chemical and physical analyses of the crystalline compound revealed its chemical structure to be S-adenosyl-$^3$-thio-α-ketobutyrate. These in vivo experiments demonstrated that S-adenosyl-L-homocysteine was not readily hydrolyzed by the S-adenosyl-L-homocysteine hydrolase previously demonstrated in cell-free liver extracts, but was deaminated and eliminated in the urine as S-adenosyl-$^3$-thio-α-ketobutyrate.

The in vitro formation of S-adenosyl-$^3$-thio-α-ketobutyrate was studied by incubating S-adenosyl-$^3$H-L-homocysteine with various rat
tissues and chromatographically analyzing the reaction mixtures for the deaminated product. The deamination of S-adenosyl-L-homocysteine occurred only in reaction mixtures in which kidney and liver extracts were used. This reaction was found to be catalyzed by the general L-amino acid oxidase which had been previously detected in the rat kidney and liver. The characteristics of the oxidative deamination of S-adenosyl-L-homocysteine by the partially purified rat kidney L-amino acid oxidase were examined and compared to those of the deamination of L-leucine and L-methionine by the same enzyme.

A survey of cell-free extracts of various rat tissues for the presence of S-adenosyl-L-homocysteine hydrolase which cleaves S-adenosyl-L-homocysteine to adenosine and L-homocysteine revealed that the liver was the only major organ containing detectable amounts of this enzyme. However, liver perfusion studies suggested an impermeability of liver cells to S-adenosyl-L-homocysteine.

S-Adenosyl-L-homocysteine administered intravenously to rats was not readily hydrolyzed to adenosine and L-homocysteine, since the only major organ of the rat which is able to catalyze this reaction appears to be impermeable to the substrate. However, S-adenosyl-L-homocysteine formed intracellularly in the liver could undergo hydrolysis to adenosine and L-homocysteine. S-Adenosyl-L-homocysteine formed in other
major organs would not undergo hydrolysis but could be oxidatively deaminated by the kidney L-amino acid oxidase and eliminated in the urine as S-adenosyl-\(\gamma\)-thio-\(\alpha\)-ketobutyrate.
APPENDIX
<table>
<thead>
<tr>
<th>Compound</th>
<th>Amount per liter of water</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>20.0 g</td>
</tr>
<tr>
<td>Ammonium sulfate</td>
<td>3.0 g</td>
</tr>
<tr>
<td>DL-Aspartic acid</td>
<td>0.2 g</td>
</tr>
<tr>
<td>Monopotassium phosphate</td>
<td>2.0 g</td>
</tr>
<tr>
<td>Calcium chloride</td>
<td>0.25 g</td>
</tr>
<tr>
<td>Magnesium sulfate·7H₂O</td>
<td>0.25 g</td>
</tr>
<tr>
<td>Boric acid</td>
<td>1.0 mg</td>
</tr>
<tr>
<td>Zinc sulfate</td>
<td>1.0 mg</td>
</tr>
<tr>
<td>Manganese chloride</td>
<td>1.0 mg</td>
</tr>
<tr>
<td>Ferric chloride</td>
<td>0.5 mg</td>
</tr>
<tr>
<td>Copper sulfate·5H₂O</td>
<td>0.1 mg</td>
</tr>
<tr>
<td>Potassium iodide</td>
<td>0.1 mg</td>
</tr>
<tr>
<td>Inositol</td>
<td>3.0 mg</td>
</tr>
<tr>
<td>Beta-Alanine</td>
<td>1.0 mg</td>
</tr>
<tr>
<td>Nicotinic acid</td>
<td>0.1 mg</td>
</tr>
<tr>
<td>Pyridoxine</td>
<td>0.02 mg</td>
</tr>
<tr>
<td>Thiamine</td>
<td>0.02 mg</td>
</tr>
<tr>
<td>Para-Aminobenzoic acid</td>
<td>0.02 mg</td>
</tr>
<tr>
<td>Biotin</td>
<td>0.01 mg</td>
</tr>
</tbody>
</table>

Adjust pH to 4.0 and sterilize at 121 °C for 12 min.
Bial's Reagent for the Determination of Pentose (13)

Orcinol (recrystallized from benzene) .............. 0.2 g
Hydrochloric acid (concentrated) ..................... 60.0 ml
10% Ferric chloride in concentrated HCl .............. 0.2 ml
Make fresh daily.

Ninhydrin Reagent for the Determination
of Amino Acids (75)

Potassium Cyanide-Methyl Cellosolve Solution

0.01 M Potassium cyanide ......................... 5.0 ml
Methyl cellosolve .................................. 245.0 ml

Methyl Cellosolve-Ninhydrin Solution

5.0% 1,2,3-triketohydrindene in methyl cellosolve

Potassium Cyanide-Methyl Cellosolve Ninhydrin Solution

Methyl cellosolve-ninhydrin solution ............... 50.0 ml
Potassium cyanide-methyl cellosolve solution ..... 250.0 ml
Store overnight before use.

Krebs-Ringers Bicarbonate Solution (67)

0.90% Sodium chloride (0.154 M) ................. 100 parts
1.30% Sodium bicarbonate (0.154 M) ............... 21 parts
1. 15% Potassium chloride (0.154 M) ........................................... 4 parts
1.22% Calcium chloride (0.110 M) ........................................... 3 parts
2.11% Monopotassium phosphate (0.154 M) .................................. 1 part
3.82% Magnesium sulfate·7H₂O (0.154 M). .................................. 1 part

The solution is gassed with a mixture of 95% oxygen and 5% carbon
doioxide for 10 min and stored at 4° until used. The final pH should
be 7.4.

**Detection Reagents Used with Paper**

or **Thin Layer Chromatography**

**Detection of Sulfur Compounds (65)**

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.002 M Platinic chloride</td>
<td>2.0 ml</td>
</tr>
<tr>
<td>1.0 M Potassium iodide</td>
<td>0.125 ml</td>
</tr>
<tr>
<td>2.0 N Hydrochloric acid</td>
<td>0.2 ml</td>
</tr>
<tr>
<td>Acetone</td>
<td>38.0 ml</td>
</tr>
</tbody>
</table>

Spray the developed paper with the above reagent and dry in air at
room temperature. Sulfur compounds will reduce the platinic iodide
and result in white areas against a pink background.

**Detection of Amino Acids (65)**

A solution of 0.25% 1,2,3-triketohydrindene (ninhydrin) in acetone is
sprayed on the developed paper. After the excess acetone has evapo-
rated at room temperature, the paper is heated at 110° for about 1 min.
Amino acids will yield blue-purple spots against a white background.

Detection of Carbohydrates (66)

Saturated aqueous solution of silver nitrate . . . . . 0.1 ml
Acetone . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . 20.0 ml
Water (add dropwise with shaking until the suspended silver nitrate redissolves)

a) Spray the developed paper with the above reagent.
b) Spray the dry paper with 0.5 N sodium hydroxide in 95% ethanol.
c) Remove the excess silver oxide by immersion of the paper in 6 N ammonium hydroxide, wash for 1 hour with running water.

Reducing sugars will form black or dark brown spots against a white background.
LITERATURE CITED


49. Nakano, M., Y. Tsutsumi, and T. S. Danowski. 1967. Crystal-
line L-amino acid oxidase from the soluble fraction of rat

of L-amino acid oxidases from snake venom and rat kidney.

51. Ratner, S. 1955. L-Amino acid oxidases (mammalian tissues and
snake venom), p. 204-211. In S. P. Colowick and N. O.
Kaplan (eds.), Methods of enzymology, vol. 2. Academic

novelle L-aminoacideoxydase, actvable par le magnesium.

of amino acid solutions by an ion retardation resin. Anal. Chem.
34:711-712.

54. Sakami, W., and I. Uktstins. 1961. Enzymic methylaction of homo-
cysteine by a synthetic tetrahydrofolate derivative. J. Biol.
Chem. 236:PC50-PC51.

55. Salvatore, F., V. Zappia, and R. Cortese. 1966. Studies on the
deamination of L-amino acids in mammalian tissues.
Enzymologia 31:113-127.


