ABSTRACT

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The purpose of this study was to develop an affinity chromatography system with S-adenosyl-L-methionine (SAM) as the ligand by a solid-phase biosynthetic reaction between agarose-ATP and L-methionine catalyzed by SAM synthetase. The SAM synthetase in preliminary experiments was a 60-80 fold purified preparation from E. coli and was provided by R. K. Morse. Because of problems with the results with E. coli SAM synthetase, attempts were made to isolate a purer SAM synthetase preparation from baker's yeast and use this in the solid-phase reaction.

The procedure followed for the solid-phase biosynthesis reaction came from unpublished work of R. K. Morse. The method followed for the extraction and purification of SAM synthetase from baker's yeast was a combination of the methods of Mudd and Cantoni (1958), Lombardini et al., (1970), and Chou and Talalay (1972).

The SAM synthetase assay method used throughout this study to monitor SAM synthetase activity was developed by Holcomb and Shapiro (1975). The assay measures activity by the separation of $^{14}CH_3$-methionine from $^{14}CH_3$-SAM on Bio-Rex 70 ion exchange resin.

Two experiments were performed with the solid-phase biosynthesis reaction using the E. coli SAM synthetase preparation with agarose-ATP having the ATP attached (1) via N^6-amino group of adenyl moiety, and (2) via the ribosyl moiety. Results from both experiments were incon-

clusive, although the data does suggest that the first type of agarose-ATP may be more promising for the biosynthesis of SAM than the second type of agarose-ATP.

Four attempts were made to isolate and purify SAM synthetase from baker's yeast, but all four attempts were unsuccessful. Therefore, as a result of this work, specific suggestions to help improve this procedure are made.

STUDIES ON THE SOLID-PHASE BIOSYNTHESIS OF S-ADENOSYL-L-METHIONINE

by

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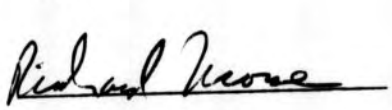

APPROVAL PAGE

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LIST OF ABBREVIATIONS

(1) ETOH: ethanol

(2) ETSH: 2-mercaptoethanol

(3) KPB: KH_2PO_4- KOH

(4) P_i: inorganic phosphate

(5) POPOP: 1,4-bis [2-(5-phenyloxazolyl)] benzene

(6) PP_i: inorganic pyrophosphate

(7) PPO: 2,5=diphenyloxazole

(8) PPPase: tripolyphosphatase

(9) PPP_i: inorganic tripolyphosphate

(10) SAH: S-adenosyl-L-homocysteine

(11) SAM: S-adenosyl-L-methionine

(12) Tris: tris (hydroxymethyl) aminomethane

CHAPTER 1

INTRODUCTION

Since the discovery of S-adenosyl-L-methionine (SAM)[1] as the biological methyl donor by Cantoni (1953), it has been identified as an important compound in the metabolic and regulatory pathways of a variety of microbial, plant, and animal systems. SAM is formed enzymatically from ATP and L-methione. The exact mechanism of the reaction will be discussed later, but it involves the complete dephosphorylation of ATP and the transfer of the adenosyl group to the sulfur atom of methionine. The stoichiometry of SAM formation is as follows:

(1) ATP + L-methionine $\longrightarrow$ S-adenosyl-L-methionine + PPi + Pi

SAM is a high energy sulfoniun compound with the sulfur atom carrying a positive charge (Lombardini and Talalay, 1971). The transmethylation reactions involving SAM are exothermic meaning that SAM is a "high energy" compound in the cell. Cantoni (1965) has shown that all three chemical groups attached to the sulfur are energetically equivalent, and therefore all three groups could be involved in a specific transfer reaction. SAM exists in two diastereomeric forms, (-) S-adenosyl-L-methionine and (+) S-adenosyl-L-methionine, with all transmethylation reactions utilizing the minus form (Lombardini and Talalay, 1971). SAM is stable in acid solution, but mild alkaline treatment results in decomposition to adenine and S-ribosylmethionine (Schlenk, 1965). However, Greene (1969) has reported that no significant degradation of SAM was observed after 30 minutes at pH 9.0 and 37° C.

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[1] Refer to page viii for a list of abbreviations used in this thesis.

A. Transmethylation Functions of SAM

The most important function of SAM is as a methyl group donor in a large number of transmethylation reactions. A list of methylated compounds include:

TABLE 1: A List of Methylated Compounds by SAM

Methylated Compounds:

Phosphatidylcholine
Cyclic fatty acids
Ergosterol
Choline
Creatine
Sarcosine
Anserine
Epinephrine
Metanephrine
1-methylhistamine
Melatonin
N-methylnicotinamide
Vitamin B_{12}
Methylated nucleotides of DNA, tRNA, rRNA, and mRNA
Methylated amino acids in proteins

No attempt will be made to discuss all of these reactions in detail, but several representative reactions will be mentioned. The slightly positive charge generated on the adjacent carbon atoms by the positive sulfonium group of SAM makes these atoms susceptible to nucleophilic attack by nitrogen, oxygen, sulfur, and carbon atoms on the acceptor molecules (Lombardini and Talalay, 1971).

(1) Small Molecules: Lipids

SAM has been shown to be the methyl donor to fatty acids to give fatty acid methyl esters and 10-methylated fatty acids. SAM is also the methyl donor in the three step methylation of phosphotidylethanolamine to phosphotidylcholine catalyzed by the enzyme phosphotidylethanolamine-N-methyltransferase.

(2) Small Molecules: Biogenic Amines

SAM is the methyl donor in transmethylation reactions involving the synthesis and metabolism of physiologically active amines. The synthesis of epinephrine, for example, involves the methylation of norepinephrine catalyzed by the enzyme phenylethanol amine-N-methyltransferase (Axelrod, 1977). The metabolic inactivation of epinephrine and norepinephrine involves methylation by SAM of the meta-hydroxyl group and is catalyzed by the enzyme catecholamine-O-methyltransferase from rat liver (Axelrod, 1977). The product of epinephrine inactivation is 3-methoxy-4-hydroxyphenyl-2-methylethanolamine (metanephrine). The inactivation of histamine is brought about by ring methylation to form 1-methylhistamine and is catalyzed by the enzyme histamine-N-methyltransferase (Axelrod, 1977). Lastly, the formation of melatonin from N-acetylserotonin involves indole methylation by SAM and is catalyzed by the enzyme hydroxyindole-O-methyltransferase (Axelrod, 1977). All of the methyltransferases thus far discussed show a high specificity for SAM.

(3) Nucleic Acid Methylation

It is known that DNA, tRNA, rRNA, and mRNA all contain methylated nucleotides and that all are methylated via SAM (Kerr and Borek, 1973). Some examples of the methylated derivatives include N^6-methyladenine, 1-methyladenine, 5-methyluridine, 5-methylcytosine, N^2, N^2-dimethylguanine, N^2-monomethylguanine, and 1-methylguanine. A number of methyltransferases specific for tRNA have been isolated and studied, although no homogeneous preparation has been characterized due to the instability of the enzymes (Nau, 1976). Methyltransferases specific for rRNA and DNA have been isolated and studied, although most of the work has been done with bacterial

and viral methyltransferases (Kerr and Borek, 1973). Eukaryotic DNA methyltransferases are membrane-bound to the nucleus which makes isolation difficult. DNA methylation is closely involved with DNA restriction with restriction enzymes also being methyltransferases (Arber, 1974). The most recent aspect of nucleic acid methylation has centered around mRNA methylation because of the discovery of a unique methylated 5'-terminal "cap" (Rottman, 1976). This "cap" was subsequently shown to contain 7-methylguanine at the 5'-terminus followed by a pyrophosphate bond to one or two O-methylated nucleotides (Desrosiers et al., 1975; Wei et al., 1975). Cellular mRNA but not viral mRNA also contains an internal N^6-methyladenine usually close to the 3' poly A tail (Desrosiers et al, 1975). Martin et al. (1975) have isolated an enzyme from vaccinia virions which catalyzes the addition of guanine to the 5'-terminus and then methylates it in the 7-position forming the 7-methylguanine cap. They have not been able to separate the two enzymatic activities. Thus far no methyltransferases specific for cellular mRNA have been isolated. The biological significance of nucleic acid methylation is only speculative at this stage and still requires more experimental data, although it has been shown that the methylated "cap" of mRNA is required for ribosomal binding and subsequent translation (Both, et al., 1975a, 1975b; Muthukrishnan, et al., 1975).

(4) Protein Methylation and Protein Methylases

Many types of proteins have been shown to contain methylated amino acids (Paik and Kim, 1971; 1975) including histones, cytochrome c in plants and fungi, encephalitogenic basic protein, ribosomal proteins, and the contractile proteins, myosin and actin. The types of methylated amino acids that have been observed include: ε -N-mono, di, and trimethyllysines,

N^G-monomethylarginine, N^G, N^G- and N^G, $N^{G'}$-dimethylarginines, and 3-N-methylhistidine (Paik and Kim, 1975). Methylation of amino acid residues occurs after protein synthesis (Allfrey, et al., 1964; Kim and Paik, 1965; Morse, et al., 1975). Allfrey, et al., (1964) and Kim and Paik (1965), using methyl-labeled methionine and SAM have shown that the incorporation of radioactivity into protein was puromycin-sensitive with methyl-labeled methionine, but not with methyl-labeled SAM. This has also been shown using cycloheximide, another protein synthesis inhibitor (Morse, et al., 1975). Morse et al.(1975) have also shown that methylation can occur while the polypeptide is still on the ribosome (i.e. as a nascent polypeptide chain still covalently attached to the tRNA).

The methylation of proteins requires SAM as methyl donor and is catalyzed by a number of different methyltransferases, the number being unknown at present (Paik and Kim, 1975). Paik and Kim (1971, 1975) have isolated and characterized three methyltransferases designated as protein methylase I (SAM: protein-arginine methyltransferase), protein methylase II (SAM: protein-carboxyl methyltransferase), and protein methylase III (SAM: protein-lysine methyltransferase). Protein methylase I activity has been found in the cytosol and nuclei of cells from rat brain, spleen, and testis, the highest activity being found in rat brain (Paik and Kim, 1969; Paik and Kim, 1975). The protein substrates are histones and encephalitogenic basic protein, and the methylated amino acid is arginine. The enzyme has been purified 120-fold from calf brain (Lee, et al., 1977). Its molecular weight is greater than 1.5×10^6, although it should be noted that the enzyme preparation used for this determination was not homogeneous (Lee, et al., 1977). Protein methylase II has been

purified 2400-fold and has been shown to have a molecular weight of approximately 25,000, although subunits have been observed (Kim, 1973). This enzyme methylates the β- and γ-carboxyl groups of glutamate and aspartate respectively, forming methyl esters. Its natural substrate is unknown, but it will methylate gelatin, ovalbumin, and ribonuclease, proteins which are not normally methylated. Protein methylase III methylates lysine residues, and unlike the other two methylases, is found only in the nuclei of cells, particularly thymus (Paik and Kim, 1970; Paik and Kim, 1975). The main protein substrate for this enzyme are histones. Paik and Kim (1970) were successful in solubilizing the enzyme, but only purified it 1.3-fold. Durban, et al. (1977) have purified a protein methylase III from Neurospora crassa which is specific for cytochrome c. The exact purification factor was not mentioned, but they did show the enzyme to be 90% pure when applied to gel electrophoresis and to have a molecular weight of 120,000.

(5) Methylation of Contractile Proteins

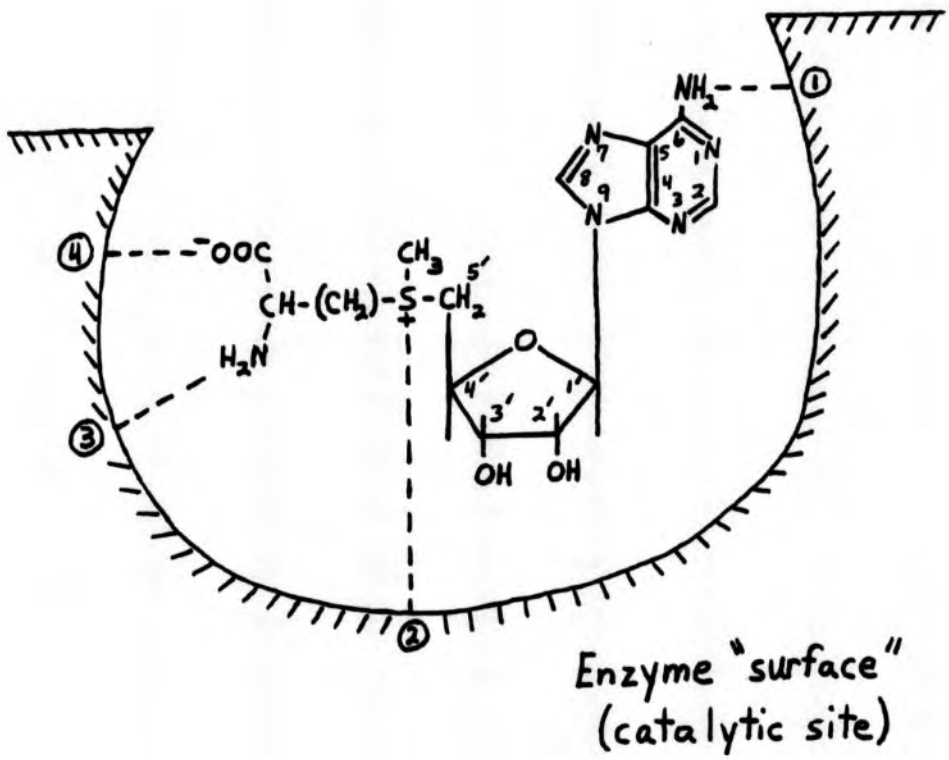
Actin and myosin have been shown to contain methylated amino acids, and that methylation is a post-translational event (Krzysik, et al., 1971; Morse, et al., 1975; Paik and Kim, 1975). The methylated amino acids that have been observed include 3-N-methylhistidine, ε-N-mono, di, and trimethyllysines, and N^G, N^G-, N^G,N^G-dimethylarginines. It is interesting to note that all methylated amino acids are located exclusively in the heavy meromyosin fragment (Krzysik, et al., 1971). The content of 3-N-methylhistidine has been shown to be absent from red muscle myosin but present in white muscle myosin; also it is absent from fetal white muscle, but appears during the first post-natal month (Trayer, et al.,

1968; Krzysik, et al, 1971). Lobley, et al. (1971) have observed that the amount of 3-N-methylhistidine is markedly reduced in myosin from vitamin E-induced dystrophic rabbits. The amounts of the other methylated amino acids remained the same. Thus far no methyltransferases for any of the methylated amino acids have been isolated that are specific for contractile proteins.

B. Specificity of SAM and SAH in Transmethylation Reactions

Many structural requirements and important binding sites for SAM in transmethylation reactions have been elucidated through the use of analogues of SAM. Zappia et al. (1969) performed a study using deaminated and decarboxylated analogues of SAM in three different methyltransferase reactions. The analogues of SAM had been deaminated at the α amino group of the methionyl moiety or at the 6-amino group of the adenyl moiety (or both) and had been decarboxylated using SAM decarboxylase (Zappia et al., 1969). The three methyltransferase systems investigated were purified preparations of histamine-N-methyltransferase, acetylserotonin-O-methyltransferase, and homocysteine-S-methyltransferase. The resulting binding sites proposed by Zappia et al. (1969) are shown in Figure 1. The α-amino group on the methionyl moiety was needed by all three methyltransferases (Zappia et al., 1969). The α-deaminated analogues were found to be inactive both as methyl donors and as inhibitors of the reaction. Thus the α-amino group of the methionyl moiety is necessary for binding to the enzyme rather than for facilitating methyl transfer (Zappia et al., 1969). The carboxyl group is necessary in N-methyl and O-methyl transfer but not in S-methyl transfer. Unlike the

Figure 1: Proposed Binding Sites for SAM in Transmethylation Reactions (Zappia et al., 1969)



α-deaminated analogues, the decarboxylated analogues exhibited significant inhibition of the histamine-N-methyltransferase and acetylserotonin-O-methyltransferase reactions (Zappia et al., 1969). The deaminated analogues at the 6-amino group of the adenyl moiety were found to be inactive with the acetylserotonin-O-methyltransferase, partially active with the histamine-N-methyltransferase, but fully active with the homocysteine-S-methyltransferase (Zappia et al., 1969). It should be noted that these methyltransferase reactions involved small molecules, i.e. no macromolecular substrates. This fact may limit the general significance of these results for protein and nucleic acid methylation.

Studies have also been done with analogues of SAH as competitive inhibitors of methyl transfer reactions involving catecholamine-O-methyltransferase, histamine-N-methyltransferase, phenylethanolamine-N-methyltransferase, and hydroxyindole-O-methyltransferase (Coward and Sweet, 1972; Borchardt and Wu, 1974; Borchardt et al., 1974). These studies showed that SAH required the same binding sites to inhibit as proposed for SAM by Zappia et al. (1969; Borchardt and Wu, 1974). In addition, Borchardt and Wu (1974) have shown that the sulfur atom was directly involved in binding to the enzyme since analogues of SAH with the sulfur in a different oxidation state (i.e. sulfoxide, sulfone, or sulfoximide) resulted in decreased binding to the enzymes tested. They postulated that the decreased binding was due to steric effects rather than electronic effects (Borchardt and Wu, 1974). Again note that only small molecular substrates are being considered here.

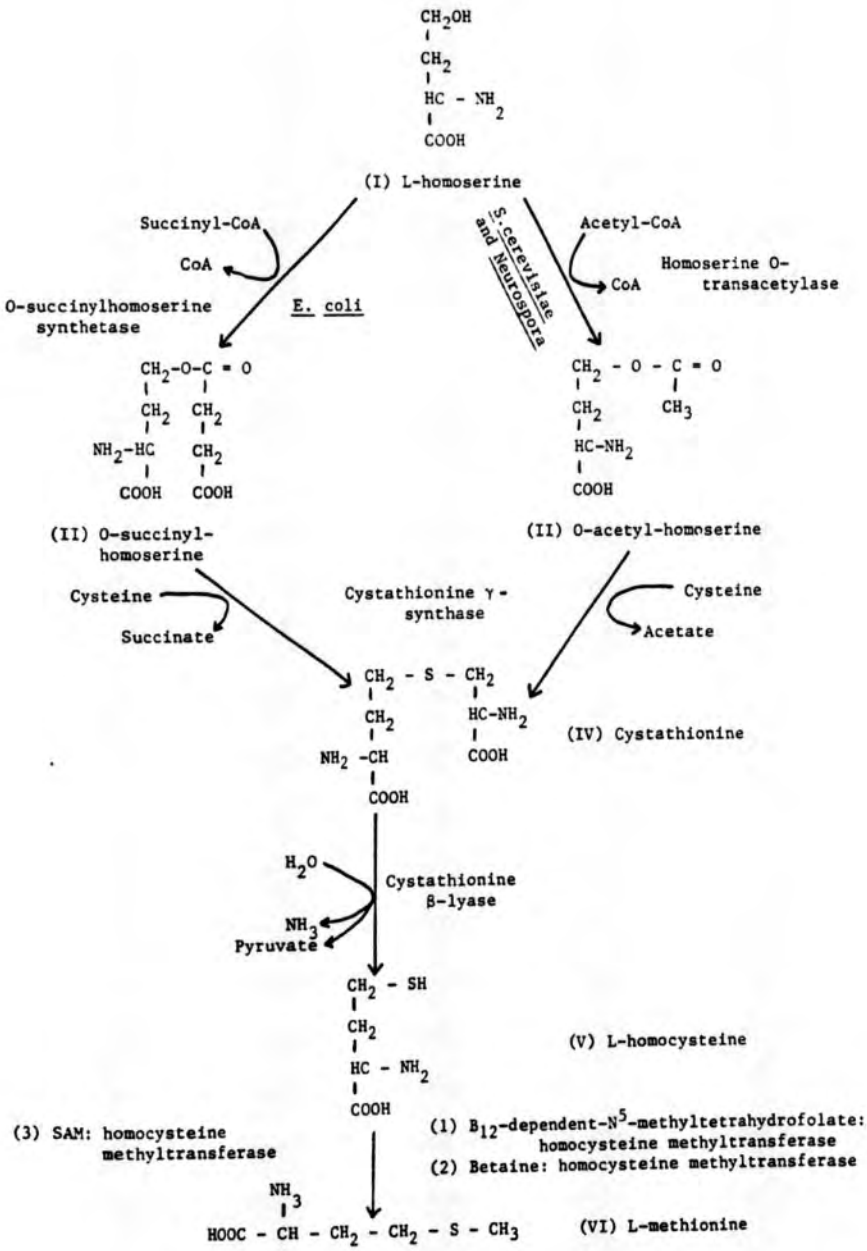
C. SAM as an Effector in Microbial Systems

SAM is involved in a number of regulatory reactions in methionine

biosynthesis. The synthesis of methionine in microorganisms is shown in Figure 2. It has been shown that the formation of O-succinyl-L-homoserine in E.coli is inhibited by high concentrations of SAM (Lee et al., 1966). Lee et al. (1966) have also shown, by using a methionine-requiring auxotroph of E.coli, that the enzyme O-succinylhomoserine synthetase is under dual negative control by low concentrations of both methionine and SAM acting together. It has been observed in Saccharomyces cerevisiae that homoserine-O-transacetylase is also under regulatory control by L-methionine and SAM (De Robichon-Szulmajster and Cherest, 1967). Enzyme activity is strongly inhibited allosterically by SAM but not by L-methionine, whereas methionine represses the synthesis of the enzyme (De Robichon-Szulmajster and Cherest, 1967; Lombardini and Talalay, 1971). Kerr and Flavin (1969) have shown that in Neurospora methionine biosynthesis is allosterically controlled by SAM at the level of cystathionine γ-synthase. They have observed that this enzyme is 50% inhibited at a SAM concentration of 3-10 μm.

The last step in methionine biosynthesis involves the methylation of homocysteine to methionine (Figure 2). In most organisms the methyl group comes from N^5-methyltetrahydrofolate, and the reaction is catalyzed by a vitamin B12-dependent enzyme, N^5-methyltetrahydrofolate: homocysteine methyltransferase (Taylor and Weissbach, 1969a; Lombardini and Talalay, 1971). Taylor and Weissbach (1969a,b) have studied this enzyme in E.coli B and have shown that the reaction requires SAM, but that SAM is not the methyl donor. Instead, they found that SAM activates the enzyme by methylating the B12 prosthetic group. The activated enzyme is then able to transfer the methyl group from N^5-methyltetrahydrofolate to homocysteine

Figure 2: The Biosynthesis of Methionine in Microbial Organisms



(Taylor and Weissbach, 1961a; 1969b). Shapiro et al.(1964) have partially purified and studied SAM: homocysteine methyltransferase from S. cerevisiae. In this case the methyl group donor to homocysteine is SAM itself. Two other compounds are known that can donate a methyl group to homocysteine. They are betaine and dimethylthetin, and their respective methyltransferases have been studied (Schlenk, 1965). SAM also regulates its own synthesis, but this will be discussed in the section on SAM synthesis.

D. Turnover of SAM and SAH in Microbial and Mammalian Systems

The turnover rate of SAM in the cell is controlled by two processes. First, the amount of SAM involved in group transfer reactions regulates its level in the cell. Secondly, SAM is subject to direct enzymatic cleavage to yield 5'-methylthioadenosine and α-aminobutyrolactone (Lombardini and Talalay, 1971). The enzyme that catalyzes this process has been partially purified from baker's yeast (Mudd, 1959). This enzyme has been observed in bacteria and yeast, but not in mammalian systems. The 5'-methylthioadenosine is further cleaved to adenine and 5'-methylthioribose, and the α-amino γ- butyrolactone is spontaneously hydrolyzed to homoserine (Lombardini and Talalay, 1971). The homoserine can then reenter the methionine biosynthetic pathway (microbial systems only).

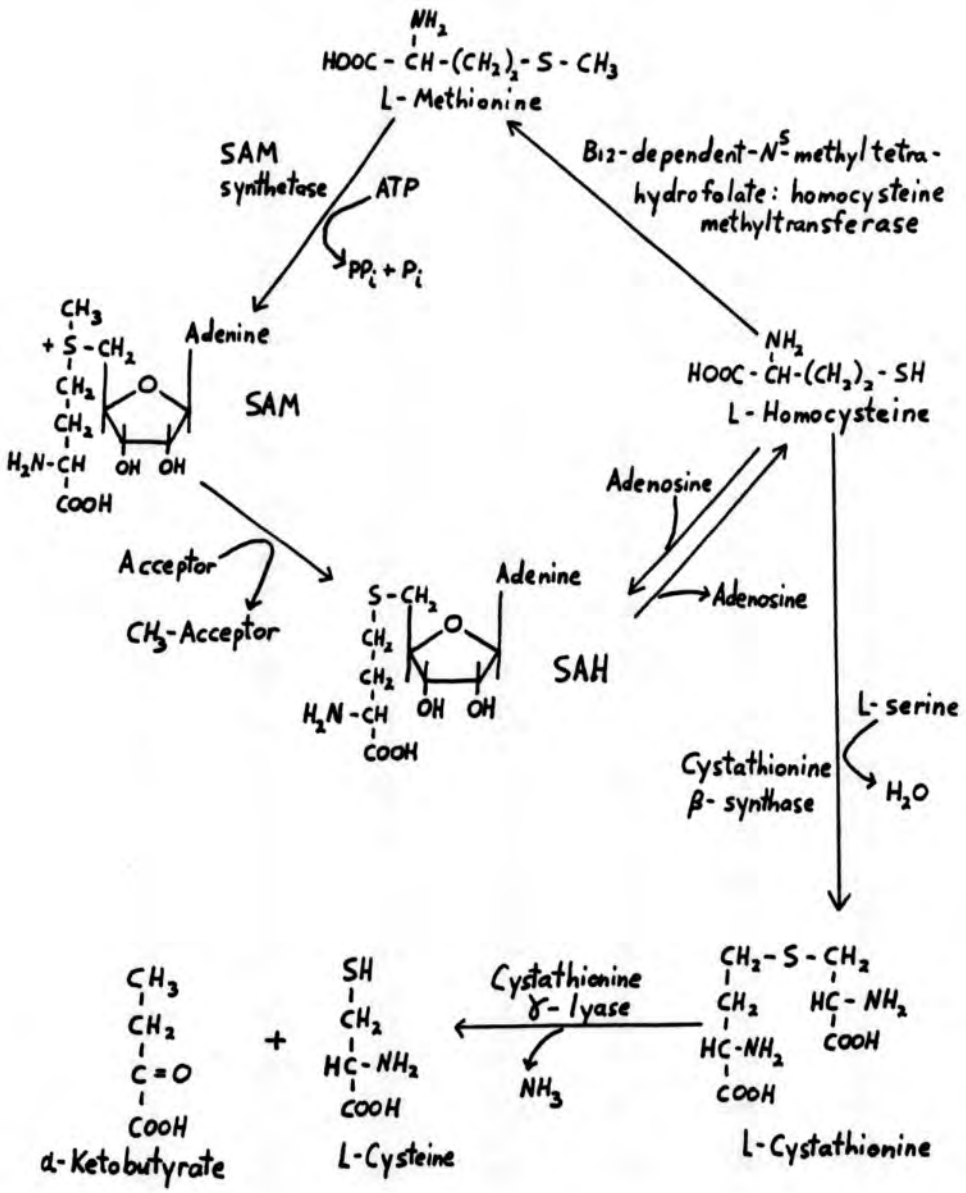
S-adenosylhomocysteine, formed from SAM after demethylation, is usually cleaved enzymatically to adenosine and L-homocysteine (de la Haba and Cantoni, 1959; Lombardini and Talalay, 1971). De la

Haba and Cantoni (1959) purified an enzyme from rat liver that catalyzed the synthesis of SAH from adenosine and L-homocysteine (Figure 3). They also observed that this enzyme had hydrolytic activity if the reaction products were subsequently removed by other enzymatic reactions. The homocysteine which is formed can follow two pathways: (1) it can be remethylated to methionine via N^5-methyltetrahydrofolate, betaine, or SAM thus conserving methionine, or (2) it can combine with L-serine to form cystathionine (via cystathionine β-synthase) which is further cleaved by cystathionine γ-lyase to L-cysteine and α-ketobutyrate (Lombardini and Talalay, 1971). It has been shown that SAH inhibits both N^5-methyltetrahydrofolate: homocysteine methyltransferase and betaine: homocysteine methyltrasnferase, but stimulates cystathionine β-synthase (Finkelstein et al., 1974). Figure 3 shows a composite diagram of the principal pathways of synthesis and metabolism of SAM in mammalian systems.

E. S-Adenosyl-L-Methionine Synthetase: Characteristics and Functions

S-adenosyl-L-methionine is formed enzymatically from ATP and L-methionine. This reaction is catalyzed by the enzyme SAM synthetase (ATP: L-methionine S-adenosyltransferase E.C.2.5.1.6). SAM synthetase activity has been found in many different types of cells, but it has been mainly isolated and characterized from E.coli, baker's yeast, and rat liver (Lombardini and Talalay, 1971). The enzyme from all three sources has been shown to have an absolute requirement for a divalent cation, usually satisfied by Mg^{++} or Mn^{++}, and a monovalent cation, usually satisfied by K^+ or NH_4^+ (Mudd and Cantoni, 1958; Greene, 1969;

Figure 3: The Utilization and Turnover of SAM and SAH in Mammalian Organisms.



Lombardini et al.,1973). SAM synthetase from baker's yeast has been shown to have a pH optimum around pH 7.6 (Mudd and Cantoni, 1958), but Greene (1969) has shown a pH optimum near 9.0 for the enzyme from the same source.

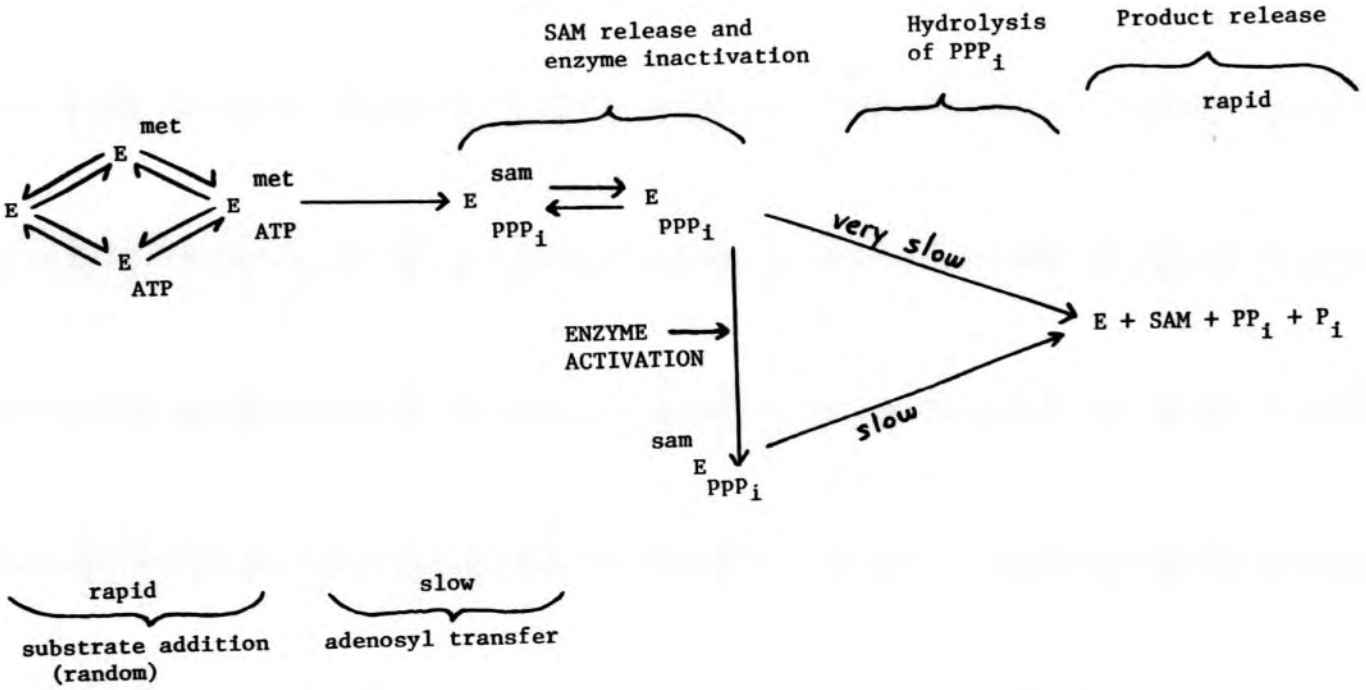
(1) Reaction Mechanism of the Yeast SAM Synthetase

Kinetic studies of the yeast enzyme have elucidated the reaction sequence in the formation of SAM (Figure 4). The reaction begins with the addition of ATP and L-methionine in a random order to form the ATP-L-Met-enzyme ternary complex (E-ATP-L-MET) (Lombardini and Talalay, 1971; Chou and Talalay, 1972). The transfer of the adenosyl group from ATP to L-methionine is considered one of the rate-limiting steps. At this point, release of SAM from the catalytic site results in the formation of a tightly bound enzyme-tripolyphosphate (PPP_i) complex which is hydrolyzed very slowly (Chou and Talalay, 1972). The enzyme has been shown to possess PPP_iase activity which is specifically activated by low levels of SAM (Mudd and Mann, 1963; Greene, 1969; Chou and Talalay, 1972). The activated enzyme then hydrolyzes the PPP_i to $PP_i + P_i$ more quickly. The hydrolytic products are less firmly bound and are released quickly from the catalytic site thus freeing the enzyme. The activation of SAM synthetase by SAM therefore is of importance to regulation (Greene, 1969; Chou and Talalay, 1972).

(2) Substrate Specificity Studies

Analogues of L-methionine and ATP have been used to study the substrate specificity and important binding sites of SAM synthetase. Analogues of L-methionine have shown that few changes of the carboxyl,

Figure 4: The Reaction Mechanism for SAM Synthetase from Baker's Yeast



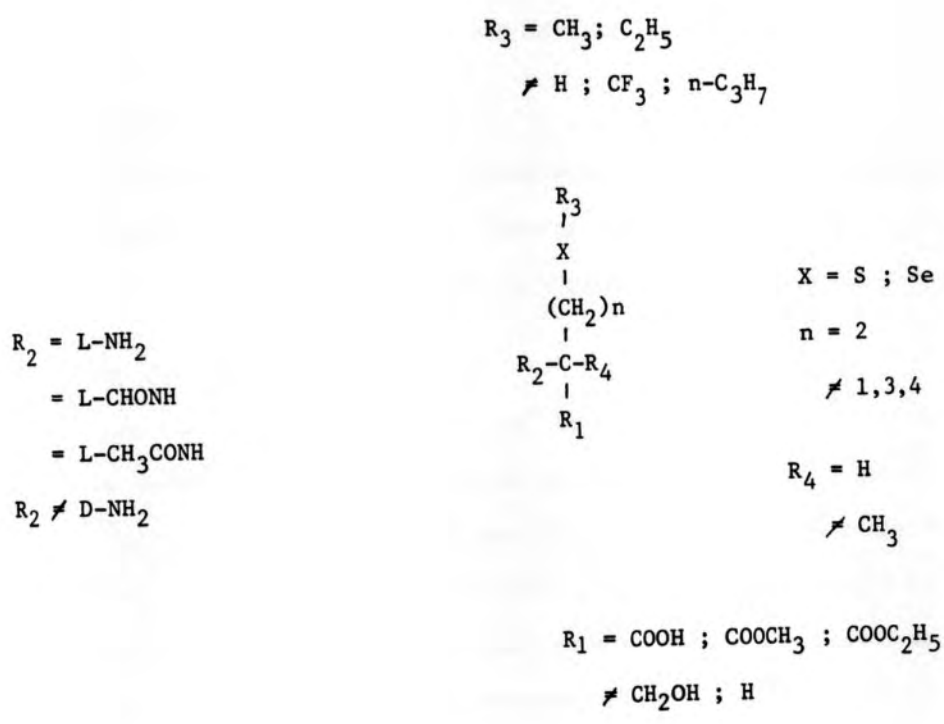
α-hydrogen, and α-amino groups are tolerated by the enzyme (Figure 5) (Lombardini et al., 1970). It should be noted that the sulfur atom must be in the correct oxidation state, the amino acid must be in the L-configuration, and the hydrocarbon bridge joining the α-carbon to the sulfur must be two carbons in length (Lombardini et al., 1970; Lombardini and Talalay, 1971).

SAM synthetase shows a very high degree of specificity for ATP (Chou and Talalay, 1973). Through the use of ATP analogues, Chou and Talalay (1973) have proposed that the triphosphate group of ATP is of primary importance and is necessary for binding to the enzyme, while the adenosyl moiety is necessary for substrate recognition but has lower affinity for the enzyme than the triphosphate group.

F. Purpose of Study

The purpose of this study was to develop an affinity chromatography system with SAM as the ligand. SAM is to be attached to agarose by a solid-phase reaction between agarose-ATP and L-methionine catalyzed by SAM synthetase. The SAM synthetase to be used was prepared from E.coli by R. K. Morse. It is also part of this study to isolate SAM synthetase from baker's yeast and use this preparation in the above solid-phase reaction. If developed, an agarose-SAM affinity column could be used to isolate methyltransferases specific for the contractile proteins, actin and myosin, as well as for other proteins, catecholamines, RNA, and DNA.

Figure 5: Structural Requirements of Methionine in the SAM Synthetase Reaction



= : active substrates

≠ : inactive substrates

Methionine : R_1 = COOH , R_2 = L-NH_2, R_3 = CH_3

R_4 = H , n = 2, X = S

CHAPTER II

MATERIALS AND METHODS

A. Chemicals

The chemicals used in this study were all reagent grade and were purchased principally from Sigma Chemical Company or Fisher Scientific Company. All solutions were made with glass-distilled water.

B. Ion Exchange Materials

The Bio-Rex 70 (50-100 mesh, Na^+ form), a weakly acidic carboxylic cation exchanger, was purchased from Bio-Rad Laboratories. Before being used, it was first converted to the H^+ form by washing the resin in bulk on a Buchner funnel first with 2N NaOH, then H_2O, and then with 6N H_2SO_4 until the OD_{256} of the effluent was less than 0.05. The resin was allowed to stand in the 6N H_2SO_4 for 30 minutes to ensure complete conversion to the H^+ form. The resin was then washed thoroughly with H_2O until the pH of the effluent was 6.5-7.0 and stored in H_2O. The columns were prepared from disposable Pasteur pipets with a small amount of sulfuric acid-washed Pyrex glass wool at the bottom to serve as a support bed. An aqueous slurry of the converted resin was poured into the Pasteur pipets to product a 0.5 X 2.5 cm column. Several volumes of H_2O were run through each column to ensure proper packing. The columns were then sealed with Parafilm on the top and a piece of rubber hose clamped with wire was placed on the bottom. They were then stored at 4^o C.

The DEAE-cellulose used in this study was purchased first from Sigma (medium mesh) and then, later, from Whatman Biochemicals (fibrous

form-fines reduced). The DEAE-cellulose from Sigma was precycled according to the method of Peterson and Sober (1962). The dry cellulose was allowed to sink into 1N NaOH and then was immediately filtered on a coarse Buchner funnel. The cellulose was washed with additional 1N NaOH until all of the greenish color was removed. Enough 1N HCl was added to make a strongly acid suspension as determined by pH paper. The acid suspension was filtered immediately and washed with H_2O. It was then suspended in 1N NaOH again, filtered, washed with H_2O and then suspended in 2-3 volumes of equilibrating buffer, filtered, and washed again with 2-3 volumes of buffer. The cellulose was then suspended in 60 ml of equilibrating buffer per gram of cellulose. The fines were removed twice by allowing the cellulose to settle for one hour.

The DEAE-cellulose purchased from Whatman Biochemicals was precycled according to their suggested procedures. The cellulose was stirred into 15 volumes of 0.5N HCl and left for 30 minutes. It was filtered and washed with H_2O until the effluent was about pH 4. The cellulose was resuspended in 15 volumes of 0.5N NaOH, left for 30 minutes, and then filtered. The NaOH treatment was repeated and then the cellulose was washed with H_2O until the effluent was near pH 7.0 and then suspended in 10 mM KPB (pH 7.0)-5 ETSH. The cellulose was degassed until no more bubbles were noticed but before vigorous boiling occurred.

C. Radioisotopes

The two radioactive compounds used in this study were [$^{14}CH_3$] - methionine and [C^3H_3]-SAM. Both compounds were purchased from Research Products International and were made by the Centre D'Etudes Nucleaires

de Saclay, France. The [$^{14}CH_3$]-methione as purchased had a specific activity of 53 mCi/mmole, and the [C^3H_3]-SAM as purchased had a specific activity of 15 Ci/mmole. The [$^{14}CH_3$]-methionine was stored in the freezer at -20°C. The [C^3H_3]-SAM, being more subject to radiolysis, was stored at -196°C in liquid nitrogen.

D. Counting Methods

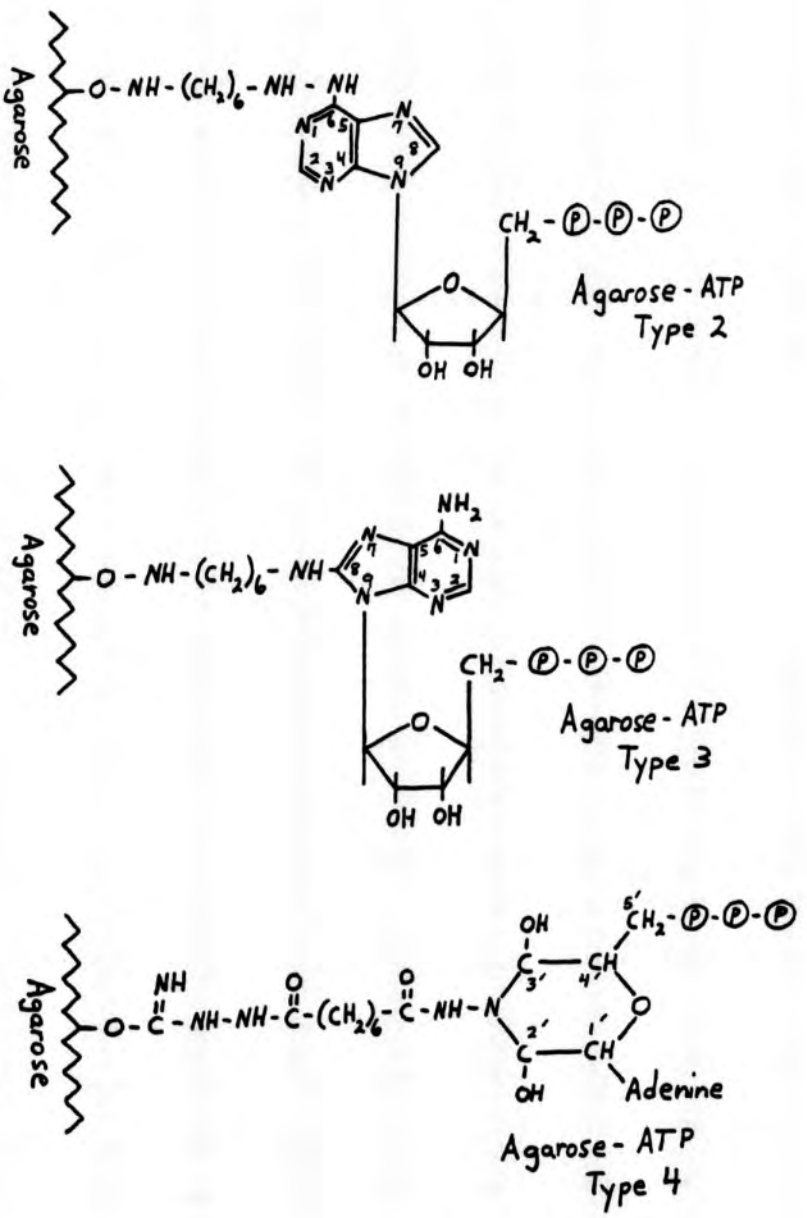
The scintillation cocktail used in this study was prepared from Triton X100-toluene (1:3 v/v) with 5.5 g PPO and 0.1 g POPOP per liter. The Triton X100 was purchased from Sigma Chemical Company or Research Products International and was reagent grade. The PPO and POPOP was obtained from Fisher Scientific Company and was their scintanalyzed purity grade. The socktail as prepared was capable of holding up to 21% aqueous solution. All samples were counted in a Beckman LS-100 liquid scintillation counter. An efficiency value for ^{14}C was calculated to be 41.1% for a 3 ml aqueous (17%) sample in 15 ml cocktail. An efficiency value for ^3H was calculated to be 49.0%.

E. Affinity Chromatography Materials

The agarose, agarose-hexane, and agarose-ATP used for the solid-phase biosynthesis experiments were purchased from P-L Biochemicals and were stored either at 4°C (agarose and agarose-hexane) or at -20°C in 50% glycerol (agarose-ATP). Two types of agarose-ATP were used in this study. They are agarose-ATP Type 2 and agarose-ATP Type 4.

The type 2 gel has the ATP attached to the agarose via a hexane spacer to the N^6-amino group of the adenyl moiety (Guilford et al., 1972). The type 4 gel has the ATP attached to the agarose via a hexane spacer to the 2', 3'-carbons of the ribosyl moiety (Lamed et al., 1973). Refer to Figure 6.

Figure 6: Attachment Structures of Agarose-ATP Types 2, 3, and 4



F. The SAM Synthetase Assay Method

The assay method used to measure the activity of SAM synthetase was developed by Holcomb and Shapiro (1975). Basically, the method measures the activity of the enzyme by determining the amount of [$^{14}CH_3$]-SAM formed from [$^{14}CH_3$] -methionine. The substrate, [$^{14}CH_3$] -methionine, was then separated from the product, [$^{14}CH_3$] -SAM, on small, disposable Bio-Rex 70 columns.

The activity of SAM synthetase was assayed in a reaction mixture containing 20 mM [$^{14}CH_3$] -methionine (1 μCi), 20 mM ATP, 25 mM $MgCl_2$, 100 mM KCL, 8 mM glutathione, and 200 mM Tris buffer (pH 8.0) in a volume of 0.17 ml. The test sample containing enzyme was added in 0.18 ml giving a final volume for the reaction mixture of 0.25 ml. For a negative control, glass-distilled water was added instead of the enzyme. Both reaction mixture and enzyme were pre-warmed separately for 5 minutes, then combined and incubated for 30 minutes at 37°C. The reaction was stopped by cooling to 0°C in an ice-water bath. The reaction mixture was applied to a Bio-Rex 70 column and allowed to settle into the bed. The unreacted methionine was removed by passing 10 ml of glass-distilled water through the column. The labelled SAM was then eluted by addition of 3 ml of 0.1 N H_2SO_4. The eluted SAM was collected directly into a scintillation vial to which 15 ml of scintillation cocktail was added. Before addition of the cocktail, the H_2SO_4 was neutralized by the addition of about 50 mg of dry $BaCO_3$. After a period of at least one hour dark adaptation, the samples were counted. The number of micromoles of SAM formed was calculated, and activity was expressed

as units per mg of protein where a unit is defined as the number of micromoles of SAM formed per 30 minutes at 37°C.

G. Extraction and Purification of SAM Synthetase from Baker's Yeast

The procedure followed for the extraction and purification of SAM synthetase from baker's yeast was a combination of the methods of Mudd and Cantoni (1958), Lombardini et al., (1970), and Chou and Talalay (1972) (Table 2). Unless otherwise stated, all procedures were carried out at 0-4°C. Estimation of protein concentration was performed by OD_{280}/OD_{260} ratio (Warburg and Christian, 1941) or by OD_{280} alone (near end of purification).

Step 1: The enzyme was obtained from baker's yeast (gift of Anhauser-Busch, Inc., Greensboro, N.C.) by autolysis. Four to five one pound cakes were used for each extraction. The fresh yeast was grated onto a large drying tray between several sheets of Whatman #1 chromatography paper and dried in this fashion for six days at room temperature. After six days the yeast was usually stored for a day at -20° C. Drying time could be reduced to three days by blowing air over the drying tray with a 8" fan. The dried yeast (600 g) was suspended in 1800 ml of 67 mM K_2HPO_4 containing 1.8 g L-methionine. It usually took about 30 minutes to get all of the yeast suspended and any lumps dispersed. The yeast suspension was then incubated for 4 h at 32°C with frequent stirring. After incubation the suspension was cooled to 0°C in an ice-water bath and centrifuged at 4900 X g for 20 minutes (7500 rpm, Sorvall GSA rotor). The supernatant fluid, which usually varied between 825-905 ml, was collected and a sample removed for assaying and protein determination.

TABLE 2: Flow Chart for the Extraction and Purification of SAM Synthetase from Baker's Yeast

Step 1 - 4-5 Yeast Cakes

Grated and dried for 6 days

Store at -20°C

Autolyze at 32°C, 4 h. in 67 mM K_2HPO_4 with 1.8 g L-methionine

Centrifuge at 4900 x g, 20 min

Prec. discarded

Step 2 - Supernatant

Add acetone to 24%, -8°C

Centrifuge at 2500 x g, 20 min. -8°C

Prec. discarded

Supernatant

Add acetone to 45%, -8°C

Centrifuge at 800 x g, 10 min. -8°C

Supernatant discarded

Precipitate suspended in 20 mM KPB (pH 6.6)

Dialyze overnight against 1 mM KPB (pH 7.0) +5 mM ETSH (one change) 25 volumes

Centrifuge at 18,000 x g, 5 min.

Prec. discarded

Step 3 - Supernatant chromatographed on DEAE-cellulose using constant volume mixing chamber and gradient from 1 mM KPB (pH 7.0) +5 mM ETSH-0.4 M KPB (pH 7.0) +5 mM ETSH

Collect 8-10 ml fractions

Step 4 - Combine fractions with high activity

Add $(NH_4)_2SO_4$ to 50%

Centirfuge at 13,200 x g, 20 min.

Prec. discarded

Supernatant

Add $(NH_4)_2SO_4$ to 75%

Centirfuge at 13,200 x g, 20 min.

Supernatant discarded

Precipitate suspended in 1 mM KPB (pH 7.0) +5 mM ETSH

Dialyze overnight against 1 mM KPB (pH 7.0) +5 mM ETSH (one change) 25 volumes

Step 5 - Adjust protein concentration to 10 mg/ml

Add bentonite 30 mg/ml

TABLE 2: Flow Chart for the Extraction and Purification of SAM Synthetase from Baker's Yeast (Cont.)

Stir for 20 min; centrifuge at 12,000 g, 20 min.

├── Prec. discarded

Supernatant

├── Add bentonite 15 mg/ml

Stir 20 min; centrifuge at 12,000 g, 20 min.

├── Prec. discarded

Step 6 - Supernatant-repeat twice

├── Add $(NH_4)_2SO_4$ to 53%

Centrifuge at 17,300 g, 20 min.

├── Prec. discarded

Supernatant

├── Add $(NH_4)_2SO_4$ to 84%

Centrifuge at 17,300 g, 20 min.

├── Supernatant discarded

Prec. suspended in 1 mM KPB (pH 7.0)+5 mM ETSH

|

Step 7 - Dialyze overnight against 1 mM KPB (pH 7.0) +5 mM ETSH (no changes) 25 volumes

├── Add ETOH to 52%

Centrifuge at 17,300 g, 20 min.

├── Prec. discarded

Supernatant

├── Add ETOH to 64%

Centrifuge at 17,300 g, 20 min.

├── Supernatant discarded

Prec. suspended in 2 mM KPB (pH 7.0) +5 mM ETSH

|

Dialyze overnight against 2 mM KPB (pH 7.0)+5 mM ETSH (no changes) 25 volumes

|

Step 8 - Adjust protein concentration to 2 mg/ml

├── Add $(NH_4)_2SO_4$ to 42%

Centrifuge at 17,300 g, 20 min.

├── Prec. discarded

Supernatant

├── Add $(NH_4)_2SO_4$ to 52%

Centrifuge at 17,300 g, 20 min.

├── Supernatant discarded

Prec. suspended in 2 mM KPB (pH 7.0)+5 mM ETSH

|

Dialyze overnight against 2 mM KPB (pH 7.0)+5 mM ETSH (no changes) 25 volumes

|

Store at $-20^{o}C$.

Step 2: Acetone was added dropwise to the crude autolysate to 24% while the solution was quickly lowered to -8°C in an alcohol-ice-water bath. The addition took about 45 minutes and after addition the solution was allowed to stand for 15 minutes. The solution was then centrifuged at 2500 X g for 20 minutes at -8°C (4000 rpm, Sorvall GSA rotor). The precipitated protein was discarded, and acetone was added dropwise to the supernatant to 45% at -8°C. The addition again took about 45 minutes. The solution was allowed to stand for 15 minutes and was centrifuged at 800 x g for 15 minutes at -8°C (2500 rpm, Sorvall GSA rotor; 2000 rpm, International rotor 284). The precipitate was collected and resuspended in 20 mM KPB (pH 6.6). The resulting enzyme solution was dialyzed overnight against 25 volumes of 1 mM KPB (pH 7.0) +5 mM ETSH (changed once). The dialysate was clarified by centrifugation at 18,000 x g for 5 minutes (12,500 rpm, Sorvall SS-34 rotor). At this stage the enzyme solution was stable for several months at -20°C and was used as a positive control in the SAM synthetase assay for the other steps.

Step 3: The clarified enzyme solution was chromatographed on a column of DEAE-cellulose with bed dimensions of 2.6 x 47 cm. The cellulose was packed in a 2.6 X 70 cm Pharmacia column. A gradient was constructed by use of a constant volume mixing chamber containing 800 ml 1 mM KPB (pH 7.0) +5 mM ETSH connected to a reservoir containing 0.4 M KPB (pH 7.0) +5 mM ETSH. The buffer reservoirs and the column were jacketed and were kept at 4°C. The column effluent went via teflon tubing into a refrigerator containing the fraction collector. Usually 135 eight to ten ml fractions were collected. A preliminary check for enzyme activity was done by removing 50 µl samples from each

of ten tubes and pooling them for a single assay. This could be performed while the column run was in progress. After narrowing the peak of enzyme activity to sets of ten fractions, the individual fractions in the sets of interest were assayed to determine the fractions with the highest acitvity in terms of units/ml. The fractions with significant activity (i.e. greater than 0.10 units/ml) were then combined for the next step.

Step 4: The enzyme in the combined fractions was further purified by fractionation between 50-75% with a saturated, neutralized $(NH_4)_2SO_4$ solution. The $(NH_4)_2SO_4$ was saturated at 0^oC and required 707 g of salt added to 1000 ml H_20. It was neutralized to pH 7.0 with 1 M KOH. $(NH_4)_2SO_4$ was added dropwise to the combined fractions to 50% saturation. The solution was allowed to stand for 15 minutes and was centrifuged at 13,200 x g for 20 minutes (10,000 rpm, Sorvall GSA rotor). The precipitate was discarded and more $(NH_4)_2SO_4$ was added to the supernatant to 75% saturation. The solution was allowed to stand for 20 minutes and was centrifuged again at 13,200 x g for 20 minutes (10,000 rpm, Sorvall GSA rotor). The precipitate was collected, resuspended in 1 mM KPB (pH 7.0) +5 mM ETSH, and dialyzed overnight against 25 volumes of the buffer (changed once). A sample was removed for assay and protein determination.

Step 5: The protein concentration was adjusted to 10 mg/ml by dilution with 1 mM KPB (pH 7.0) +5 mM ETSH before a series of negative bentonite adsorptions. The adsorptions were performed in four stages. Stage one involved adding 30 mg bentonite per ml of enzyme solution.

The bentonite was suspended as evenly as possible and then the solution was stirred gently for 20 minutes. The suspension was centrifuged at 12,000 x g for 20 minutes (10,000 rpm, Sorvall SS-34 rotor), and the precipitate discarded. This bentonite adsorption step was repeated three more times except 15 mg of bentonite was added per ml. At each stage enzyme activity was monitored and protein concentration determined.

Step 6: The supernatant from the last bentonite adsorption was further purified by fractionation between 53-84% with saturated, neutralized $(NH_4)_2SO_4$. The $(NH_4)_2SO_4$ was added dropwise to 53%, allowed to stand for 20 minutes and centrifuged at 17,300 x g for 20 minutes (12,000 rpm, Sorvall SS-34 rotor). The precipitate was discarded and more $(NH_4)_2SO_4$ to 84% saturation was added to the supernatant. It was allowed to stand for 20 minutes and was centrifuged at 17,300 x g for 20 minutes (12,000 rpm, Sorvall SS-34 rotor). The precipitate was collected and resuspended in 1 mM KPB (pH 7.0) +5 mM ETSH and dialyzed overnight against 25 volumes of the buffer (no changes).

Step 7: The dialysate was further purified by fractionation with ethanol between 52-64% (v/v). Ethanol (at 25°C) was added dropwise to 52%, the solution was allowed to stand for 15 minutes,and was centrifuged at 17,300 x g for 20 minutes (12,000 rpm, Sorvall SS-34 rotor). The precipitate was discarded, and ethanol was added dropwise to the supernatant to 64%. It was allowed to stand for 20 minutes and was then centrifuged at 17,300 x g for 20 minutes (12,000 rpm, Sorvall SS-34 rotor). The precipitate was collected, resuspended in 2 mM KPB (pH 7.0) +5 mM ETSH, and dialyzed overnight against 25 volumes of the same buffer (no changes).

Step 8: For the last step, the protein concentration was adjusted to 2 mg/ml. Saturated, neutralized $(NH_4)_2SO_4$ was added dropwise to the dialysate to 42% saturation. It was allowed to stand for 20 minutes and was centrifuged at 17,300 x g for 20 minutes (12,000 rpm, Sorvall SS-34 rotor). The precipitate was discarded, and $(NH_4)_2SO_4$ was added to the supernatant to 52% saturation. Again it was allowed to stand for 20 minutes and was then centrifuged at 17,300 x g for 20 minutes (12,000 rpm, Sorvall SS-34 rotor). The precipitate was collected, resuspended in 2 mM KPB (pH 7.0) +5 mM ETSH, and dialyzed overnight against 25 volumes of the same buffer (no changes). At this point, the enzyme preparation could be stored at -20°C until ready for use.

H. Solid-Phase Biosynthesis of SAM on Agarose-ATP

The procedure for the solid-phase biosynthesis of SAM on agarose-ATP comes from unpublished work of my research advisor, Dr. Richard Morse. Suggestions to modify the procedure will be made as a result of this study. The types of agarose-ATP used in these experiments are agarose-ATP Type 2 and agarose-ATP Type 4 (see Materials and Methods). Agarose and agarose-hexane were used as control gels for these experiments.

Two 0.1 ml samples of each agarose type were taken and washed twice with 5 ml of cold, distilled water (Table 3). The washing was done by placing the sample into a conical centrifuge tube, adding the 5 ml of water, and centrifuging at 319 x g (1200 rpm, International rotor #269) for 5 minutes and subsequently discarding the supernatant. The following solutions were added to the twice-washed agarose samples: 20mM $[^{14}CH_3]$ - methionine (2 µCi) and unlabelled methionine, 25 mM $MgCl_2$, 100 mM KCL, 200 mM Tris buffer (pH 8.0) (100µl); 20 mM ATP (20 µl); and 8 mM

TABLE 3: Flow Chart for the Solid-Phase Biosynthesis of SAM on Agarose-ATP

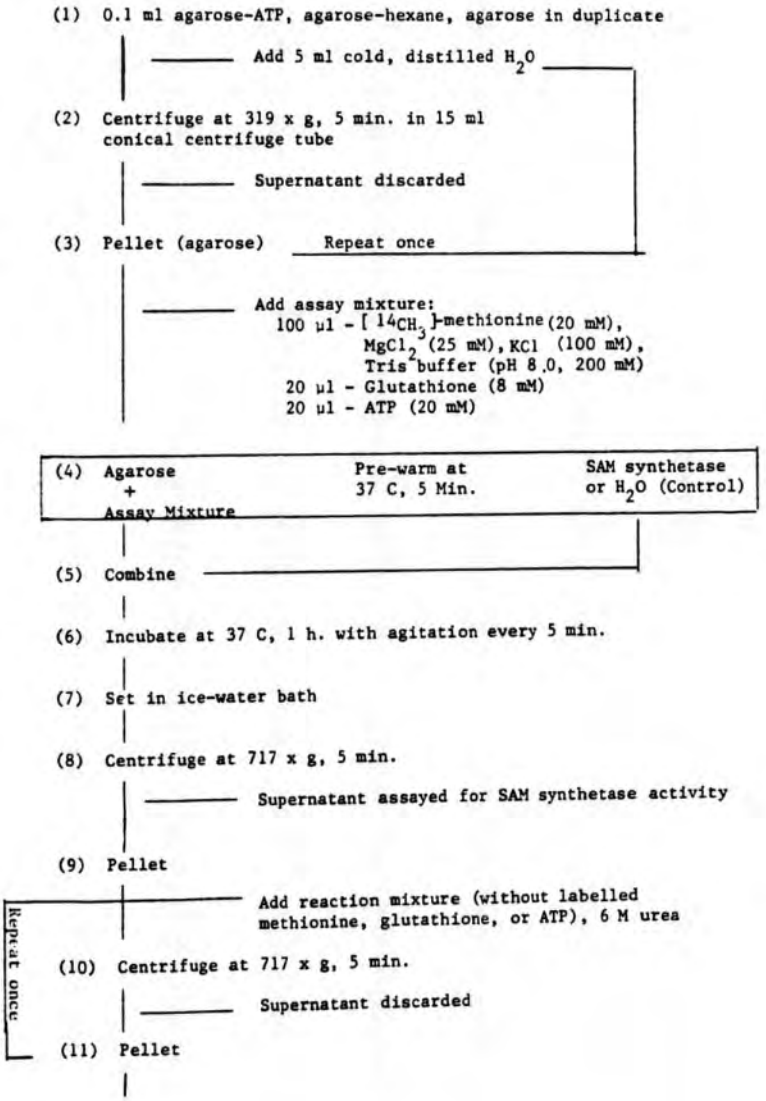
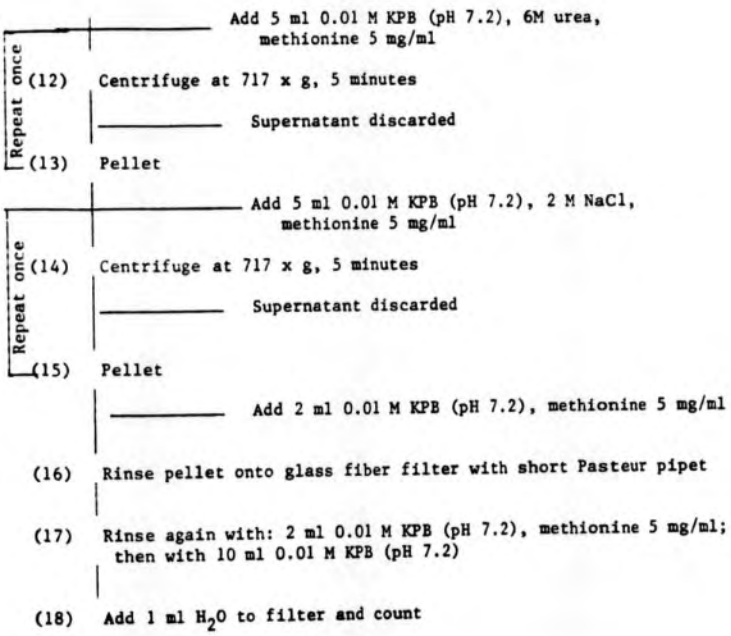


TABLE 3: Flow Chart for the Solid-Phase Biosynthesis of SAM on Agarose-ATP (Cont.)



glutathione (20 µl). The samples were then pre-warmed at 37°C for 5 minutes. To one sample of each agarose type, 0.36 ml SAM synthetase preparation was added (360 ug; 4.74 units/mg). To the other sample was added 0.36 ml distilled water as a control. Both enzyme and water had been pre-warmed for 5 minutes. After mixing the preparations were incubated for one hour at 37°C with agitation every 5 minutes to keep the agarose beads suspended. The preparations were cooled to 0°C and centrifuged at 717 x g (1800 rpm, International rotor #269) for 5 minutes. The supernatant was removed and assayed for methionine and SAM as described above.

The agarose samples were then washed twice with each of the following solutions in the order listed: 5 ml reaction mixture (without labelled methionine, glutathione, or ATP); 5 ml 0.01 M KPB (pH 7.2), 6 M urea, methionine 5 mg/ml; 5 ml 0.01 M KPB (pH 7.2), 2M NaCl, methionine 5 mg/ml. The agarose was centrifuged at 717 x g (1800 rpm, International rotor #269) for 5 minutes between each wash and the supernatant discarded. After the last wash, the agarose was transferred to a glass fiber filter by adding 2 ml 0.01 KPB (pH 7.2) + methionine 5 mg/ml and then transferring the agarose with a short Pastuer pipet. On the filter the agarose was washed once with 2 ml 0.01 KPB (pH 7.2) + methionine 5 mg/ml and twice with 5 ml KPB (pH 7.2). The filter and agarose were moistened with one ml of water and counted as described above.

CHAPTER III

RESULTS

A. The Sam Synthetase Assay Method

Two control experiments were performed in order to check reproducibility of the separation of methionine from SAM on Bio-Rex 70 (H^+ form). In the first experiment, [$^{14}CH_3$]-methionine in a total volume of 0.25 ml was added to a column of Bio-Rex 70 and was eluted as shown in Table 4. Results show that >99% of the total radioactivity was removed in the first 10 ml of H_2O, whereas less than 0.1% came off in the first 3 ml of H_2SO_4.

In the second experiment involving [C^3H_3]-SAM, problems were encountered with impurities in the SAM sample, probably caused by radiolytic breakdown products. Research Products International reported a 10-15%/year decomposition rate for their [C^3H_3]-SAM when stored in 0.1 N H_2SO_4 and at -196°C. (Manufacturer's information sheet). At room temperature the decomposition rate is 10-15%/week. No attempt was made to identify the decomposition products. The presence of impurities in the H_2O fraction produced results inconsistent with Holcomb and Shapiro (1975). At least 98% of the [C^3H_3]-SAM should be in the first 3 ml of the H_2SO_4 fraction. (Table 5)

Neutralization of the 0.1 N H_2SO_4, in which the [C^3H_3]-SAM was stored, also posed problems. I first tried neutralization with an equal volume of 0.1 N NaOH, but the NaOH apparently created localized aklaline conditions long enough to break down some of the SAM (cf. Schlenk, 1965). No attempt was made to analyze the decomposition products, but again

TABLE 4: Elution Profile of $[^{14}CH_3]$ -Methionine on Bio-Rex 70 (H^+ Form)

Elution Fraction	Methionine Eluted	
	CPM	Percent
H_2O, 1st ml	724583	82.8
" , 2nd ml	147950	16.9
" , 3rd ml	6081	.7
" , 5th ml	183	<.1
" , 10th ml	61	<.1
" , 15th ml	47	<.1
" , 20th ml	42	<.1
0.1N H_2SO_4, 1st ml	441	<.1
" , 2nd ml	672	<.1
" , 3rd ml	299	<.1
" , 4th ml	87	<.1
" , 6th ml	53	<.1

The $[^{14}CH_3]$- methionine (875,583 CPM) was added in a total volume of 0.25 ml. All CPM expressed are minus background. The elution percent was calculated by comparing the counts obtained from a 0.25 ml sample of labelled methionine placed directly in a scintillation vial.

TABLE 5: Elution Profile of [C^3H_3]-SAM on Bio-Rex 70 (H^+ form)

Elution Fraction	SAM Eluted	
	CPM	Percent
H_2O, 1st ml	167738	23.1
" , 2nd ml	3389	.5
" , 3rd ml	759	.1
" , 5th ml	472	< .1
" , 10th ml	320	< .1
" , 15th ml	264	< .1
" , 20th ml	218	< .1
0.1N H_2SO_4, 1st ml	22687	3.1
" , 2nd ml	471589	64.8
" , 3rd ml	33035	4.5
" , 4th ml	5472	.8
" , 6th ml	946	.1

The [C^3H_3]-SAM (727,382 CPM) was added in a total volume of 0.1 ml. Each fraction was added to 10 ml of scintillation cocktail and counted. All CPM expressed are minus background. The elution percent was calculated by comparing the counts obtained from a 0.1 ml sample of labelled SAM placed directly in a scintillation vial.

their presence produced results inconsistent with Holcomb and Shapiro (1975) as shown in Table 6. The problem of SAM sensitivity to high pH was solved by (1) diluting the [C^3H_3] SAM with 0.1 M KPB (pH 6.9) before applying it to the column, and (2) taking a sample from the first ml H_2SO_4 fraction, diluting it with 1 M KPB (pH 7.2) and rechromatographing this sample immediately. [C^3H_3]-SAM in a total volume of 0.15 ml was applied to a Bio-Rex 70 column and eluted as shown in Table 7. A 50 µl sample was taken from the first ml H_2SO_4 fraction, diluted 1:5 with 1 M KPB (pH 7.2), and eluted as before (Table 8). Results show that 98.5% of the total radioactivity was eluted in the first 3 ml of 0.1 N H_2SO_4 and that only 0.9% was eluted in the first 10 ml of H_2O.

The SAM synthetase assay was first tested using the enzyme preparation from E. coli provided by R. Morse (Tabor and Tabor, 1971). The only change from the method of Holcomb and Shapiro (1975) was that 10-180 µg of protein was added instead of 1-3 mg as they had specified. The difference is due to the fact that Holcomb and Shapiro used a crude enzyme extract, whereas the enzyme from E. coli had been purified about 60-fold. Refer to Table 9 for the results of the assay and the calculation of activity units.

B. Solid-phase Biosynthesis of SAM on Agarose-ATP

All experiments connected with the solid-phase biosynthesis of SAM on agarose were performed with the E. coli SAM synthetase preparation. In the first experiment, all procedures as outlined in Materials and Methods were followed exactly (Table 10). Results show no difference in the CPM between the experiments with enzyme and without enzyme for the two agarose-ATP types, even though the CPM in the supernatant (i.e. re-

TABLE 6: Elution Profile of [C^3H_3]-SAM on Bio-Rex 70 (H^+ form) after Neutralization with 0.1 N NaOH

Elution Fraction	Sam Eluted	
	CPM	Percent
H_2O, 1st 10 ml	7620	52.6
" , 2nd 10 ml	40	.3
0.1N H_2SO_4, 1st ml	3605	24.9
" , 2nd ml	946	6.5
" , 3rd ml	277	1.9
" , 10th ml	9	<.1

A 0.1 ml sample of [C^3H_3]-SAM (14,492 CPM) was neutralized with an equal volume of 0.1 N NaOH and applied to the column. Each fraction was counted in 10 ml of scintillation cocktail. The elution percent was calculated by comparing the counts obtained from a 0.1 ml sample of labelled SAM placed directly in a scintillation vial. All CPM expressed are minus background.

TABLE 7: Elution Profile of [C^3H_3]-SAM on Bio-Rex 70 (H^+ form) after Neutralization with 0.1 M KPB (pH 6.9)

Elution Fraction	SAM Eluted	
	CPM	Percent
H_2O, 1st 10 ml	15660	4.4
" , 2nd 10 ml	1720	.5
0.1N H_2SO_4, 1st ml	250640	71.0
" , 2nd ml	48220	13.7
" , 3rd ml	13623	3.9
" , 10th ml	1719	.5

A 0.1 ml sample of [C^3H_3]-SAM (353,085 CPM) was neutralized with an equal volume of 0.1 M KPB (pH 6.9) and applied to the column. Each fraction was counted in 10 ml of scintillation cocktail. The elution percent was calculated in the same manner as Table 6. All CPM are minus background.

TABLE 8: Elution Profile of [C^3H_3]-SAM on Bio-Rex 70 (H^+ form) after Rechromatography

Elution Fraction	Sam Eluted	
	CPM	Percent
H_2O, 1st 10 ml	108	.9
" , 2nd 10 ml	9	.1
0.1N H_2SO_4 , 1st ml	11028	89.1
" , 2nd ml	943	7.6
" , 3rd ml	225	1.8
" , 10th ml	31	.3

A 50 µl sample of [C^3H_3]-SAM (12,376 CPM) was taken from the 1st ml H_2SO_4 fraction from Table 7 and diluted with 0.20 ml of 1 M KPB (pH 7.2) and rechromatographed immediately. Each fraction was counted in 10 ml of scintillation cocktail. The elution percent was calculated in the same manner as in Table 6. All CPM are minus background.

TABLE 9: Results from SAM Synthetase Assay Using E. coli SAM Synthetase and Calculation of Activity Units

CPM in SAM Fraction = 168,903

Calculation of Activity Units:

Efficiency value for $[^{14}CH_3]$-methionine = 44.1%

Specific activity of $[^{14}CH_3]$-methionine in assay = $\frac{200\ \mu Ci}{mmole}$

$$\frac{200\ \mu Ci}{mmole} \times 2.220 \times 10^6 \frac{DPM}{\mu Ci} = 4.44 \times 10^8 \frac{DPM}{mmole}$$

$$\frac{CPM}{mmole} = 0.441 \quad 4.44 \times 10^6 \frac{DPM}{mmole} = 1.96 \times 10^8 \frac{CPM}{mmole}$$

$$\frac{168{,}903\ CPM}{1.96 \times 10^6 \frac{CPM}{mmole}} \times \frac{1000\ \mu mole}{mmole} = \frac{0.862\ \mu mole}{30\ min.} = \frac{0.862\ units}{0.180\ ml}$$

$$= 4.79 \frac{units}{ml}$$

$$\text{Specific activity of enzyme} = \frac{4.79 \frac{units}{ml}}{1.01 \frac{mg}{ml}} = 4.74 \frac{units}{mg\ protein}$$

TABLE 10: Results from Solid-Phase Biosynthesis Experiment #1

Agarose Type	Conditions	CPM in SAM Fraction	Filter CPM
Agarose-ATP Type 2	w/ enzyme	141092	865
Agarose-ATP Type 2	w/o enzyme	4092	582
Agarose-ATP Type 4	w/ enzyme	134887	788
Agarose-ATP Type 4	w/o enzyme	3922	937
Agarose-hexane	w/ enzyme	149942	198
Agarose-hexane	w/o enzyme	4122	90
Agarose	w/ enzyme	323150	71
Agarose	w/o enzyme	4481	79

All CPM are minus background. In the conditions calling for no enzyme, distilled H_2O was substituted.

action mixture after removal of agarose beads) reflected the presence or absence of enzyme.

Agarose-ATP Type 2 contains 3.3 µmoles of ATP/ml of agarose (0.33 µmoles/0.1 ml), and agarose-ATP Type 4 contains 7.5 µmoles of ATP/ml of agarose (0.75 µmoles/01 ml). Since there are 10 µmoles of ATP in the solution and counts were low on the filters, it is possible that the ATP in solution were competing for the available enzyme. A second set of experiments was performed in order to check this possibility. In the first experiment, the ATP concentration in the reaction mixture was zero, and for the second experiment the ATP concentration was 20 µM. All procedures remained the same as before (Table 11). Results show a difference between agarose-ATP Type 2 and agarose-ATP Type 4 with Type 2 having approximately twice the number of CPM as Type 4. Again there was no difference between experiments with enzyme and without enzyme for the two agarose-ATP types. It should be noted that the CPM in the SAM fraction in each case are not reliable since these experiments were done before the SAM synthetase assay method was tested and proved reproducible.

C. Extraction and Purification of SAM Synthetase from Baker's Yeast

Four attempts were made to purify SAM synthetase from autolyzed baker's yeast, but all four attempts were unsuccessful. During the first attempt, all procedures as outlined in Materials and Methods were followed closely. The only difference came at the level of the first $(NH_4)_2SO_4$ fractionation (Table 2, Step 4). The first time it was performed, I suspended the 50-75% precipitate in a volume of 1 mM KPB (pH 7.0) +5 mM ETSH which results in an enzyme solution less than 10

TABLE 11: Results from Solid-Phase Biosynthesis Experiments After Varying ATP Concentration

Series A: Zero ATP in Reaction Mixture

Agarose Type	Conditions	CPM in SAM Fraction	Filter CPM
AGarose-ATP Type 2	w/ enzyme	8112	3575
Agarose-ATP Type 2	w/o enzyme	2656	3851
Agarose-ATP Type 4	w/ enzyme	5834	1478
Agarose-ATP Type 4	w/o enzyme	2921	1744
Agarose-hexane	w/ enzyme	10420	165
Agarose-hexane	w/o enzyme	-	130
Agarose	w/ enzyme	10418	69
Agarose	w/o enzyme	-	77

Series B: 20 μM ATP in Reaction Mixture

Agarose Type	Conditions	CPM in Sam Fraction	Filter CPM
Agarose-ATP Type 2	w/ enzyme	7151	3303
Agarose-ATP Type 2	w/o enzyme	2621	5028
Agarose-ATP Type 4	w/ enzyme	8176	1892
Agarose-ATP Type 4	w/o enzyme	3180	1720
Agarose-hexane	w/ enzyme	11441	160
Agarose-hexane	w/o enzyme	-	141
Agarose	w/ enzyme	12404	65
Agarose	w/o enzyme	-	85

All CPM are minux background. In the conditions calling for no enzyme, distilled H_2O was substituted.

mg/ml for the negative bentonite series. To alleviate this problem, the enzyme solution was first re-precipitated by adding saturated, neutralized $(NH_4)_2SO_4$ to 75%. The precipitate obtained was resuspended in a smaller amount of buffer resulting in a higher protein concentration.

DEAE-cellulose chromatography was performed at a flow rate of about 5 ml/min. Before the column was run, a test of the gradient produced by the constant volume mixing chamber was performed. One hundred 8 ml fractions were collected and the conductivity (in mmhos) of every fifth fraction was measured. A series of standards ranging from 1 mM to 0.4 M KPB (pH 7.0) +5 mM ETSH were made and their conductivity also measured. The test of the gradient is plotted in Figure 7. The enzyme solution (8.3 g total protein) from the preceding acetone fractionation step was applied to the column, and 0.76 g of total protein with significant enzyme activity was recovered which corresponds to a 9.2% recovery. No attempts were made to measure the conductivity during a column run to determine at what concentration the enzyme eluted. All enzyme activity was lost during the third $(NH_4)_2SO_4$ fractionation. The results of the first attempt are summarized in Table 12.

During the second attempt, problems were encountered with the column step due to overloading the DEAE-cellulose with protein. The enzyme solution (13.3 g total protein) was applied to the column, and 4.1 g of total protein was recovered. This corresponds to a 30.8% recovery. Even though a large amount of total protein was removed, the column did not increase specific activity significantly. A second column step was added between the second $(NH_4)_2SO_4$ fractionation and the ethanol fractionation in hopes of increasing specific activity. The second column

Figure 7: Test of the Elution Gradient Produced by the Constant Volume Mixing Chamber

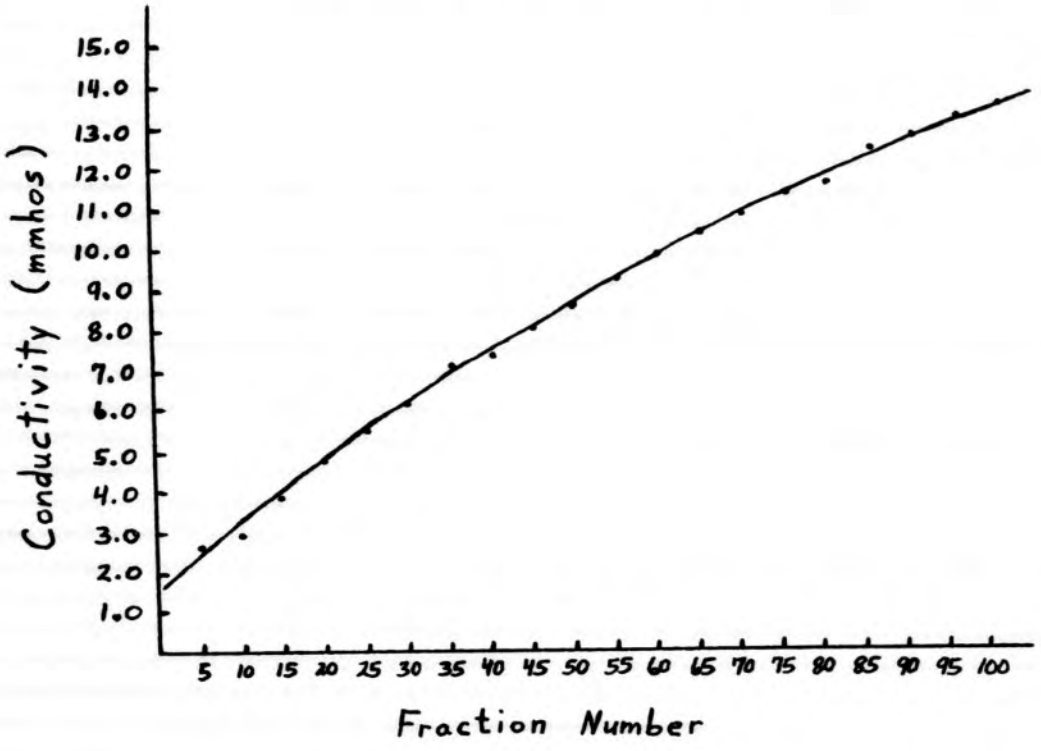


TABLE 12: Purification of SAM Synthetase from Baker's Yeast: Attempt #1

Procedure	Fraction No.	Volume (ml)	Units / ml	Total Units	Protein mg/ml	Total Protein	Units / mg prot.	Yield %	Purification
Autolysate	-	835		54.6	27.8	23.2 g	.0024[1]	-	-
Acetone (24-45%)	-	182	.152	28.4	47.4	8.6 g	.0032[1]	-	-
DEAE-cellulose	79-86; 92-114	220	.066	14.4	3.5	770 mg	.019[1]	-	-
1st $(NH_4)_2SO_4$ (50-75%)	-	168	1.33	223	2.0	336 mg	0.67	100[4]	-
Re-prec. 1st $(NH_4)_2SO_4$	-	18.2	5.68	103	19.0	346 mg	0.299	46	1.0[4]
Negative Bentonite[2]									
Stage #1	-	29	3.53	102	5.9	171 mg	0.598	46	2.0
Stage #2	-	27	3.26	88	5.6	151 mg	0.582	39	2.0
Stage #3	-	25	3.17	79	4.3	108 mg	0.737	35	2.5
Stage #4	-	22	2.77	61	3.3	73 mg	0.839	27	2.8
2nd $(NH_4)_2SO_4$ (53-84%)	-	10.2	4.56	47	4.4	45 mg	1.04	21	3.5
ETOH (52-64%)	-	10.2	4.14	42	1.3	13 mg	3.18	19	10.6
3rd $(NH_4)_2SO_4$[3] (42-52%)	-	3	.039	.118	.025	.08 mg	1.56	.05	5.2

[1]First three steps are not valid since they were done before the SAM synthetase assay was proved reproducible.

[2]Protein concentration adjusted to 10 mg/ml at this point

[3]Enzyme activity lost at this point

[4]Assuming 4th step to be 100% yield and 5th step to be 1.0 purification factor due to the invalid nature of preceeding three steps.

step only increased activity slightly while cutting total enzyme yield in half. Again enzyme activity was lost during the third $(NH_4)_2SO_4$ fractionation. The results of the second attempt are summarized in Table 13.

As a result of the third attempt, I discovered that the DEAE-cellulose column step was an essential part of the purification process and had to be the third step. Its main purpose is to remove a large amount of total protein from the enzyme preparation. Two batches of yeast were processed through the acetone fractionation step and then combined. At this point the column step was skipped, and the combined acetone fractionation solution was then carried through the next three steps. From the results in Table 15, it can be seen that without the column step specific activity decreased significantly. Therefore, after the second $(NH_4)_2SO_4$ fractionation, half of the resulting enzyme solution (3.5 g total protein) was applied to the DEAE-cellulose column in hopes of increasing specific activity. One hundred 10 ml fractions were collected, but no enzyme activity eluted off the column. The results of the third attempt are summarized in Table 14.

During the fourth and last attempt, all steps were followed closely with two changes. At the column step, half of the acetone fractionation solution (6.2 g total protein) was applied to the column. One hundred and six 10 ml fractions were collected at a flow rate of about 3.1 ml/min. and 0.46 g of total protein was recovered with corresponds to a 7.4% recovery. The second change occurred at the negative bentonite step. Since the protein concentration after the first $(NH_4)_2SO_4$ fractionation was 5.2 mg/ml and the volume only 39 ml, only one negative bentonite adsorption using 15 mg bentonite per ml of solution was per-

TABLE 13: Purification of SAM Synthetase from Baker's Yeast: Attempt #2

Procedure	Fraction No.	Volume (ml)	Units ml	Total Units	Protein mg/ml	Total Protein	Units mg prot.	Yield %	Purification
Autolysate	-	825	1.54	1269	60.9	50.2 g	0.025	100	-
Acetone (24-45%)	-	365[1]	2.22	809	45.1	16.5 g	0.049	64	2.0
DEAE-cellulose	24-46; 50-63	505	.422	213	8.2	4.1 g	0.051	17	2.0
1st $(NH_4)_2SO_4$ (50-75%)	-	53	2.95	97	37.2	1.2 g	0.079	7.6	3.0
Negative Bentonite[2]									
Stage #1	-	112	1.14	128	6.7	750 mg	0.170	10	7.0
Stage #2	-	106	1.11	117	5.5	580 mg	0.202	9	8.0
Stage #3	-	102	1.07	109	4.6	470 mg	0.233	8.6	9.0
Stage #4	-	97	1.01	98	3.9	380 mg	0.259	7.7	10.0
2nd $(NH_4)_2SO_4$ (53-84%)	-	18	3.50	63	19.1	340 mg	0.183	5.0	7.3
2nd DEAE-column step	-	40	.935	37	4.4	180 mg	0.213	2.9	9.0
ETOH (52-64%)	-	14	2.23	31	2.5	30 mg	0.892	2.4	36.0
3rd $(NH_4)_2SO_4$[3] (42-52%)	-	3	.172	.52	.30	.9 mg	0.573	.04	22.9

[1]Enzyme solution was concentrated to 120 ml with polyethylene glycol.

[2]Protein concentration adjusted to 10 mg/ml at this point.

[3]Enzyme activity lost at this point

TABLE 14: Purification of SAM Synthetase from Baker's Yeast: Attempt #3

Procedure	Fraction No.	Volume (ml)	Units ml	Total Units	Protein mg/ml	Total Protein	Units mg prot.	Yield %	Purification[1]
Autolysate Batch I	-	895	1.43	1280	43.0	38.5 g	0.033	100	-
Autolysate Batch II	-	905	1.38	1250	49.2	44.5 g	0.028	100	-
Acetone (24-45%) I	-	309	1.72	532	57.3	17.7 g	0.030	42	-
Acetone (24-45%) II	-	342	1.38	472	55.3	18.9 g	0.025	38	-
Combined Acetone I & II	-	640	1.53	981	53.5	34.2 g	0.029	39	-
1st $(NH_4)_2SO_4$ (50-75%)	-	235	2.73	643	63.9	15.0 g	0.043	25	-
Negative Bentonite[2]	-	206	2.37	489	57.7[2]	11.9 g	0.041	19	-
Stage #1	-	192	2.34	449	56.7	10.9 g	0.041	18	-
Stage #2	-	180	2.23	402	53.4	9.6 g	0.042	16	-
Stage #3	-	168	2.24	377	52.9	8.9 g	0.042	15	-
Stage #4	-								
2nd $(NH_4)_2SO_4$ (53-84%)	-	184	.815	150	38.9	7.2 g	0.020	6	-
DEAE-cellulose[3]	-	-	0	0	0	0	0	0	-

[1] No purification factors were calculated for this attempt.

[2] Protein concentration was not adjusted to 10 mg/ml at this point.

[3] No enzyme activity eluted off the column.

formed. Enzyme activity was lost during the ethanol fractionation step. The results of the fourth attempt are summarized in Table 15.

TABLE 15: Purification of SAM Synthetase from Baker's Yeast: Attempt #4

Procedure	Fraction No.	Volume (ml)	Units / ml	Total Units	Protein mg/ml	Total Protein	Units / mg prot.	Yield %	Purification
Autolysate	-	905	.568	514	30.2	27.3 g	0.019	100	-
Acetone (24-45%)	-	285	1.53	435	44.5	12.7 g	0.034	85	1.8
DEAE-cellulose[1]	81-106	289	.284	82	1.6	460 mg	0.178	31	9.4
1st $(NH_4)_2SO_4$ (50-75%)	-	39	1.20	47	5.2	200 mg	0.231	18	12.2
Negative Bentonite[2]	-	34	1.23	42	3.1	110 mg	0.397	16	20.9
2nd $(NH_4)_2SO_4$ (53-84%)	-	16.5	1.89	31	4.9	80 mg	0.386	12	20.3
ETOH (52-64%)[3]	-	7.0	.013	.09	.35	2.5 mg	0.037	.02	1.9

[1]One-half of the enzyme solution from the acetone fractionation was applied to the column.

[2]Only one bentonite step was performed at this point due to a low protein concentration and low volume.

[3]Enzyme activity lost at this point.

CHAPTER IV

DISCUSSION

It was the purpose of thie study to develop an agarose-SAM affinity system by the enzymatic formation of SAM on agarose-ATP. Since most of the results have been negative, the bulk of this discussion will be devoted to specific suggestions to improve the experiments and thus the results.

A. The Sam Synthetase Assay Method

The SAM synthetase assay method used throughout this study is a simple and highly reproducible assay for SAM synthetase activity and needs no further modifications. The assay is able to detect the conversion of 0.1% of substrate to product with an accuracy of $\pm$ 5% (Holcomb and Shapiro, 1975). The assay is efficient in that it allows the virtual quantitative recovery of the undegraded SAM in the first three ml of 0.1 N H_2SO_4. This also makes counting more efficient. Since the columns are disposable, assays can be performed quickly. The amount of time per assay, including disposable column preparation and actual performance of the assay, was estimated to be approximately 45 minutes. If a supply of disposable columns are prepared ahead of time, this time per assay can be further reduced to 30 minutes per assay.

B. Solid-Phase Biosynthesis of SAM

The experiments involving the solid-phase biosynthesis of SAM, though negative at this stage, may show promise with a few modifications. In-

stead of rinsing the agarose onto a glass fiber filter, I suggest that the agarose be carried through all the rinses as outlined in Table 3 while still in the conical centrifuge tube. After the last rinse, 5 ml of 0.01 M KPB (pH 7.2) is added to the agarose which is then added to 10 ml of scintillation cocktail to form a transparent gel. The agarose beads would then be counted while suspended throughout the gel instead of at the bottom of the vial as before. This would improve counting efficiency.

The series of rinses as they stand need not be changed for they serve specific functions. For example the presence of cold methionine in the rinses serve to dilute out the labelled methionine after the reaction has finished. Likewise, the presence of 6 M urea and 2 M NaCl serve to denature the enzyme present and inhibit any ionic interactions between the enzyme and the agarose.

The agarose-ATP Type 2 and Type 4 used in the solid-phase reactions have been described in Materials and Methods. Recently, P-L Biochemicals has introduced a Type 3 agarose-ATP which has the ATP attached via the C^8 of the adenyl moiety. (Lee et al., 1974; see Figure 6). This type was not used because it was not commercially available at the time this study was done. Results from this study give preliminary evidence that Type 2 is more effective for solid-phase biosynthesis of SAM on agarose than Type 4. Since Type 3 is attached similiarly to Type 2 (i.e. C^8 vs N^6-amino group of adenyl moiety), it should also give good results when tested. As discussed in the Introduction, Chou and Talalay (1973) have shown that SAM synthetase presents a high specificity for ATP and that the triphosphate group is required for substrate recognition. Since this is the case, then it can be hypothesized that the agarose-ATP Types 2 and

3 would be the types of choice for the solid-phase biosynthesis of SAM since both types would leave the triphosphate group completely free and the adenosyl group relatively free to react with the SAM synthetase. It is possible that with the ATP attached to the agarose via the ribosyl moiety (i.e. Type 4) the ATP is not presented in the proper configuration for binding to the enzyme's catalytic site. This may be one possible explanation for the lower CPM observed with the Type 4 agarose-ATP.

If a SAM-agarose affinity system could be produced, the SAM attached via the C^8-adenyl moiety (Type 3) would probably be the most promising in isolating methyltransferases. This would be the case because SAM attached via the C^8-adenyl moiety would have all of the important binding sites unmodified. The studies done by Zappia et al. (1969) and Borchardt et al. (1974) with structural analogues of SAM and SAH in transmethylation reactions would tend to support this speculation (Introduction, Figure 2).

The need for a pure SAM synthetase preparation constitutes a major suggestion and therefore prompted the four attempts at purifying it from baker's yeast. As mentioned earlier, the E. coli preparation was purified only 60-80 fold. Chou and Talalay (1972) reported purifying SAM synthetase from yeast approximately 1953-fold and observed no phosphatase activity in their preparation. Phosphatase activity would be detrimental to the agarose-ATP. Also methyltransferase activity would interfere with the solid-phase reaction if a methyl acceptor was present in the enzyme preparation. The presence of contaminating enzymes may be a partial explanation for the ambiguous results observed during the solid-phase reaction using the E. coli SAM synthetase preparation. The purity of a final enzyme preparation should be tested before its use by subjecting a sample to polyacrylamide-gel electrophoresis.

C. Purification of SAM Synthetase from Baker's Yeast

The procedure for the extraction and purification of SAM synthetase from Baker's yeast requires a number of suggested changes over the published methods of Mudd and Cantoni (1958), Lombardini et al. (1970), and Chou and Talalay (1972). When performing organic solvent fractionations, the acetone or ethanol should be cooled to -20°C before addition to the enzyme extract. Organic solvents have less of a denaturing effect on proteins when the solvent and solutions are kept at temperatures well below 0°C. All saturated $(NH_4)_2SO_4$ solutions should be cooled to 0°C before addition. When doing $(NH_4)_2SO_4$ fractionations, the solution should be allowed to stir very gently (at 0°C) for 30 minutes during the first $(NH_4)_2SO_4$ fractionation, for 45 minutes during the second, and for one hour during the third. Likewise, during the ethanol fractionation, the solution should be allowed to stir very gently for 30 minutes at -8°C. It was discovered that frequent freezing and thawing of the enzyme preparations, particularly at low protein concentrations, resulted in spontaneous denaturation of the enzyme. According to Mudd and Cantoni (1958), Lombardini et al. (1970), and Chou and Talalay (1972), the enzyme is stable after the acetone fractionation, the second $(NH_4)_2SO_4$ fractionation, and the third $(NH_4)_2SO_4$ fractionation. At any other time the enzyme solution should not be frozen.

Holcomb and Shapiro (1975) have shown that SAM synthetase specific activity can be increased 70% if the yeast is cultured in the presence of threonine. Therefore to increase enzyme yield, it might be advantageous to culture the yeast in a minimal essential medium containing 5 mM L-threonine for 16 h before drying. Since threonine represses homoserine

kinase (Umbarger, 1969), it is theorized that more homoserine would become available for methionine biosynthesis which, in turn, will induce SAM synthetase at low concentrations (Holloway et al., 1970; Holcomb and Shapiro, 1975).

Recently, Chiang and Cantoni (1977) have reported the purification of SAM synthetase from baker's yeast to homogeneity and have observed the separation of the enzyme into two forms, tentatively called SAM synthetase I and II. Both forms have been shown to have a molecular weight of 110,000 with two subunits of 55,000 and 60,000 respectively. It should be noted that a molecular weight of 110,000 is in agreement with Greene (1969) who reported a molecular weight of 100,000, but is in disagreement with a value of 150,000 reported by Mudd (1963) and a value of 45,000 reported by Chou and Talalay (1972). The reasons for these discrepancies are not understood. The reason for two enzymatic forms is also not understood, but Chiang and Cantoni (1977) have shown that the two forms differ with respect to their PPPase activity, i.e. SAM synthetase I PPPase activity is independent of any exogeneous SAM, whereas SAM synthetase II PPPase activity is completely dependent on added SAM.

D. Future Protocol for Experiments

In conclusion, I would suggest the following protocol for future experiments involving the solid-phase biosynthesis of SAM on agarose: (1) isolate SAM synthetase from baker's yeast according to Table 1 with suggested modifications and using the SAM synthetase assay to follow each step, (2) test the final enzyme preparation by analyzing a sample on polyacrylamide-gell electrophoresis, (3) perform the solid-phase reaction using the purified SAM synthetase and agarose-ATP Types 2, 3, and 4 with

suggested modifications and using agarose and agarose-hexane as controls, and (4) test the usefulness of SAM as an affinity ligand by isolating a known methyltransferase as a standard and testing its retention and elution characteristics on the agarose-SAM affinity column. If developed and tested for reproducibility, an agarose-SAM affinity column would be useful in the purification of methyltransferases specific for a wide range of proteins, nucleic acids, lipids, biogenic amines, and other biologically important molecules.

BIBLIOGRAPHY

Allfrey, V.G., R.Faulkner, and A.E.Mirsky (1964) Acetylation and methylation of histones and their possible role in the regulation of RNA synthesis. Proc.Nat.Acad.Sci. 51: 786-794.

Arber, W. (1974) DNA modification and restriction. Prog.Nucleic Acid Res.Mol.Biol. 14: 1-37.

Axelrod, J. (1977) Physiological and Pharmacological Actions of Adenosylmethione, in The Biochemistry of Adenosylmethionine (F.Salvatore, E.Borek, V.Zappia, H.G.Williams-Ashman, and F.Schlenk, eds.), Columbia University Press, pp. 539-554.

Borchardt, R.T. and Y.S.Wu (1974) Potential inhibitors of S-adenosylmethionine-dependent methyltransferases. 1.Modification of the amino acid portion of S-adenosylhomocysteine. J.Med.Chem. 17: 862-873.

Borchardt, R.T., J.A. Huber, and Y.S.Wu (1974) Potential inhibitors of S-adenosylmethionine-dependent methyltransferases.2.Modification of the base portion of S-adenosylhomocysteine. J.Med.Chem. 17: 868-873.

Borek, E. (1977) Modification of nucleic acids in relation to differentiation. Trends Biochem. Sciences 2: 3-6.

Both, G.W., A.K.Banerjee, and A.J.Shatkin (1975a) Methylation-dependent translation of viral messenger RNAs in vitro. Proc.Nat.Acad.Sci. 72: 1189-1193.

Both, G.W., Y.Furuichi, S.Muthukrishnan, and A.J.Shatkin (1975b) Ribosome binding to reovirus mRNA in protein synthesis requires 5'-terminal 7-methylguanosine. Cell 6: 185-195.

Canaani, D., M.Ravel, and Y.Groner (1976) Translational discrimination of 'capped' and 'non-capped' mRNAs: Inhibition by a series of chemical analogs of m^7GpppX. FEBS Lett. 64: 326.

Cantoni, G.L. (1953) S-adenosylmethionine: a new intermediate formed enzymatically from L-methionine and adenosinetriphosphate. J.Biol.Chem. 204: 403-416.

Cantoni, G.L. (1965), in S.K.Shapiro and F.Schlenk (1965), pp. 21-32.

Chiang, P.K. and G.L.Cantoni (1977) Activation of methionine for transmethylation. Purification of the S-adenosylmethione synthetase of baker's yeast and its separation into two forms. J.Biol.Chem. 252: 4506-4513.

Chou, T.C. and P.Talalay (1972) The mechanism of S-adenosyl-L-methionine synthesis by purified preparations of baker's yeast. Biochem. 11: 1065-1073.

Chou, T.C. and P.Talalay (1973) Inhibition of ATP: L-methionine S-adenosyltransferase of baker's yeast by structural analogues of ATP. Biochem.Biophys.Acta 321: 467-474.

Coward, J.K. and W.D.Sweet (1972) Analogs of S-adenosylhomocysteine as potential inhibitors of biological transmethylation. Synthesis and biological activity of homocysteine derivatives bridged to adenine. J.Med.Chem. 15: 381-384.

Coward, J.K. and E.P.Slisz (1973) Analogs of S-adenosylhomocysteine as potential inhibitors of biological transmethylations. Specificity of the S-adenosylhomocysteine binding site. J.Med.Chem. 16: 460-

De la Haba, G. and G.L.Cantoni (1959) Tne enzymatic synthesis of S-adenosyl-L-homocysteine from adenosine and homocysteine. J.Biol. Chem. 234: 603-608.

De la Haba, G., G.A.Jamieson, S.H.Mudd and H.H.Richards (1959) S.-adenosyl-methionine. The relation of configuration at the sulfonium center to enzymatic reactivity. J.Amer.Chem.Soc. 81: 3975-3980.

De Robichon-Szulmajster, H. and H.Cherest (1967) Regulation of homoserine O-transacetylase. First step in methionine biosynthesis in Saccharomyces cerevisiae. Biochem. Biophys.Res.Communs. 28: 256-262.

Desrosiers, R.C., K.H.Friderici, and F.M.Rottman (1975) Characterization of Novikoff hepatoma mRNA methylation and heterogeneity in the methylated 5'-terminus. Biochem. 14: 4367-4374.

Dubin, D.T. and R.H.Taylor (1975) The methylation state of poly A-containing-mRNA from cultured hamster cells. Nucleic Acids Res. 2: 1653-1668.

Duerre, J.A. (1968) In vivo and in vitro metabolism of S-adenosyl-L-homocysteine by Saccharomyces cerevisiae. Arch.Biochem.Biophys. 124: 422-430.

Durban,E., W.K.Paik, S.Nochumson, and S.Kim (1977) Purification and characterization of cytochrome c-specific protein methylase III from Neurospora crassa. Fed.Proc. 36: 717 (Abstract #2345).

Finkelstein, J.D., W.E.Kyle, and B.J.Harris (1974) Methionine metabolism in mammals: Regulatory effects of S-adenosylhomocysteine. Arch. Biochem.Biophys. 165: 774-779.

Greenberg, J. and A.I.Krasna (1976) Effect of enzymatic methylation on the chemical, physical, and biological properties of DNA. Arch. Biochem.Biophys. 177: 468-479.

Greene, R.C. (1969) Kinetic studies of the mechanism of S-adenosylmethionine synthetase from yeast. Biochem. 8: 2255-2265.

Guilford, H., P.O.Larsson, and K.Mosbach (1972) Adenine nucleotides for affinity chromatography. Chemica Scripta 2: 165-170 from Chem. Abstracts 78: 528 (Abstract #43900w) (1973)

Holcomb, E.R. and S.K.Shapiro (1975) Assay and regulation of S-adenosylmethionine synthetase in Saccharomyces cerevisiae and Candida utilis. J.Bact. 121: 267-271.

Holloway, C.T., R.C.Greene, and C.H.Su (1970) Regulation of S-adenosylmethionine synthetase in Escherichia coli. J.Bact. 104: 734-747.

Kerr, D. and M.Flavin (1969) Inhibition of cystathionine γ-synthetase by S-adenosylmethionine: A control mechanism for methionine synthesis in Neurospora. Biochem.Biophys.Acta 177: 177-179.

Kerr, S.J. and E.Borek (1973) Enzymic methylation of natural polynucleotides. The Enzymes (ed. P.D.Boyer), 9: 167-195.

Kim, S. (1973) Purification and properties of protein methylase II. Arch.Biochem.Biophys. 157: 476-484.

Kim, S. and W.K.Paik (1965) Studies on the origin of ε-N-methyl-L-lysine in protein. J.Biol.Chem. 240: 4629-4634.

Krzysik, B., J.P.Vergnes, and I.R.McManus (1971) Enzymatic methylation of skeletal muscle contractile proteins. Arch.Biochem.Biophys. 146: 34-45.

Lamed, R., Y.Levin, and M.Wilchek (1973) Covalent coupling of nucleotides to agarose for affinity chromatography. Biochim.Biophys.Acta 304: 231-235.

Lee, H.W., S.Kim, and W.K.Paik (1977) S-adenosylmethionine-protein: arginine methyltransferase-purification and mechanism of enzyme. Biochem. 16: 78-84.

Lee, L.W., J.M.Ravel, and W.Shive (1966) Multimetabolite control of a biosynthetic pathway by sequential metabolites. J.Biol.Chem. 241: 5479-5480.

Lee,C.Y., D.Lappi, B.Wermuth, J.Everse, and N.O.Kaplan (1974) 8-(6-amino-hexyl)-amino-adenine nucleotide derivatives for affinity chromatography. Arch.Biochem.Biophys. 163: 561-565.

Lobley, G.E., S.V.Perry, and D.Stone (1971) Structural changes in myosin induced by vitamin E dystrophy. Nature 231: 317-318.

Lombardini, J.B., A.W.Coulter, and P.Talalay (1970) Analogues of methionine as substrates and inhibitors of the methionine adenosyltransferase reaction. Mol.Pharmacol. 6: 481-499.

Lombardini, J.B. and P.Talalay (1971) Formation, functions, and regulatory importance of S-adenosylmethionine. Adv.Enzyme Regulation 9: 349-384.

Lombardini, J.B., T.C.Chou, and P.Talalay (1973) Regulatory properties of adenosine triphosphate-L-methionine S-adenosyltransferase of rat liver. Biochem.J. 135: 43-57.

Martin, S.A., E.Paoletti, and B.Moss (1975) Purification of mRNA guanylyltransferase and mRNA (guanine-7)-methyltransferase from vaccinia virions. J.Biol.Chem. 250: 9322-9329.

Morse,R.K., J.P.Vergnes, J.Malloy, and I.R.McManus (1975) Sites of biological methylation of proteins in cultures chick muscle cells. Biochem. 14: 4316-4325.

Mudd, S.H. (1959) Enzymatic cleavage of S-adenosylmethionine. J.Biol. Chem. 234: 87-92.

Mudd,S.H. (1963) Activation of methionine for transmethylation. VI. Enzyme-bound tripolyphosphate as an intermediate in the reactions catalyzed by the methionine-activating enzyme of baker's yeast. J.Biol.Chem. 238: 2156-2163.

Mudd, S.H. and G.L.Cantoni (1958) Activation of methionine for transmethylation. III. The methionine-activating enzyme of baker's yeast. J.Biol.Chem. 231: 481-492.

Mudd,S.H. and J.D.Mann (1963) Activation of methionine for transmethylation.VII. Some energetic and kinetic aspects of the reaction catalyzed by the methionine-activating enzyme of baker's yeast. J.Biol. Chem. 238: 2164-2170.

Muthukrishnan, S., G.W.Both, Y.Furuichi, and A.J.Shatkin (1975) 5'-Terminal 7-methylguanosine in eukaryotic mRNA is required for translation. Nature 255: 33-37.

Nau, F. (1976) The methylation of tRNA. Biochimie 58: 629-645.

Paik, W.K. and S.Kim (1969) Protein methylation in rat brain in vitro J.Neurochem. 16: 1257-1261.

Paik, W.K. and S.Kim (1970) Solubilization and partial purification of protein methylase III from calf thymus nuclei. J.Biol.Chem. 245: 6010-6015.

Paik, W.K. and S. Kim (1971) Protein methylation. Science 174: 114-119.

Paik, W.K. and S.Kim (1975) Protein methylation: chemical, enzymological, and biological significance. Adv.Enzymol. 42: 227-286.

Peterson, E.A. and H.A.Sober (1962) Column chromatography of proteins: substituted celluloses. Methods Enzymol. 5: 3-27.

Rottman, F.M. (1976) Methylation of messenger RNA. Trends Biochem. Sciences 1: 217-219

Schlenk, F. (1965) in S.K.Shapiro and F.Schlenk (1965) pp.

Shapiro, S.K., D.A.Yphantis, and A.Almenas (1964) Biosynthesis of methionine in Saccharomyces cerevisiae. Partial purification and properties of S-adenosylmethionine: homocysteine methyltransferase. J.Biol.Chem. 239: 1551-1556.

Shapiro, S.K. and F.Schlenk, eds.(1965) Transmethylation and Methionine Biosynthesis, University of Chicago Press, Chicago.

Shatkin, A.J. (1976) Capping of eukaryotic mRNAs. Cell 9: 645-654.

Tabor, H. and C.W.Tabor (1971) Enzymatic synthesis of radioactive S-adenosylmethionine. Methods Enzymol. 17: 393-397.

Taylor, R.T. and H.Weissbach (1969) Escherichia coli B N^5-methyltetrahydrofolate-homocysteine methyltransferase: sequential formation of bound methylcobalamin with S-adenosyl-L-methionine and N^5-methyltetrahydrofolate. Arch.Biochem.Biophys. 129: 728-744.

Taylor, R.T. and H.Weissbach (1969) Escherichia coli B N^5-methyltetrahydrofolate-homocysteine methyltransferase: activation with S-adenosyl-L-methionine and the mechanism of methyl group transfers. Arch.Biochem.Biophys. 129: 745-766.

Trayer, I.P., C.I.Harris, and S.V.Perry (1968) 3-Methyl-histidine in adult and foetal forms of skeletal muscle myosin. Nature 217: 452-453.

Tyihak, E., B.Szende, and K.Lapis (1977) Biological significance of methylated derivatives of lysine and arginine. Life Sci. 20: 385-392.

Umbarger, H.E. (1969) Regulation of amino acid metabolism. Ann.Rev. Biochem. 38: 323-370.

Wei,C.M., A.Gershowitz, and B.Moss (1975) Methylated nucleotides block 5'-terminus of HeLa cell messenger RNA. Cell 4: 379-386.

Zappia, V., C.R.Zydek-Cwick, and F.Schlenk (1969) The specificity of S-adenosylmethionine derivatives in methyl transfer reactions. J.Biol.Chem. 244: 4499-4509.