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(Signed).....William A Dodd

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THE UNIVERSITY OF ALBERTA

STUDIES OF THE SYNTHESIS AND METABOLISM OF S-ADENOSYL-L-METHIONINE IN PEA SEEDLINGS

by

© WILLIAM ALFRED DODD

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES

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OF DOCTOR OF PHILOSOPHY

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EDMONTON, ALBERTA

SPRING 1969

UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled STUDIES ON THE SYNTHESIS AND METABOLISM OF S-ADENOSYL-L-METHIONINE IN PEA SEEDLINGS submitted by William Alfred Dodd in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

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ABSTRACT

The reactions leading to the biosynthesis of S-adenosyl-L-methionine in pea seeds during germination were examined *in vivo* and *in vitro*. The tissue content of S-adenosylmethionine and S-adenosylhomocysteine was assayed during the first three days of germination. There is net synthesis of both of these compounds during this period.

Tissue slices of pea cotyledons incubated with methionine, labelled in the methyl group with ^{14}C or ^{3}H or with methionine-^{35}S, rapidly incorporated radioactivity into S-adenosylmethionine. This labelled pool of S-adenosylmethionine was found to be in an active state of turnover. Kinetic studies showed that sulphur labelled and methyl labelled S-adenosylmethionine had quite different rates of turnover. Such differences were related to the possible recycling of sulphur and transmethylation.

Experiments with cell-free extracts were carried out to determine the possible sequence of reactions for regeneration of S-adenosylmethionine. Attempts to demonstrate the remethylation of S-adenosylhomocysteine were unsuccessful. Experiments showed that S-adenosyl-8-^{14}C-L-homocysteine was readily cleaved to give adenosine and homocysteine. Homocysteine could be methylated by S-methylmethionine, S-adenosylmethionine or 5-methyltetrahydrofolate. It is concluded that these latter three compounds are likely to be the most important sources of methyl groups in germinating pea seeds.

Studies with partially purified enzymes showed the

presence of two distinct enzymes, capable of transferring methyl groups to homocysteine. One of these enzymes was able to transfer methyl groups from 5-methyltetrahydrofolate, while the other enzyme could transfer methyl groups from both S-adenosylmethionine and S-methylmethionine. The catalytic properties of these two partially purified enzymes were studied using isotopic and microbiological assays.

The properties and possible relationships of these two methyltransferases to the synthesis of methionine and S-adenosylmethionine in germinating pea seeds are discussed.

ACKNOWLEDGEMENTS

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I would also like to thank Mrs. Jennifer Weston for her work on the methionine microbiological assays, Mr. Ben Blawacky for his help on numerous occasions and Mrs. E. Ford for typing this thesis.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

$5CH_3H_4PteGlu$	: 5-methyltetrahydrofolate monoglutamate
$5CH_3H_4PteGlu_3$	: 5-methyltetrahydrofolate triglutamate
SAM	: S-adenosyl-L-methionine
SAHC	: S-adenosyl-L-homocysteine
MTA	: 5-methylthioadenosine
DPT	: dimethyl-β-propiothetin
SMM	: S-methyl-L-methionine
mCi	: millicurie
μCi	: microcurie
dpm	: disintegrations per minute
cpm	: counts per minute
ATP	: adenosine triphosphate
TRIS	: tris (hydroxymethyl) aminomethane
EC.	: Enzyme Commission
DEAE-cellulose	: diethylaminoethyl cellulose
EDTA	: disodium ethylenediamine tetra-acetic acid
THFA	: tetrahydrofolic acid

LITERATURE REVIEW

Transmethylation reactions have been studied in organisms of sufficient diversity to justify the conclusion that S-adenosyl-L-methionine (SAM) is the major biological methyl donor (Mudd and Cantoni, 1964). This generalization appears to be valid although the specificity of SAM as a methyl donor has not been investigated in some cases. However, it has now been established that SAM functions as a specific methyl donor in at least sixty different metabolic reactions (Meister, 1965; Lederer, 1965).

The importance of transmethylation as a biochemical process was established by considerable work, based on the discovery by du Vigneaud, *et al.*, (1939), that choline and homocystine could replace methionine in the diet of rats. This work, reviewed by du Vigneaud and Rachele (1965), showed that choline donated an intact methyl group to homocysteine forming methionine. It was also suggested that the reaction would be reversible and that methionine could be a source of methyl groups for choline synthesis by transmethylation. Subsequently it has been shown that where-ever methyl groups have been derived from methionine, it is usually the activated form of methionine, namely SAM, which is the actual methyl donor.

The Discovery and Biosynthesis of SAM

SAM was discovered by Cantoni (1953) and was later shown to be an important methyl group donor in a variety of tissues (Mudd and Cantoni, 1964). In plants, SAM is essential for the synthesis of many metabolites including

chlorophyll (Radmer and Bogorad, 1967), lignin (Byerrum, *et al.* 1954), and pectin (Sato, *et al.* 1964). The mechanism of enzymatic synthesis of SAM was investigated using partially purified enzymes derived from rabbit liver (Cantoni and Durell, 1957), bakers' yeast (Mudd and Cantoni, 1958), barley seedlings (Mudd, 1960) and *Escherichia coli* (Tabor and Tabor, 1960). In each case the reaction was found to be similar and had a requirement for methionine, Mg^{2+} ions and ATP (reaction 1).

$$\text{Methionine} + \text{ATP} \xrightarrow{Mg^{2+}} \text{SAM} + \text{pyrophosphate} + \text{Pi} \qquad (1)$$

The equilibrium of this enzyme catalyzed reaction strongly favours the formation of SAM. In plants, all studies to date, have shown that SAM synthesis proceeds by this same reaction (Mudd, 1960; Davies, 1966). However, Schlenk and Ehninger (1964) have reported tracer studies showing that the yeast *Candida utilis* is able to regenerate SAM from methylthioadenosine (reaction 2).

$$\text{Methylthioadenosine} + \text{4 carbon amino acid} \longrightarrow \text{SAM} \qquad (2)$$

Other Biologically Important Methyl Donors

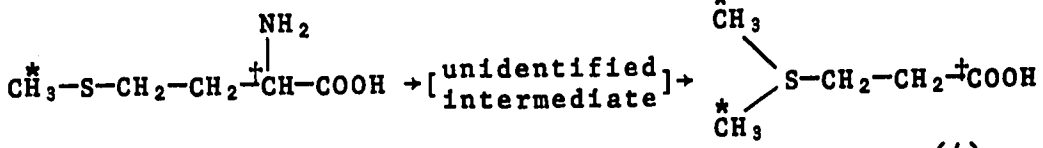
The classical work of du Vigneaud, *et al.* (1939) showed that choline was a donor of methyl groups in the rat and subsequently a number of other methyl donors were recognized in animal tissues. However, all available evidence suggests that in plants, SAM, S-methylmethionine (SMM) and dimethylpropiothetin are the only methyl donors, (Thompson, 1967) except for the primary synthesis of

methionine from $5CH_3H_4PteGlu$.

S-methylmethionine (SMM) was first discovered in plants by McRorie, *et al.* (1954) and is now known to be of widespread occurrence in plant tissues (Sato, *et al.* 1958, Greene and Davis, 1960; Karr, Tweto and Albersheim, 1967). SMM is also present in bacteria (McRorie, *et al.* 1954; Shapiro, 1956) and in animal tissues (Shapiro, 1956). In all organisms so far investigated, the synthesis of SMM is catalyzed by the enzyme S-adenosylmethionine:L-methionine S-methyltransferase (reaction 3).

$$\text{SAM} + \text{methionine} \longrightarrow \text{S-methylmethionine} + \text{SAHC} \quad (3)$$

Dimethylpropiothetin (DPT) was the first sulphonium compound to be unequivocally identified in nature (Challenger and Simpson, 1948). The biosynthesis of DPT was studied by Green (1962) who showed that methionine was an efficient precursor of DPT in the marine alga, *Ulva lactuca*. Although the interconversion is not clear, the amide of methylthiopropionic acid may be an intermediate (Green, 1962). Reaction 4 shows that the methyl group and sulphur of methionine are both incorporated into DPT.



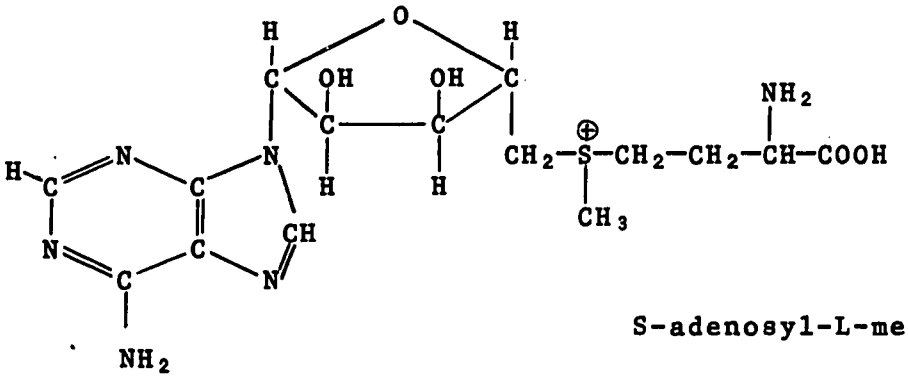
(4)

Challenger, *et al.* (1957) reported that DPT was present in many groups of marine algae, however this compound is apparently seldom found in higher plants or in animals.

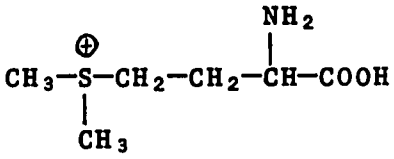
Both SMM and DPT have been shown to be potentially good methyl group donors (Abrahamson and Shapiro, 1965). Their involvement in transmethylation will be discussed under homocysteine dependent transmethylase reactions.

The Mechanism of Biological Methyl Transfer

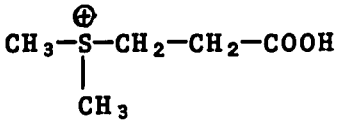
Examination of the structures of the more important methyl donors (Figure 1) shows that an onium pole is the significant common feature, and all transmethylation reactions that have been studied in detail, proceed from an onium pole (Mudd and Cantoni, 1964). It has become apparent that only methyl groups, originally attached to a sulphonium or ammonium atom, are transferred enzymatically from that site to an acceptor site. The presence of an onium centre places a partial positive charge upon the carbon atoms immediately adjacent to it, making these atoms susceptible to nucleophilic attack. The acceptor molecules contain nucleophilic sites in which a nitrogen, oxygen, sulphur, selenium, or carbon atom, spearheads the nucleophilic attack. The overall result is the displacement of the sulphur or nitrogen onium atom and its remaining substituents from the methyl carbon atom. There is a concomitant formation of a new bond between the nucleophilic acceptor and the methyl carbon. Cantoni (1965) has shown that methyl transfers from a sulphonium pole are exergonic. A proton is released, which at physiological pH makes a major contribution to a favorable free energy change. These experiments have shown that the onium poles do supply the



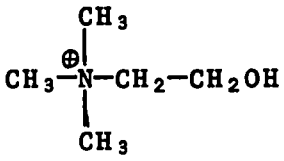
S-adenosyl-L-methionine



S-methyl-L-methionine



dimethyl-β-propiothetin



choline

Figure 1. Structures of the more important methyl donors, showing the presence of an onium pole which makes these molecules vunerable to nucleophylic attack and allows transfer of the methyl group.

driving force for transmethylation reactions.

The Concept of Transmethylation

Transmethylation is now accepted as a basic biochemical process (du Vigneaud and Rachele, 1965) and the enzymic reactions involved in transfer of methyl groups to many acceptors have now been studied in a wide range of organisms. In particular, the methylation of homocysteine, forming methionine, has now been studied extensively using enzyme preparations from many sources. The use of homocysteine as the methyl acceptor in these studies has been necessary since all the recognized methyl donors will transfer methyl groups to homocysteine to form methionine. Some generalizations can now be made concerning the biosynthesis of methionine from homocysteine.

Homocysteine Dependent Transmethylase Reactions

The formation of methionine from homocysteine involves the addition of a methyl group from a methyl donor via a transmethylase enzyme. Although some of these transmethylase enzymes have been partially purified, there is insufficient information to make a detailed comparison of their common features (Cantoni, 1965). However, those studied do not appear to require a prosthetic group. For example, there have been a number of reports that metal chelating agents will inhibit the homocysteine transmethylases from certain microorganisms (Shapiro and Yphantis, 1959) pea seeds (Turner and Shapiro, 1961) and yeast (Shapiro, *et al.* 1965). Such inhibition could be reversed by divalent cat-

ions, particularly Zn^{2+} (Shapiro and Yphantis, 1959), however there is no evidence for cation activation in animal homocysteine transmethylases.

Most of the homocysteine transmethylases investigated have a pH optimum in the range 7.5 to 8.0 and in most studies the transmethylases show inhibition due to the product, methionine, although the extent of this inhibition varies with enzymes from different sources. There is strong evidence in the only case investigated, that there is no methylated enzyme formed in the course of the reaction (Durell and Cantoni, 1959). The homocysteine transmethylases may be divided into two categories.

(1) Homocysteine dependent transmethylation where the donor is 5-methyltetrahydrofolate. In mammalian liver (Sakami and Ukstins, 1961; Mangum and Scrimgeour, 1962), and in *E. coli* (Guest, *et al.* 1962; Kisliuk, 1963), 5-methyltetrahydrofolate triglutamate donates a methyl group to homocysteine (reaction 5). This system is vitamin B_{12} dependent.

$$5CH_3H_4PteGlu + \text{homocysteine} \xrightarrow{\text{Vit } B_{12}} \text{methionine} + THFA \quad (5)$$

Escherichia coli, *Aerobacter aerogenes* and *Salmonella typhimurium* have two systems for methionine formation. One utilizes vitamin B_{12} and one does not. In the vitamin B_{12} independent pathway, the methyl donor is the

triglutamate form of 5-methyltetrahydrofolate while in the B_{12} system either the monoglutamate or triglutamate forms are effective (Thompson, 1967). A catalytic amount of SAM is necessary in the B_{12} system, apparently to methylate the enzyme (Taylor and Weissbach, 1966).

In higher plants, both Woods, Foster and Guest (1965) and Elford, *et al.* (1965) state that only the triglutamate derivative of 5-methyltetrahydrofolate is able to donate methyl groups to homocysteine. These reactions are claimed for pea leaves and spinach leaves but both statements are unsupported by data. There has been some speculation (Woods, *et al.*1965) that vitamin B_{12} may be a coenzyme in higher plants. However, while there is some conflicting evidence, the data of Bowen, *et al.* (1962) indicate that vitamin B_{12} is an unlikely normal constituent of higher plant tissues.

(2) Homocysteine dependent reactions where the methyl group is not derived from the THFA pool.

In this category *de novo* synthesis of methyl groups cannot occur since the methyl donor originally derived its methyl groups from

methionine. Choline (reaction 6) has been shown as a methyl donor in animal tissues (du Vigneaud and Rachele, 1965) and in plant tissues (Cromwell and Rennie, 1954).

Choline + homocysteine ⟶ methionine + dimethyl-aminoethanol (6)

Furthermore, all tissues examined to date have the ability to use SAM as a methyl donor for methionine synthesis (reaction 7).

SAM + homocysteine ⟶ methionine + SAHC (7)

In addition, microorganisms and higher plants are able to use both SMM and DPT (Guest, *et al.* 1962; Kisliuk, 1963; Abrahamson and Shapiro, 1965; and Turner and Shapiro, 1961), as a source of methyl groups for methionine formation (reactions 8 and 9).

SMM + homocysteine ⟶ 2 methionine (8)

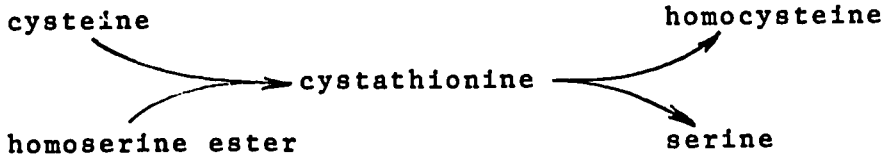
DPT + homocysteine ⟶ methionine + β-thiomethyl-propionic acid (9)

All available evidence, (Mudd and Cantoni, 1965) suggests that SMM, SAM and DPT are the only significant methyl donors in plants, except for the possibility that the primary synthesis of methionine occurs from the THFA pool. The significance of dimethylpropiothetin as a methyl

donor is, however, uncertain, although it is of frequent occurrence in marine plants (Challenger, *et al.* 1957). The central role of methionine in carbon-1 metabolism is obvious since the major methyl group donors, namely SAM, SMM and DPT, all derive their methyl groups from methionine. However, it would appear that an apparent paradox exists, for if methionine is essential for synthesis of the methyl donor, then no net synthesis of methionine can result when these donors methylate homocysteine. Clearly for net synthesis of these methylated compounds, the necessary methyl groups would have to be derived from compounds such as $5CH_3H_4PteGlu$.

The Origin of Homocysteine

One of the principal biochemical roles of homocysteine is as a precursor of methionine. In higher plants, fungi and bacteria, homocysteine is formed as a result of transsulphuration reactions (Thompson, 1967). The first steps in homocysteine formation involve the progressive reduction of inorganic sulphur to form cysteine, using energy derived from hydrolysis of ATP. The sulphur moiety from cysteine is transferred to homocysteine as shown in reaction 10.



(10)

Transsulphuration was the term originally applied to the transfer of the sulphur atom from homocysteine to cysteine in animals. The term has logically been extended to

include the reverse transfer which occurs in organisms which are autotrophic for sulphur. Transsulphuration is unidirectional in plants, leading to formation of homocysteine. Fungi, although they are autotrophic for sulphur, can carry out transsulphuration in both directions. The synthesis of cystathionine in plants proceeds when a homoserine ester, (o-acetylhomoserine or o-succinylhomoserine) is present, but not with homoserine alone (Thompson, 1967). While the pathway for homocysteine synthesis in plants is via transsulphuration, homocysteine may also be formed from SAHC in yeast (Duerre, 1968).

Where SAM is involved in transmethylation reactions it is generally agreed that SAHC is formed (reaction 7). Although earlier workers suggested that SAHC would be remethylated to form SAM, (Duerre and Schlenk, 1959; Pigg, Spence and Parks, 1962), this reaction has not been demonstrated *in vitro*. In 1962, Duerre showed the presence of an enzyme in three different bacteria which actively cleaved SAHC forming adenine and ribosylhomocysteine. However, the pathway for SAHC degradation in yeast is different, for Duerre (1968) showed that *Saccharomyces cerevisiae* contained an enzyme which degraded SAHC, forming homocysteine and adenosine. The resulting homocysteine could then serve as a methyl acceptor to form methionine. The fact that the primary methyl acceptor, homocysteine may be regenerated when SAM participates in transmethylation reactions points to a very close relationship between trans-

methylation and sulphur metabolism.

Methionine Biosynthesis and Transmethylation

The main nutritional source of methionine in animal metabolism is plant protein and it seems important that the mechanism of methionine biosynthesis in plants be investigated to clarify the pathways involved. There have been many studies on methionine biosynthesis in animals and microorganisms, but relatively few in higher plants. It has been established that there are at least five methyl donors that are able to methylate homocysteine to form methionine. While it is clear that *de novo* synthesis of methyl groups can only occur from the THFA pool, it is not clear why there should be at least four sulphonium compounds which are also active in this synthesis. The reason for this may be a necessity for using methyl groups from different pathways. However, before this enigma is solved, more detailed enzyme studies are needed. To date there have been no studies where an enzyme has been examined for its ability to transfer methyl groups derived from all of the three methyl donors, SMM, SAM and 5-methyltetrahydrofolate. In higher plants at least, these may be considered as the most important methyl donors. In marine algae, DPT may also be of importance in this synthesis, (Challenger, *et al.* 1957).

Enzymes capable of catalyzing transfer of methyl groups to homocysteine have been examined by a number of workers. For example, Shapiro, *et al.* (1964) using a 500-fold purification of an enzyme from *Torulopsis utilis* and *Saccharomyces*

cerevisiae showed that the enzyme was highly specific for homocysteine as the methyl acceptor and that SMM and SAM were equally effective as donors. Abrahamson and Shapiro (1965), using a partially purified enzyme (250-fold) from jack bean meal, showed that SMM, SAM and DPT were effective donors of methyl groups. Taylor and Weissbach (1967) partially purified (290-fold) an enzyme from *Escherichia coli* and showed that it catalysed the transfer of methyl groups from methyl-B_{12}, SAM and 5-methyltetrahydrofolate. The latter two required a reducing system for transmethylation to occur.

Of these studies only those of Shapiro, *et al.* (1964) presented unequivocal evidence that one enzyme was active with two substrates. From their data, the relative activity with both SMM and SAM as methyl donors, remained constant throughout a variety of purification steps. There is some indication that methionine may control the operation of the homocysteine methyl transferases by end product inhibition (Balish and Shapiro, 1967), but clearly further detailed studies are needed before the relative importance of these methyl donors in methionine biosynthesis can be assessed.

The Present Investigation

In recent years a great deal of attention has been focused on methionine biosynthesis in microorganisms and mammalian liver (Elford, *et al.* 1965). Although the major nutritional source of methionine for animals is derived from plant protein, relatively few studies have been conducted on

the mechanism of methionine production in plants. Studies that have been made, have not clarified the metabolic roles of the four potential methyl donors in plants. Certainly the most important methyl donor is SAM (Mudd and Cantoni, 1964), and yet no studies have been made which show the content of this compound in metabolically active tissues. In fact, there are few reported assays of the SAM contents of any tissues and only one report of the levels of SAHC (Salvatore, Zappia and Shapiro, 1968). Preliminary studies were, therefore, aimed at a quantitative estimation of the SAM content of pea seeds during the early stages of germination. Pea seeds were selected for the present work, as various aspects of the C_1 metabolism of this tissue have been examined in this laboratory. (Cossins and Sinha, 1965; Roos, Spronk and Cossins, 1968; Wong and Cossins, 1966). In addition, the levels and metabolism of amino acids in pea seedlings are well documented (Lawrence and Grant, 1963; Larson and Beevers, 1965). The close biochemical relationships between SAM and SAHC made it necessary to measure tissue contents of both compounds in order to obtain an estimate of their net synthesis during germination.

The fate of the SAM skeleton (as SAHC) after transmethylation is also uncertain. It was hoped that feeding experiments, carried out under *in vitro* and *in vivo* conditions would provide data which would either support or refute the theory of Duerre and Schlenk (1962), that SAHC is remethylated to form SAM. Experiments were also conducted

with partially purified cell free extracts, in order to study enzyme catalysed reactions of importance in the synthesis and turnover of SAM.

MATERIALS and METHODS

Chemicals

S-methyl cysteine, D,L-homoserine and L-methyl methionine sulphonium iodide were obtained from the California Corporation for Biochemical Research, California, U.S.A. D,L-homocysteine thiolactone and potassium borohydride were obtained from Sigma Chemical Company, St. Louis, Missouri, U.S.A.All other amino acids, bases and nucleosides were purchased from Nutritional Biochemicals Corporation, Cleveland, Ohio, U.S.A. Sephadex gels and dextran blue were obtained from Pharmacia Canada Ltd., Montreal. Scintillation grade 2,5-diphenyloxazole (PPO) and 1,4-bis-[2-(5-phenyloxazolyl)] benzene (POPOP) were purchased from Nuclear-Chicago, Des Plaines, Illinois. Special enzyme grade ammonium sulphate was obtained from Mann Research Laboratory Inc., New York. All other chemicals were obtained from Fisher Scientific Company, Edmonton. In every instance the chemicals used were of highest purity available. All labelled compounds were obtained from the Radiochemical Centre, Amersham, England.

Preparation of Compounds

The following compounds were not available commercially and were, therefore, prepared using the following methods. The method of Kereztesy and Donaldson (1961) was used to prepare 5-methyltetrahydrofolate. This chemical synthesis involved the addition of formaldehyde to tetrahydrofolic acid at neutral pH. The product of this reaction 5,10-methylenetetrahydrofolate, was then reduced to 5-methyl-

tetrahydrofolate using potassium borohydride as a reducing agent. The concentration and purity of the 5-methyltetrahydrofolate produced, were determined by column chromatography of samples on DEAE-cellulose columns (Sotobayashi, *et al.* 1966) and by assaying the fractions with *Lactobacillus casei* (A.T.C.C.7469), and *Streptococcus faecalis* (A.T.C.C.8043) by the "aseptic plus ascorbate" technique of Bakerman (1961). Growth was measured by titration of the lactic acid produced after 70 hours of incubation at 37°C. Figures 2 and 3 illustrate the radiochemical purity and microbiological growth response given by this compound when prepared from formaldehyde-^{14}C. The differential microbiological assay confirmed that only the 5-methyltetrahydrofolate derivative was present, since this compound supports the growth of *L. casei* but not the growth of *S. faecalis* (Stockstad and Koch, 1967). The data shown in Figure 2 are similar to those of Roos, *et al.* (1968), which clearly showed that 5-methyltetrahydrofolate is collected in fractions 62-66 under these conditions. Radioactivity was confined to 5-methyltetrahydrofolate, apart from a small contamination from formaldehyde-^{14}C and one of its labelled polymers (Figure 3).

Methyl-^{14}C-methionine sulphonium was prepared by chemical synthesis from methyl-^{14}C-iodide and L-methionine using the method of Toennies and Kolb (1945). After vacuum distillation of the reaction products, methylmethionine sulphonium was separated and purified by recrystallization

FIGURE 2

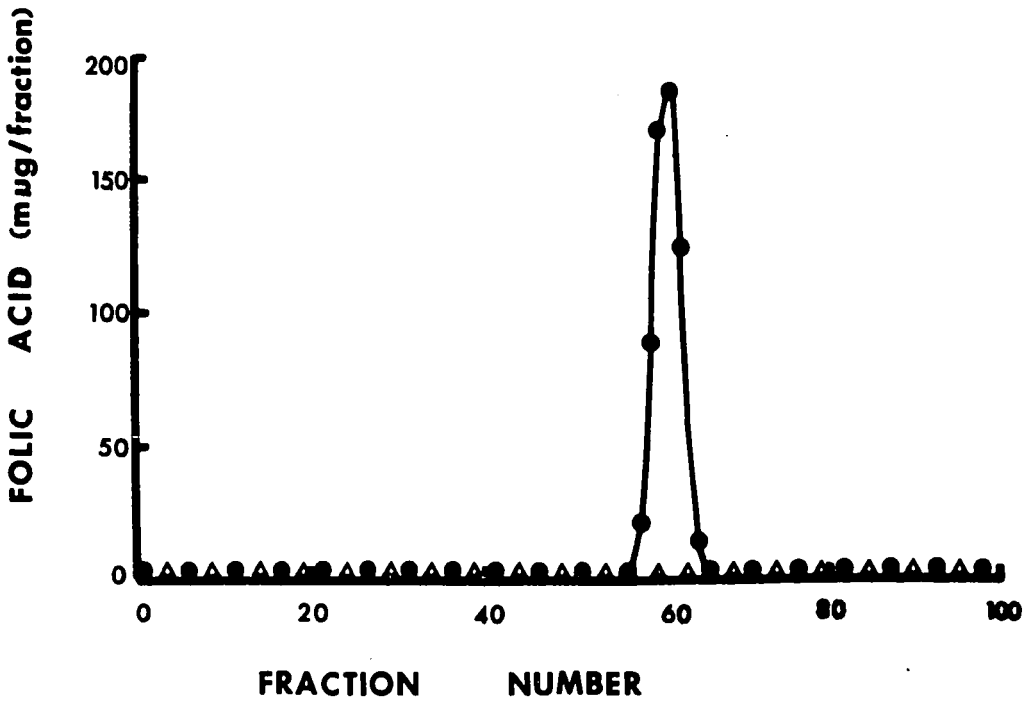
Chromatography of 5-methyl-^{14}C-tetrahydrofolate on DEAE-cellulose

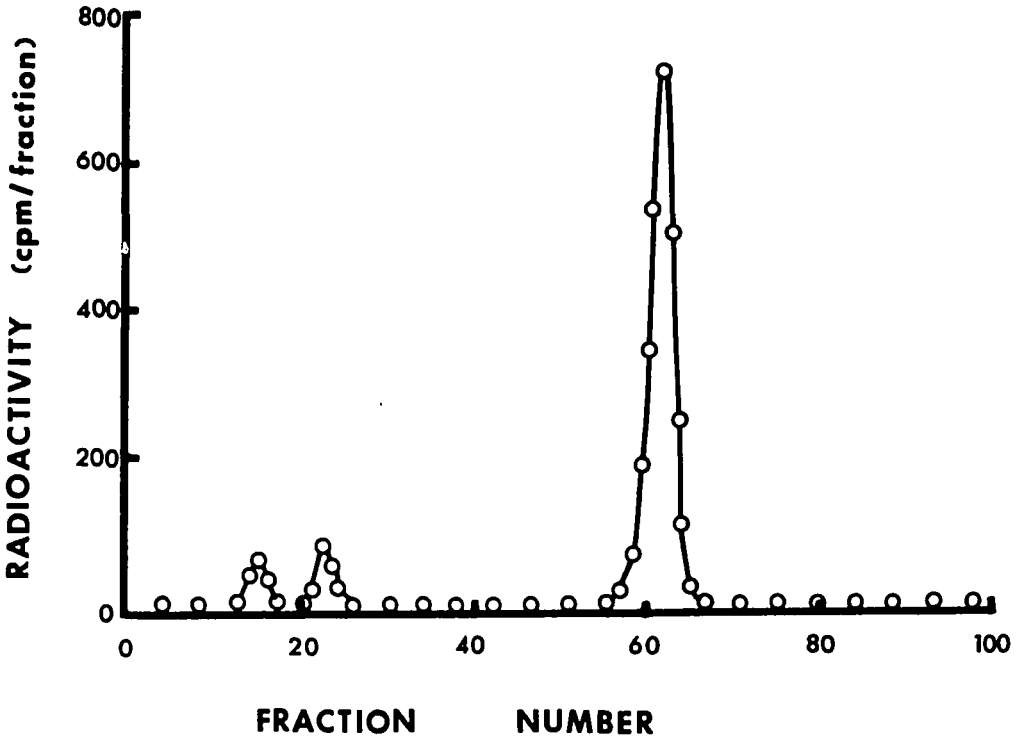
Preparations of 5-methyl-^{14}C-tetrahydrofolate were passed through the DEAE-cellulose column and the fractions (3 ml) were assayed with *L. casei* (● - ●) and *S. faecalis* (Δ - Δ).

FIGURE 3

Radiochemical purity of 5-methyl-^{14}C-tetrahydrofolate determined by chromatography on DEAE-cellulose

The same preparation which was assayed microbiologically (Figure 2) was assayed for radioactivity by counting an 0.1 ml aliquot from each 3 ml fraction by liquid scintillation. The main peak of radioactivity was found in the fractions which contained 5-methyltetrahydrofolate. Two minor contaminants (tentatively identified as formaldehyde-^{14}C and one of its polymers) amounted to 3% of total radioactivity applied to the column. Thus the folate positively identified as 5-methyltetrahydrofolate was radiochemically 97% pure.


FOLIC ACID (mμg/fraction)
200
150
100
50
0
0
20
40
60
80
100
FRACTION NUMBER


RADIOACTIVITY (cpm/fraction)
800
600
400
200
0
0
20
40
60
80
100
FRACTION NUMBER

from 80% (v/v) ethanol. The resulting product had a high degree of purity as determined by co-chromatography with authentic SMM in two solvent systems, n-propanol:water (70:30 v/v) and ethanol:acetic acid:water (65:1:34 by volume). Autoradiography showed that greater than 99% of total radioactivity recovered, was present in SMM.

Although isotopically labelled SAM (^{3}H and ^{14}C) of high purity was readily available, commercial samples of reagent grade SAM were found to be of variable quality. Considering this problem of quality, and the fairly large quantities of SAM required in this study, it was necessary to prepare this compound in the laboratory. The method used (Schlenk and Depalma, 1953) takes advantage of the fact that yeast, grown in the presence of methionine will accumulate large quantities of SAM in the vacuole. Bakers' yeast was incubated at 30°C for 48 hours in the medium used by Schlenk and Depalma (1953). After completion of the culture period, a perchloric acid extraction removed SAM which was then purified by phosphotungstic acid precipitation and recrystallization (Schlenk and Depalma, 1953). SAM labelled with ^{35}S was not available commercially and attempts to prepare this compound from yeast were unsuccessful, so the following method was used. Five dry pea seeds were allowed to imbibe 1 mCi of L-methionine-^{35}S (specific radioactivity 112 µCi/µmole) in 0.2 ml of distilled water. After the radioisotope had been taken up, three more aliquots of 0.2 ml of distilled water were added to prevent the seeds from drying out. Following

complete imbibition, the seeds were germinated in moist vermiculite in the dark at 25°C. After 48 hours, ^{35}S labelled SAM was extracted from the seeds using perchloric acid, followed by column chromatography as described by Shapiro and Ehninger (1966). The labelled product was finally purified by phosphotungstic acid precipitation and thin layer chromatography as described below. Although the yield of ^{35}S-SAM was only 18% in terms of L-methionine-^{35}S fed, the purity by autoradiography was always at least 99% and the specific radioactivity was quite high (10 µCi/µmole).

S-adenosylhomocysteine was prepared by the method of Duerre (1962), which involves the enzymic condensation of adenosine and homocysteine. The condensing enzyme was extracted from two fresh rat livers, by homogenizing with 3 volumes of 0.01 M acetic acid. The supernatant solution obtained after centrifuging the homogenate was stored at -15°C and used as a source of the condensing enzyme (de la Haba and Cantoni, 1959). Adenosine (4.0 mgm) and L-homocysteine (8.1 mgm) were buffered at pH 6.5 with 0.1 M potassium phosphate in an atmosphere of nitrogen. Under these conditions 0.5 ml of the condensing enzyme catalyzed the production of SAHC without detectable formation of the corresponding sulphoxide. The SAHC was purified by chromatography on Dowex 50W-X8 (H^+ form) resin, using the method mentioned below. Precipitation by phosphotungstic acid, and separation with organic solvents (Schlenk and Depalma, 1952) gave a product with the same absorption spectrum, and

characteristics on thin layer chromatography as given by Duerre (1962) for SAHC.

Plant Material

Pea seeds (*Pisum sativum* L. var. Homesteader) were obtained from Steele Robertson Ltd., Edmonton. When the material was being grown for enzyme studies, the seeds were soaked in deionized water for 24 hours. When tissue was required for assays of SAM and SAHC contents, or for feeding experiments, the seeds were planted in moist vermiculite and germinated in the dark at 25°C for periods of up to 4 days. Under these conditions the approximate stage of germination on each day can be described as follows:

1-day-old - seeds fully imbibed, no marked growth of embryos

2-day-old - radicles emerging and up to 1 cm long

3-day-old - radicles 1-2 cm long, epicotyls emerging

4-day-old - radicles 3-4 cm long, epicotyls 0.5-1 cm long

Preparation of Ion Exchange Resins

Analytical grade cation exchange resin, Dowex 50W-X8 (H^+ form) 100-200 mesh and anion exchange resin, AG1-X10 (Cl^- form) 200-400 mesh were obtained from Bio Rad Laboratories, Richmond, California. Before use, the cation exchange resin was made into a thin slurry using deionized water and the fines were removed by suction. This operation was repeated a number of times. The wet resin was then washed in an Erlenmeyer flask with 6N H_2SO_4. (Four

washes of 100 ml of acid were used for each 100 ml of wet resin). After acid washing, the resin was rinsed several times with deionized water, before being packed into a large column (approximately 30 x 2.2 cm). It was found to be necessary to carry out the acid and water washes in flasks to avoid the shattering of columns caused by rapid changes in resin volume. The resin was then washed with deionized water until the pH of the effluent was at least 5.0. Resin required in the sodium form was washed with 800 ml of 4N NaCl followed by 200 ml of 0.1 N NaCl, (these volumes were necessary to convert 100 ml of wet resin from the H^+ to Na^+ form). The converted resins were then stored at 4°C until required.

Resin required in the lithium form was prepared from Dowex 50W-X8 (H^+ form) by washing each 50 ml of resin with 150 ml of 2N LiOH in a 30 x 2.2 cm column. The column was then washed with 150 ml of deionized water and the resin was stored at 4°C until required.

Chloride resin was washed in deionized water and the fines were removed by suction. The resin was then stored at 4°C until required.

Method for Assay of S-adenosylmethionine and S-adenosylhomocysteine

The column chromatographic method used was basically that of Shapiro and Ehninger (1966). The method involved sequential elution of the nucleosides from Dowex 50W-X8 resin in the Na^+ and H^+ forms using H_2SO_4. In later exper-

washes of 100 ml of acid were used for each 100 ml of wet resin). After acid washing, the resin was rinsed several times with deionized water, before being packed into a large column (approximately 30 x 2.2 cm). It was found to be necessary to carry out the acid and water washes in flasks to avoid the shattering of columns caused by rapid changes in resin volume. The resin was then washed with deionized water until the pH of the effluent was at least 5.0. Resin required in the sodium form was washed with 800 ml of 4N NaCl followed by 200 ml of 0.1 N NaCl, (these volumes were necessary to convert 100 ml of wet resin from the H^+ to Na^+ form). The converted resins were then stored at 4°C until required.

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iments the method was modified to use HCl instead of H_2SO_4 as the eluting acid. Using this modification the eluting acid was completely removed *in vacuo* at 40°C. The residue was then taken up in distilled water, allowing the radioactivity of aqueous aliquots to be counted by liquid scintillation without the high degree of quenching normally encountered with strongly acidic solutions.

For the assay of SAM and SAHC contents of germinating pea seeds, approximately 15 gm of tissue were ground in a pestle at 4°C with 5 ml of 1.5 N perchloric acid and a small quantity of acid-washed sand. After centrifugation at 10,000 x g for 10 minutes the residue was washed with about 10 ml of ice cold distilled water. The pH of the combined supernatants was then adjusted to 6.3 by addition of solid $KHCO_3$. A further centrifugation at 10,000 x g for 10 minutes prepared the extract for ion exchange chromatography. The supernatant was divided into two equal parts each of which was added to columns of Na^+ and H^+ resins respectively.

Violent bubbling resulted when perchlorate extracts were added directly to H^+ columns, so after centrifugation the extracts were placed in 125 ml Erlenmeyer flasks with 6 ml of wet resin and agitated at intervals for 20 minutes at 4°C. The extract and resin were then poured into columns (1.2 cm diameter) containing a 5 mm layer of glass beads, (Virtis homogenizing beads) overlaying a small glass wool plug. Experiments with this type of column showed recover-

ies of 95%-106% for SAM and SAHC with H^+ resin and 94%-101% for SAM with Na^+ resin. Elution experiments with plant extracts (Figure 4) showed that 100 ml of each elutant was sufficient to remove any compounds displaced from the H^+ column. The elution pattern of plant extracts on the Na^+ column is similar to Figure 4 except that SAHC is recovered in 2N fraction.

Based on the information from Figure 4, columns containing the extracts were routinely eluted at room temperature with 100 ml of deionized water, followed by 100 ml of 2N acid and finally 100 ml of 6N acid. SAM was recovered in the 6N fraction from both Na^+ and H^+ column. The quantities of SAM and SAHC present in the column effluents were assayed spectrophotometrically using a Beckman DB-G spectrophotometer. The quantity of SAHC was then calculated by difference. The molar extinction coefficients for SAM and SAHC in 6N acid eluates were taken as Em=14,700 at 260 nm. (Shapiro and Ehninger, 1966).

Sephadex Column Chromatography

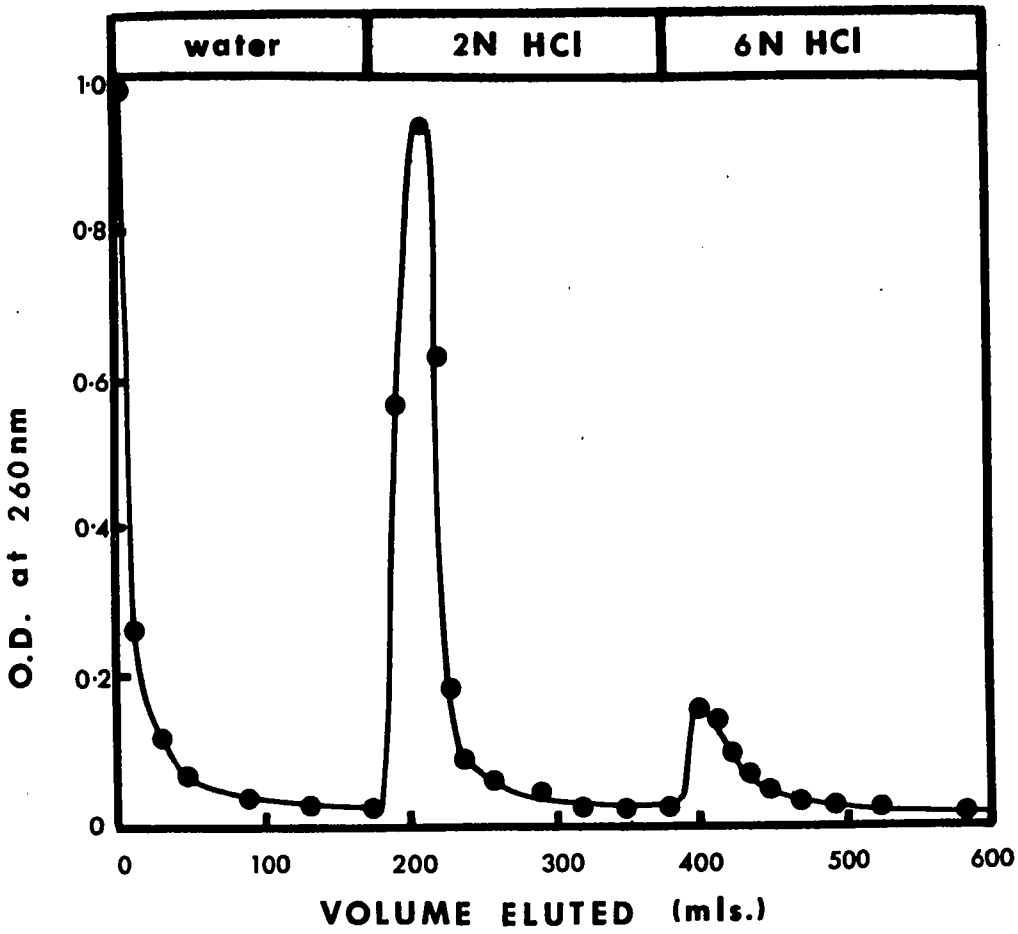
Sephadex G-10 was suspended in distilled water and boiled for 1 hour with stirring. The fines were removed by decantation and the gel was then placed *in vacuo* to remove air bubbles and packed into a column by gravity to give a final bed of 1.8 x 40 cm. The Sephadex was washed with 200 ml of 0.01 M potassium phosphate buffer at a pH of 6.9 and stored at 4°C until required.

Sephadex G-50 was soaked at room temperature in 0.05 M

FIGURE 4

The elution pattern of pea extracts on Dowex 50W-X8 (H^+ form)

A perchloric extract prepared from 1-day-old pea seeds was placed on a Dowex 50W-X8 (H^+ form) column and sequentially eluted with deionized water, 2N HCl and 6N HCl. The 5 ml fractions were scanned for absorption at 260 nm. The 6N fraction was examined on TLC and was found to contain only SAM and SAHC.


water
2N HCl
6N HCl
1·0
0·8
0·6
0·4
0·2
0
O.D. at 260nm
0
100
200
300
400
500
600
VOLUME ELUTED (mls.)

potassium phosphate buffer (pH 6.4) for 20 hours with occasional stirring. After the fines had been decanted, the gel was placed *in vacuo* and then packed into a jacketed column to give a final volume of 1.5 x 60 cm.

Sephadex G-100 was soaked in 0.05 M potassium phosphate buffer (pH 6.4) for 3 days at 4°C with occasional stirring. The fines were removed by decantation, air was removed *in vacuo* and the gel was packed into a jacketed column giving a final resin volume of 2.8 x 51 cm. The Sephadex G-100 column was then stored at 4°C until required.

Pulse Feeding Experiments

10-20 µCi of L-methionine-^{35}S, L-methionine ($^{14}CH_3$) or L-methionine (C^3H_3) were fed to 1-day-old cotyledon slices (approximately 80 gm) which were approximately 2 mm in thickness. The specific radioactivity was usually 5 µCi/µmole and the labelled solution had a total volume of 150 ml. During incubation with the labelled methionine solutions the tissue slices were gently agitated with a stream of filtered air. After 7.5 hours in the feeding solution, the tissue slices were removed, washed in the buffer solution to remove exogenous radioisotope and then placed into 150 ml of unlabelled buffered solution of same pH, ionic strength and containing the same concentration of L-methionine as the original labelled feeding solution. Samples, (approximately 7 gm fresh weight) were taken at intervals and assayed for SAM and SAHC content and for radioactivity as described elsewhere in this thesis.

Preparation of Cell-free Extracts

25 gms of cotyledons from 1-day-old germinating pea seeds were homogenized in a Waring blendor with 50 ml of 0.1 M potassium phosphate buffer (pH 6.4) containing 0.005 M 2-mercaptoethanol. All operations were carried out at temperatures less than 5°C. The homogenate was filtered through cheesecloth to remove cell debris and centrifuged at 12,000 x g for 15 minutes. The supernatant was fractionated with solid ammonium sulphate and protein precipitating in the range of 20%-80% of saturation was collected by centrifugation and dissolved in 4 ml of 0.05 M potassium phosphate buffer (pH 6.9). A 2 ml sample of this solution containing approximately 20 mgm protein was then applied to a Sephadex G-50 column (1.5 x 40 cm) and eluted with 0.05 M potassium phosphate buffer (pH 6.9) containing 0.005 M 2-mercaptoethanol. Most of the protein was eluted from the column in a volume of 10 ml and was used in experiments as the cell-free extract.

Chromatography

All compounds were identified by thin layer chromatography using either silica gel GF_{254} (Merck), or a mixed layer of silica gel GF_{254} and cellulose powder MN300. These materials were obtained from Canadian Laboratory Supplies, Edmonton. The mixed layers were prepared by homogenizing 12 gm of cellulose powder with 6 gm of silica gel GF_{254} in 100 ml deionized water in a Virtis homogenizer for 60 seconds. The layers (300μ thick) were spread on

20 cm x 20 cm glass plates using a Desaga spreader. The layers were allowed to stand on the spreader until surface moisture had disappeared (20 minutes). The plates were then dried horizontally in an oven at 90°C-100°C for one hour, and stored in a cabinet containing a silica gel desiccant.

Silica gel layers were prepared by homogenizing 30 gm of silica gel GF_{254} with 60 ml of deionized water for 60 seconds. Layers (300μ thick) were spread on 20 cm x 20 cm glass plates and dried at 90°C-100°C for one hour. Thicker layers, (500μ) were used to purify SAM and SAHC preparations. After drying, the plates were stored in a cabinet over a desiccant.

It was found that the silica gel GF_{254} was the best medium for detection of nucleosides, bases and other compounds which absorb in the UV region. The mixed layer was also very suitable for autoradiography, as the layer was not readily disturbed upon contact with the X-ray film.

Thin layer chromatography was carried out using a number of solvent systems. The most useful were n-butanol: acetic acid:water (60:15:25 by volume), n-propanol:water (70:30 v/v), ethanol:acetic acid:water (65:1:34 by volume) and n-propanol:ammonium hydroxide:water (70:9:21 by volume). Table I lists the R_f values on silica gel and mixed layers, for the majority of the compounds used in this work. Compounds were visualized by their fluorescence under UV light, or by their reaction with platinic iodide (Toennies and

TABLE I

Values of R_f(X100) for some of the compounds used in this investigation

The compounds were dissolved in water wherever possible and the amount applied to the chromatogram was 0.01-0.05 µmoles.

Solvent 1 = n-butanol:acetic acid:water (60:15:25 by volume)
Solvent 2 = n-propanol:water (70:30 v/v)
Solvent 3 = ethanol:acetic acid:water (65:1:34 by volume)
Solvent 4 = n-propanol:ammonium hydroxide:water (70:9:21 by volume)

	SILICA GEL GF_{254}				MIXED LAYERS			
Solvent-	1	2	3	4	1	2	3	4
SAM	10	01	25	18	19	01	24	17
SAM sulphoxide	2	0	20	14	18	01	34	16
SAHC	37	40	54	53	36	36	45	53
SAHC sulphoxide	20	27	--	40	43	29	47	--
adenosine	57	64	85	71	65	75	79	71
adenine	57	64	82	82	70	77	74	70
methionine	46	57	82	54	61	69	77	65
methionine sulphoxide	21	24	57	52	36	45	70	56
methionine sulphone	33	39	72	56	40	50	70	58
S-methylmethionine	05	02	10	10	17	03	34	17
α-methylmethionine	53	65	87	--	71	76	88	73
homocysteine thiolactone	47	60	72	62	58	75	79	65
homocysteine	15	30	65	48	41	37	46	37
homocystine	26	27	62	47	50	--	--	36
cysteine	14	15	27	32	14	17	27	37
S-methylcysteine	41	51	80	56	53	61	73	65
homoserine	31	60	70	43	43	53	70	50
serine	29	36	66	36	35	45	71	50
cystathionine	15	12	58	25	17	31	--	32
ethionine	55	65	84	56	69	75	83	67
valine	47	56	84	53	63	70	81	67
alanine	33	42	77	37	45	52	70	47

Kolb, 1945), ninhydrin or aniline-xylose sprays (Block, Durrum and Zweig, 1955).

Autoradiography

Labelled compounds were detected on thin layer plates by autoradiography. The thin layer plates were placed in close contact with Kodak No-Screen X-ray film in a light-tight cassette and exposed for periods of up to one week. Areas on the plates containing 600 dpm of ^{14}C could be readily detected after 5 days exposure. The exposed films were developed using G.E. Supermix X-ray developer and fixer (General Electric X-ray Company, Edmonton).

Counting of Radioactive Samples

A 4Π Actigraph (Nuclear Chicago Corp., Des Plaines, Illinois) was used in routine scanning of thin layer chromatograms. Thin layer chromatograms were sprayed with methyl cellulose, (see below) and strips were cut for scanning. In all quantitative work radioactivity was measured in a liquid scintillation counter (Nuclear Chicago Corp., Unilux II model). With aqueous samples a dioxane-based fluor system was used. This fluor contained 12.0 gm of PPO and 0.5 gm of POPOP to each litre of a mixture of dioxane:anisole:dimethoxyethane, (6:1:1 by volume). Usually 100 µlitres of sample were added to 15 ml of fluor. A counting efficiency of approximately 65% was obtained as determined by the channels ratio method.

Mixed thin layer chromatograms were usually used when autoradiography was necessary. After detection of the

radioactive areas by autoradiography, the chromatogram was covered with two coats of 2% methyl cellulose in chloroform: ether (1:1 v/v) and allowed to dry, (Caballero, 1969). The radioactive spots were then cut out with a scalpel blade and placed in a scintillation vial containing 15 ml of a toluene-based counting solution. This counting solution contained 12.0 gm of PPO and 0.5 gm POPOP to each litre of toluene. The toluene had been previously dried by addition of metallic sodium ribbon and redistilled. Counting efficiency by the channels ratio method was 72%. Geometry of the chromatogram did not affect this efficiency.

Estimation of Protein Nitrogen

The protein content of enzyme preparations was estimated colorimetrically using the method of Lowry, *et al.* (1951). Crystalline egg albumin was used as a reference standard.

Methionine Assay

Methionine produced by transmethylation reactions *in vitro* was assayed microbiologically using *Streptococcus faecalis* (A.T.C.C.8043) by the method of Clandinin (1948). Growth was measured by titration of the lactic acid produced after 70 hours of incubation at 37°C. Reference curves were constructed using authentic L-methionine (Figure 5).

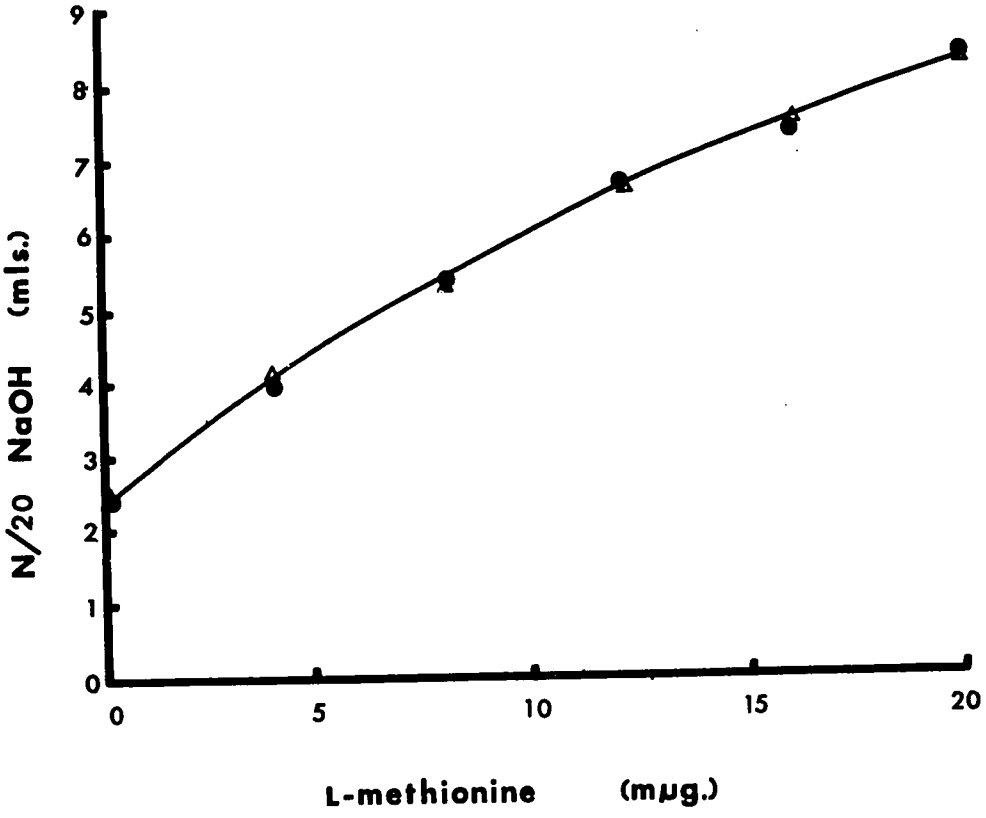
Extraction and Partial Purification of Methyl Transferases Catalysing Methionine Biosynthesis.

All procedures were carried out at 4°C. The testas

FIGURE 5

Standard curve for the microbiological assay of methionine

The curve was constructed using duplicate samples of authentic L-methionine containing up to 20 mμg/ml. Growth of *Streptococcus faecalis* (A.T.C.C.8043) was measured by titration of the lactic acid produced after 70 hours of growth at 37°C.


N/20 NaOH (mls.)
0
1
2
3
4
5
6
7
8
9
0
5
10
15
20
L-methionine (mμg.)

were removed from 1-day-old pea seeds and a 50 gm sample of cotyledons was then homogenized with 100 ml of 0.05 M potassium phosphate buffer (pH 6.9) containing 0.005 M 2-mercaptoethanol, in a Waring blendor for one minute at full speed. The homogenate was squeezed through 8 layers of cheesecloth to remove cell debris. The extract was then centrifuged at 10,000xg for 20 minutes. The supernatant, in a volume of 50 ml, was brought to 20% of saturation, by addition of solid $(NH_4)_2SO_4$ at 4°C. After stirring at 4°C for thirty minutes the precipitated protein was removed by centrifugation for 10 minutes at 10,000xg. The supernatant was brought to 60% of saturation by further addition of solid $(NH_4)_2SO_4$. After stirring for thirty minutes the precipitated protein was removed by centrifugation as before.

This precipitated protein (20-60% fraction) was then dissolved in about 8 ml of 0.05 M potassium phosphate buffer (pH 6.9) containing 0.005 M 2-mercaptoethanol. Half of this volume, containing approximately 60 mg protein, was applied to a Sephadex G-50 column (2.0 x 50 cm) and elution was carried out at 4°C using the same phosphate buffer containing 0.005 M 2-mercaptoethanol. Fractions of 3 ml were collected at a flow rate of 40 ml per hour, the bulk of the applied protein was found in fractions 12-19. These fractions were pooled and an assay for enzyme activity showed that approximately 90% of the initial enzyme activity was recovered from these fractions. The protein present in the pooled fractions was precipitated by addition of sold $(NH_4)_2SO_4$

to achieve 60% of saturation. After stirring for 20 minutes at 4°C the precipitated protein was removed by centrifugation at 10,000xg for 10 minutes. The precipitated protein was dissolved in the same buffer used in previous steps, to give a final volume of approximately 3 ml. Half of this volume, containing approximately 30 mgm of protein, was applied to a column of Sephadex G-100 (2.8 x 50 cm) and eluted with the same buffer as before. Fractions of 3 ml were collected every 20 minutes using a refrigerated Buchler fraction collector. Each tube was assayed for protein content using the method of Lowry, *et al.* (1951) and for methyltransferase activity in reaction systems containing SAM, SMM or 5-methyltetrahydrofolate ($5CH_3H_4PteGlu$) as methyl donors. (See below)

Assay of Methyltransferase Activity

The following reaction systems were used in assaying for homocysteine-dependent methyltransferase activities. The partially purified enzymes were incubated with 4 µmoles of L-homocysteine freshly prepared from the thiolactone, 2 µmoles of the methyl donor and 50 µmoles of potassium phosphate buffer (pH 6.9) containing 5 µmoles of 2-mercaptoethanol, in a total volume of 1 ml. The methionine produced during the reaction was assayed microbiologically using *Streptococcus faecalis* (A.T.C.C.8043) by the method mentioned above.

An alternative method was to use SAM (^{14}C methyl) or $CH_3H_4PteGlu$ (^{14}C methyl) as the methyl donor followed by

assay of the ^{14}C-methionine produced. The standard 0.5 ml assay mixture consisted of 0.1 ml of the enzyme solution, 1 µmole of L-homocysteine and 1 mµmole of the labelled donor (specific radioactivity 1.5×10^5 dpm/mµmole) and 50 µmole of potassium phosphate buffer pH 6.9. The reaction was terminated by rapid cooling in an ice bath and an aliquot of 0.1 ml of the cooled reaction mixture was placed on an ion exchange column. When SAM (^{14}C methyl) was used as the donor, the 0.1 ml aliquot was placed on a column (0.5 x 2.5 cm) of Dowex 50W-X8 resin in the Li^+ form. The Li^+ column was then quantitatively eluted with six washings, each of 0.2 ml distilled water. This method is basically that of Abrahamson and Shapiro (1965) but the elution pattern was modified to ensure quantitative recovery of labelled methionine. The labelled methyl donor, (SAM), was adsorbed by the Li^+ column while the product of the reaction (methionine) was collected with the effluent as illustrated in Figure 6. The column washings were collected in scintillation vials and 15 ml of dioxane scintillation fluid was added. Since thin layer chromatography confirmed that methionine was the only radioactive product present, the amount of radioactivity in the eluates was a direct measure of transmethylase activity.

When $CH_3H_4PteGlu$ (^{14}C methyl) was used as the methyl donor, 0.1 ml of the reaction mixture was placed on a column (0.5 x 2.5 cm) of Dowex AG1-X10 resin in the Cl^- form. The column was eluted with six washings each of 0.2 ml distilled

FIGURE 6

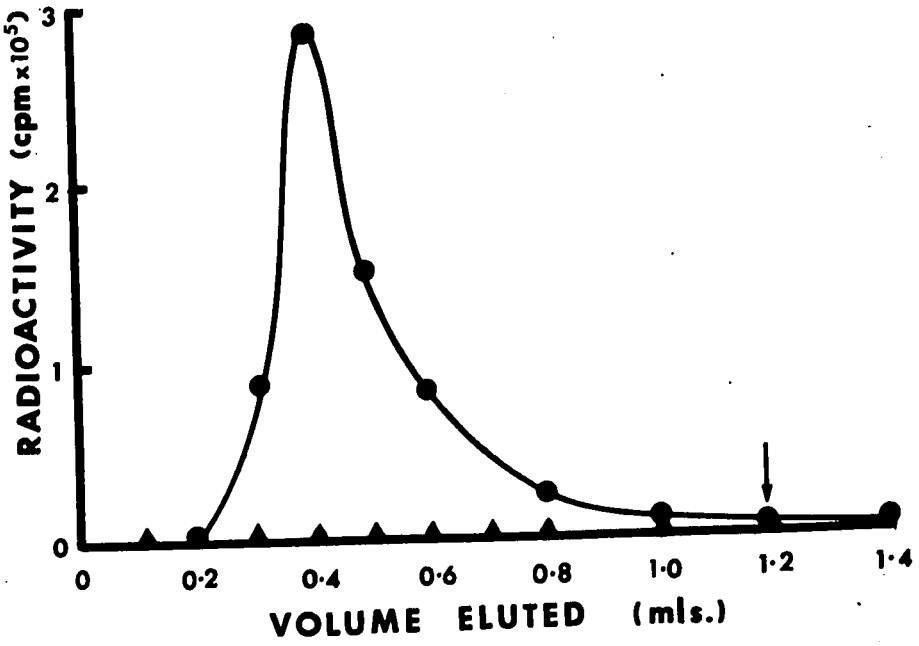
The efficiency of Dowex 50W-X8 (Li^+ form) in recovering the labelled product of transmethylation

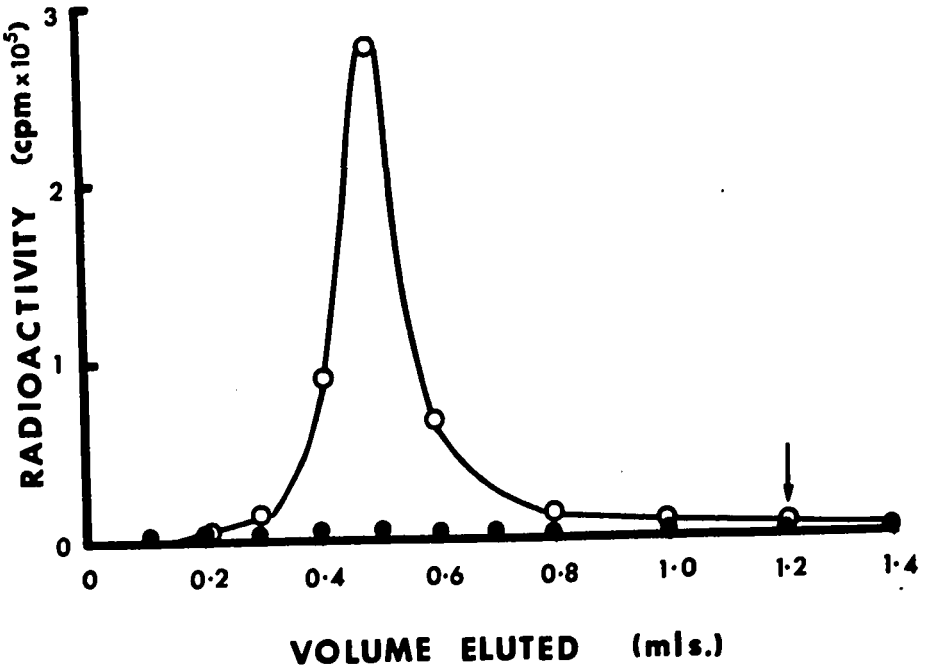
L-methionine ($^{14}CH_3$; 0.3μCi; o - o) and SAM ($^{14}CH_3$; 0.3μCi; ▲ - ▲) were applied to a Li^+ column (0.5 x 2 cm) and elution carried out with aliquots of deionized water. The aliquots were collected in scintillation vials, 15 ml of dioxane based fluor was added and radioactivity was determined by liquid scintillation counting. At the point indicated by the arrow, 99.7% of radioactivity in L-methionine added to the column, had been recovered.

FIGURE 7

The efficiency of Dowex AG1-X10 (Cl^- form) in recovering the labelled product of transmethylation

L-methionine (^{14}C methyl; 0.3μCi; o - o) and $5CH_3H_4$ PteGlu ($^{14}CH_3$; 0.3μCi; • - •) were applied to a Cl^- column (0.5 x 2 cm) and elution carried out using aliquots of deionized water. The aliquots were collected in scintillation vials, 15 mls of dioxane based fluor was added and radioactivity was determined by liquid scintillation counting. At the point indicated by the arrow, 99.4% of radioactivity in L-methionine had been recovered.


RADIOACTIVITY (cpm x 10⁵)
3
2
1
0
0
0·2
0·4
0·6
0·8
1·0
1·2
1·4
VOLUME ELUTED (mls.)


RADIOACTIVITY (cpm x 10⁵)
3
2
1
0
0
0·2
0·4
0·6
0·8
1·0
1·2
1·4
VOLUME ELUTED (mls.)

water. Under these conditions the labelled donor $CH_3H_4PteGlu$ adhered to the Cl^- column while labelled methionine was quantitatively eluted (Figure 7). Thin layer chromatography and autoradiography showed that methionine was the only labelled product of the reaction. After collection of the eluates and addition of 15 ml of dioxane scintillation fluid, the radioactivity in the reaction product was measured by liquid scintillation counting. As with the Li^+ column, the amount of radioactivity in the eluates was a direct measure of transmethylase activity.

A quench curve was used to estimate the efficiency of counting in each sample. This allowed the activity of the enzyme to be calculated from the known specific radioactivity of the methyl donor.

One unit of enzyme activity is defined as the amount of enzyme producing 1 µmole of methionine in 1 minute at 30°C under the reaction conditions specified. Specific enzyme activity is expressed as units of enzyme activity per mg protein.

RESULTS

Levels of SAM and SAHC in Germinating Pea Seeds

There is now ample evidence that the sulphonium compound, S-adenosylmethionine is of prime importance in transmethylation reactions in all organisms investigated so far, as reviewed in the introduction. SAM is an essential methyl donor to important cellular constituents in plants, such as chlorophyll (Radmer and Bogorad, 1967), pectin (Sato, *et al.* 1964) and other methylated compounds (Meister, 1965). However, despite the recognized importance of SAM, little is known of the levels of this compound or of SAHC in higher plant tissues. The only work in this area has been reported by Davies (1966) where it was shown that the pool of SAM in turnip storage tissues was not metabolically active.

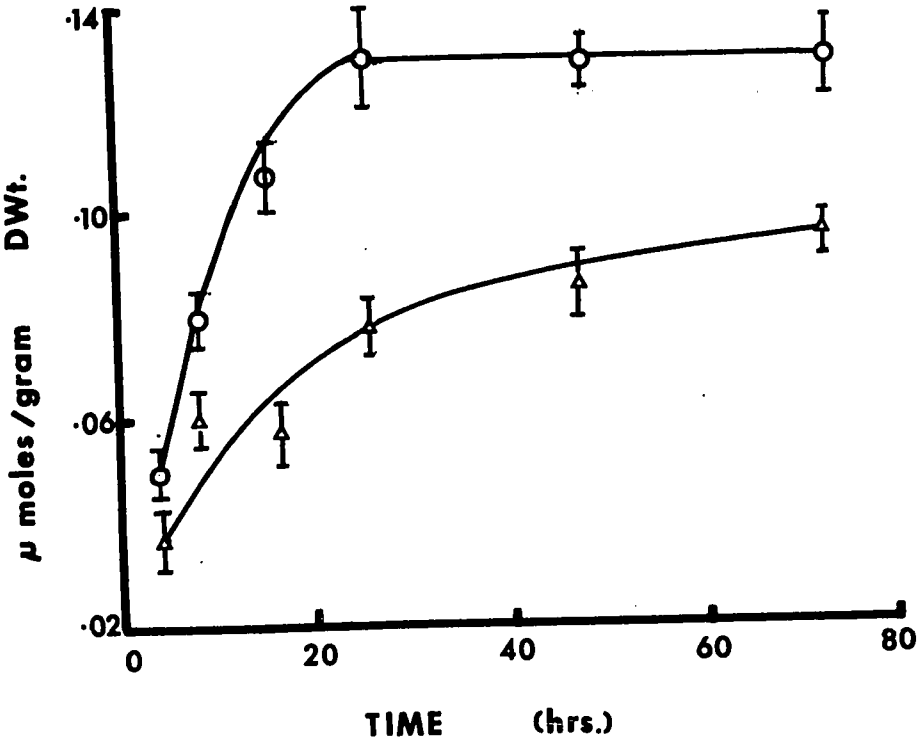
In view of the importance of synthetic reactions in germinating seeds one would expect SAM to play an important role in these reactions and consequently marked changes in the size of the pool of SAM would be expected to occur. Since there is a close metabolic relationship between SAM and SAHC, it is necessary to account for the concentrations of both compounds if one is to show that net synthesis of SAM occurs during germination.

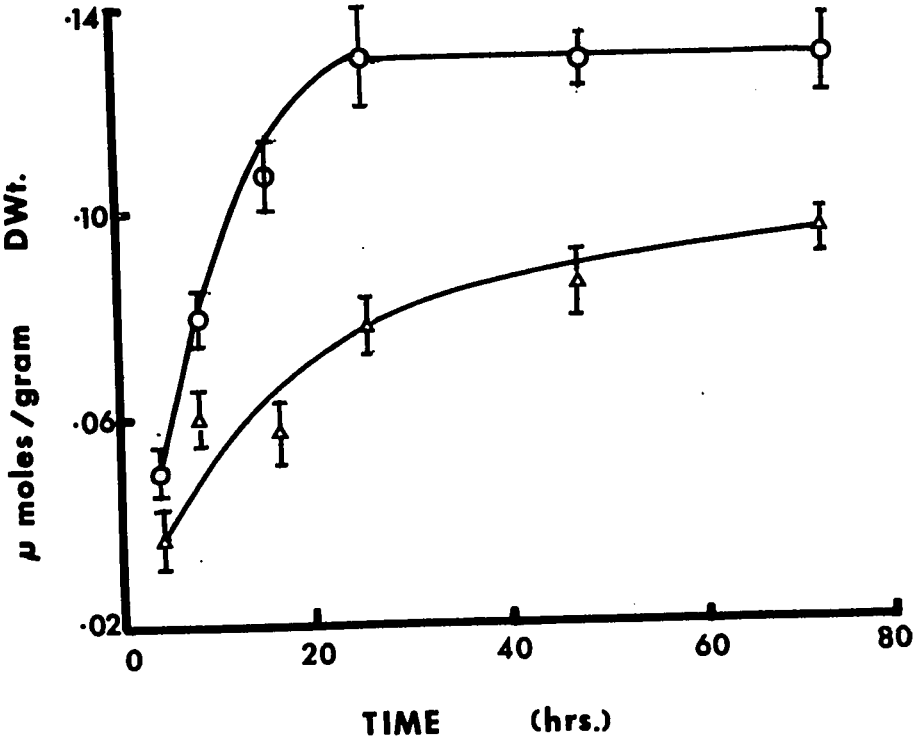
The SAM and SAHC contents of germinating pea seeds were assayed using the methods described in Materials and Methods. Seeds were sampled over a 72 hour germination period. The results of these experiments (Figure 8) show that both the SAM and SAHC contents of pea seeds increase during the

FIGURE 8

Changes in SAM and SAHC content during germination

Pea seeds were germinated at 25°C. The tissues were assayed for SAM (o - o) and SAHC (Δ - Δ) as described in the text. The vertical lines indicate the standard error of the mean, each data point representing the mean of at least six separate determinations.


·14
·10
·06
·02
µ moles / gram DWt.
0
20
40
60
80
TIME (hrs.)


·14
·10
·06
·02
µ moles/gram DWt.
0
20
40
60
80
TIME (hrs.)

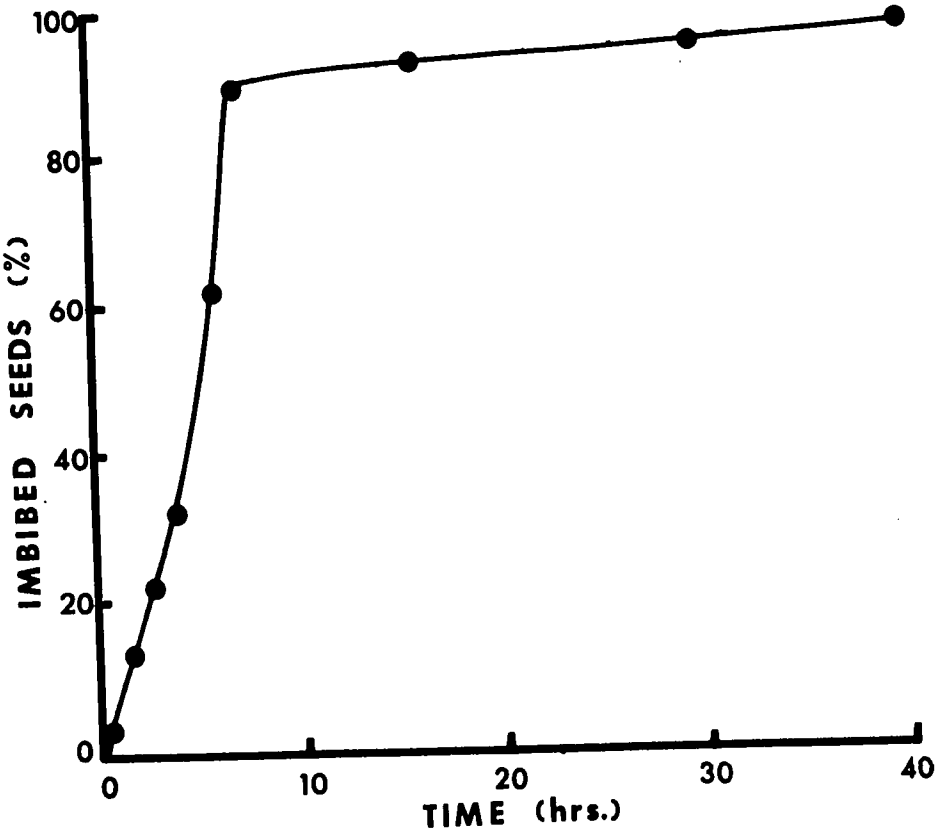
early stages of germination. These results show considerable variation between replicates for levels of both SAM and SAHC. This variation in the levels of SAM and SAHC does not appear to be inherent in the method used, since recoveries of authentic SAM and SAHC, subjected to the experimental procedure, were within the range of 95-106 per cent. This suggested that the observed variation was probably due to variability inherent in the tissue used. One indication of this was that germination and growth of seedlings was rather irregular in samples of seeds used in these experiments.

It appeared likely that some of the variation shown in Figure 8 could be due to the different rates of imbibition of water by dry seeds. To check this possibility, five replicates, each containing 100 seeds were placed in deionized water, and the time for each seed to start imbibing water was recorded. Figure 9 illustrates the results of this experiment. It is clear that there is considerable variation in the ability of individual seeds to imbibe water. These variable rates of imbibition would affect the physiological condition of the tissue sampled and clearly could be a contributing factor to the variation observed in Figure 8. In all subsequent studies, seeds were soaked in deionized water for thirty minutes and seeds which had started to imbibe water by this time, were used as the experimental material. Seeds selected using this method showed considerably more uniform germination than those selected by the earlier procedure. Although the same

FIGURE 9

Percentage imbibition of pea seeds as a function of soaking time at 22°C

Five replicates each containing 100 pea seeds were placed in deionized water at 22°C. The time taken for seeds to start imbibing water, was recorded and plotted as shown.


100
80
60
40
20
0
IMBIBED SEEDS (%)
0
10
20
30
40
TIME (hrs.)

variety of peas (Homesteader) was obtained from one supplier, there were, never the less, distinct differences in rate of water uptake between one line of seed and another.

It was convenient during this study, to have a factor available to interconvert fresh weight and dry weight values. The data in Figure 10 show the rate of uptake of deionized water by pea seeds. Since the increase in weight during water uptake is for 1 gm of dry seeds, these values may be used directly as a factor for interconverting dry and fresh weights at any stage during germination. Using this factor, the data from Figure 8 may be converted to a fresh weight basis and in this form the levels of SAM and SAHC in germinating pea seeds and in other tissues may be compared.

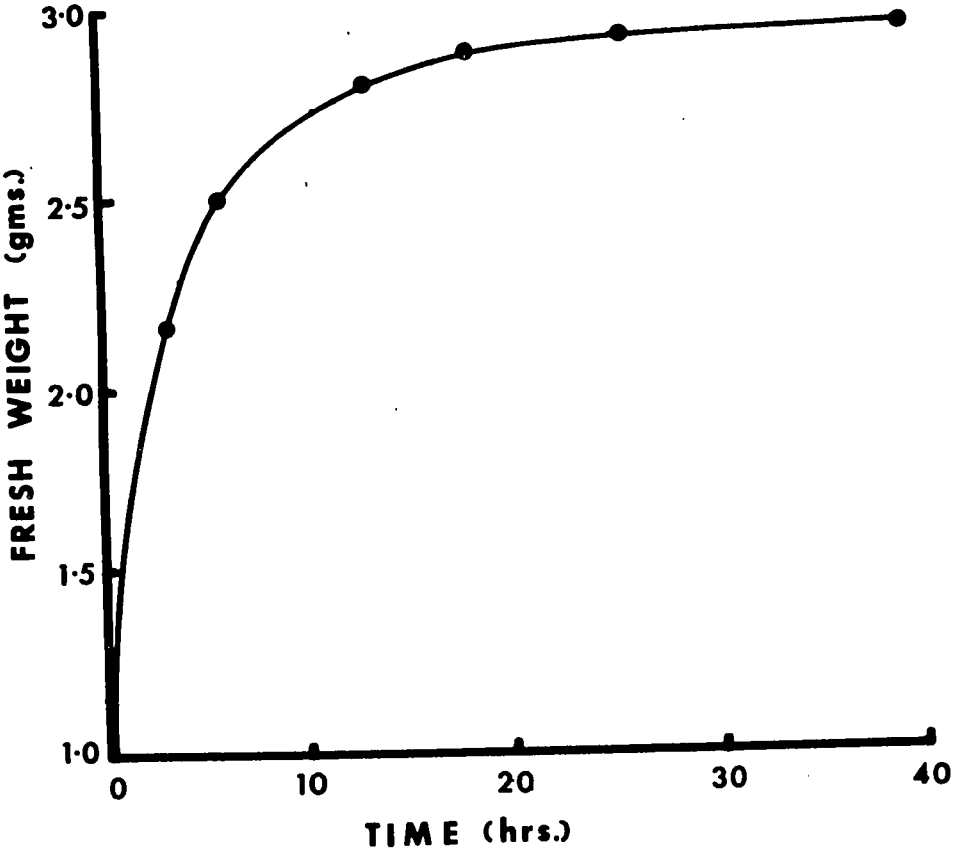
The Pathway of SAM Synthesis in Pea Seeds

As outlined in the introduction, a number of different mechanisms for the biosynthesis of SAM have been demonstrated. Several of these involve the methylation of SAHC but do not appear to be applicable in germinating pea seeds, since the pool of SAHC is very small initially (Figure 8) and therefore, would be unlikely to account for the net synthesis of SAM. Other mechanisms for SAM synthesis, not involving the participation of SAHC, have been shown. For example, Schlenk and Ehninger (1964) showed that in the yeast *Candida utilis*, SAM was formed from methylthioadenosine and a 4-carbon amino acid, tentatively identified as homoserine. Cantoni and Durell (1957) and Mudd and Cantoni (1958) established that SAM was formed by the methionine activating

FIGURE 10

Increase in fresh weight as a function of soaking time

Pea seeds were soaked in deionized water at 22°C. Of the seeds which started to imbibe water during the first five minutes, 10 gm were transferred to another vessel of deionized water. Their increase in weight was observed for the first 40 hours of germination.


3·0
2·5
2·0
1·5
1·0
FRESH WEIGHT (gms.)
0
10
20
30
40
TIME (hrs.)

enzyme from ATP and methionine.

In order to determine the pathway of SAM biosynthesis in germinating pea seeds, feeding experiments were conducted with tissue slices and with cell-free extracts. Both methionine-$^{14}CH_3$ and methionine-^{35}S showed equal incorporation into SAM when fed to either tissue slices or cell-free extracts and the results of these experiments were pooled to give the data presented in Tables II and III. It is clear that there is a rapid incorporation of methionine into SAM, both *in vitro* and *in vivo*. From Table III it can be seen that ATP:L-methionine S-adenosyltransferase is the enzyme implicated because formation of SAM is dependent on presence of ATP and Mg^{2+}. On the basis of these feeding experiments it is conceivable that the bulk of endogenous SAM in pea seeds is formed by the activation of methionine.

Feeding of uniformly labelled homoserine-^{14}C (specific radioactivity 1 µCi/µmole) to one-day-old pea cotyledon slices in potassium phosphate buffer, (0.1M and pH 6.4) showed little incorporation of ^{14}C into SAM or SAHC. This result is perhaps not surprising as Lawrence and Grant (1963) showed that homoserine is not present at high levels in pea seeds until the third day of germination and, furthermore, Larson and Beevers (1965) concluded on the basis of homoserine-^{14}C feeding experiments that this compound is not readily metabolized. Also MTA was not detected during ion exchange column chromatography and it is concluded that a significant formation of SAM from homoserine and MTA is

TABLE II

Synthesis of SAM by pea cotyledon slices

Incubation Time (hrs.)	^{35}S incorporated into SAM (dpm/gm F.wt.)	Specific Activity SAM-^{35} (μCi/μmole)	$^{14}CH_3$ incorporated into SAM (dpm/gm F.wt.)	Specific Activity SAM-$^{14}CH_3$ (μCi/μmole)
0.5	18,000	0.20	16,500	0.18
1	36,000	0.41	35,500	0.40
2	69,000	0.78	70,000	0.79
3	100,000	1.14	93,000	1.06
5	154,000	1.75	156,000	1.77
7	178,000	2.02	175,000	1.99

Methionine-$^{14}CH_3$ or methionine-^{35}S (specific radioactivity 2.2 μCi/μmole) was fed to 60 gm of 1-day-old cotyledon slices (2mm thick) in 0.1M potassium phosphate buffer (pH 6.9), and incubated at 30°C. Samples (about 10 gm) were taken at the times indicated and assayed for radioactivity and for content of SAM.

TABLE III

Synthesis of SAM by cell-free extracts

*Omission from reaction system	SAM formed (µmoles)
None	0.72
ATP	0.02
Mg^{++}	0.40
2-mercaptoethanol	0.70
cell-free extract	nil

*The complete assay system contained 8 µmole L-methionine (methyl ^{14}C) specific activity 100,000 dpm/µmole, 0.4 ml cell-free extract (containing 300 µgm protein) 8 µmole ATP, 5 µmole of 2-mercaptoethanol and 100 µmole of potassium phosphate buffer (pH 6.4) in a total volume of 2 ml. The reaction mixtures were incubated for 90 minutes at 30°C.

unlikely in germinating pea seeds.

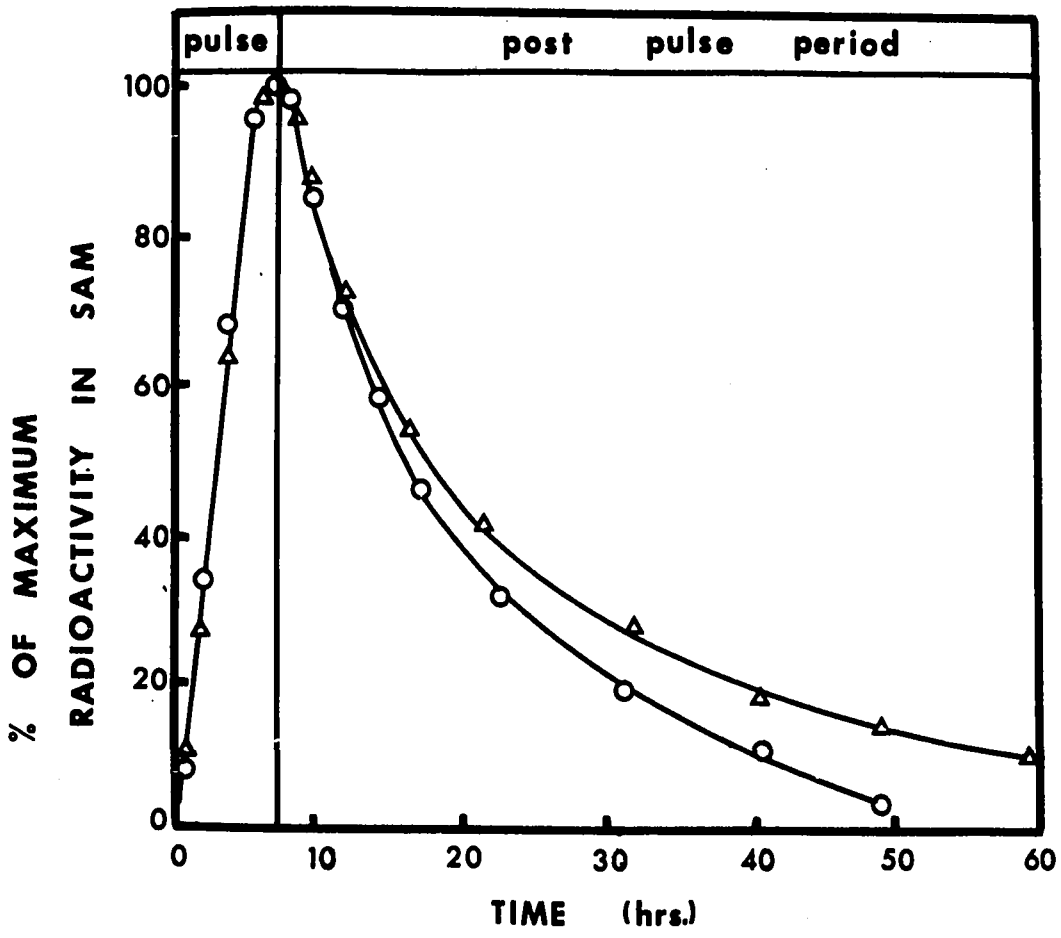
Synthesis and Turnover of Labelled SAM in Cotyledon Slices

Davies (1966) has shown that the SAM present in turnip storage tissue was metabolically inactive. It was therefore, of interest to determine the metabolic state of SAM in pea seeds. Further experiments were designed to determine the rates of turnover of the SAM pool during germination. Labelled methionine was pulse fed to cotyledon slices from one-day-old pea seeds using the methods outlined earlier. One-day-old pea seeds were selected because SAM production reached a maximum at this stage during germination (Figure 8). S-adenosylmethionine was rapidly labelled (Figure 11), and after 7 hours had a specific activity approaching that of the methionine fed. When such tissue slices were transferred to an unlabelled methionine solution (post pulse period), the radioactivity in the SAM pool was rapidly depleted (Figure 11). This loss was most pronounced when the methyl group of SAM was labelled with either ^{3}H or ^{14}C. In experiments using ^{35}S labelled SAM, the decline in activity during the post pulse period was less marked. This loss of radioactivity could be due to metabolic turnover of SAM or to loss of this compound to the external solution. The latter possibility was examined by measuring the levels of labelled SAM in the external solution at various times during the post pulse incubation period. It was found that such radioactivity accounted for not more than 5% of the radioactivity lost from SAM. Although this loss from the

FIGURE 11

Synthesis and turnover of labelled SAM in pea cotyledon slices

L-methionine labelled in the methyl group with ^{3}H and ^{14}C (o - o) and methionine-^{35}S (Δ - Δ) was pulse fed for 7½ hours.


pulse
post pulse period
100
80
60
40
20
0
% OF MAXIMUM RADIOACTIVITY IN SAM
0
10
20
30
40
50
60
TIME (hrs.)

tissue may be due to an intercellular transport mechanism, the possibility of simple leakage cannot be ruled out, since experiments with metabolic inhibitors (sodium azide 1.0 mM, 2-4-dinitrophenol 0.1 mM and potassium cyanide 2.0 mM) failed to show the presence of an energy-dependent transport mechanism. Apart from this small loss of labelled SAM to the external solution, most of the loss of radioactivity is conceivably due to the participation of SAM in transmethylation reactions.

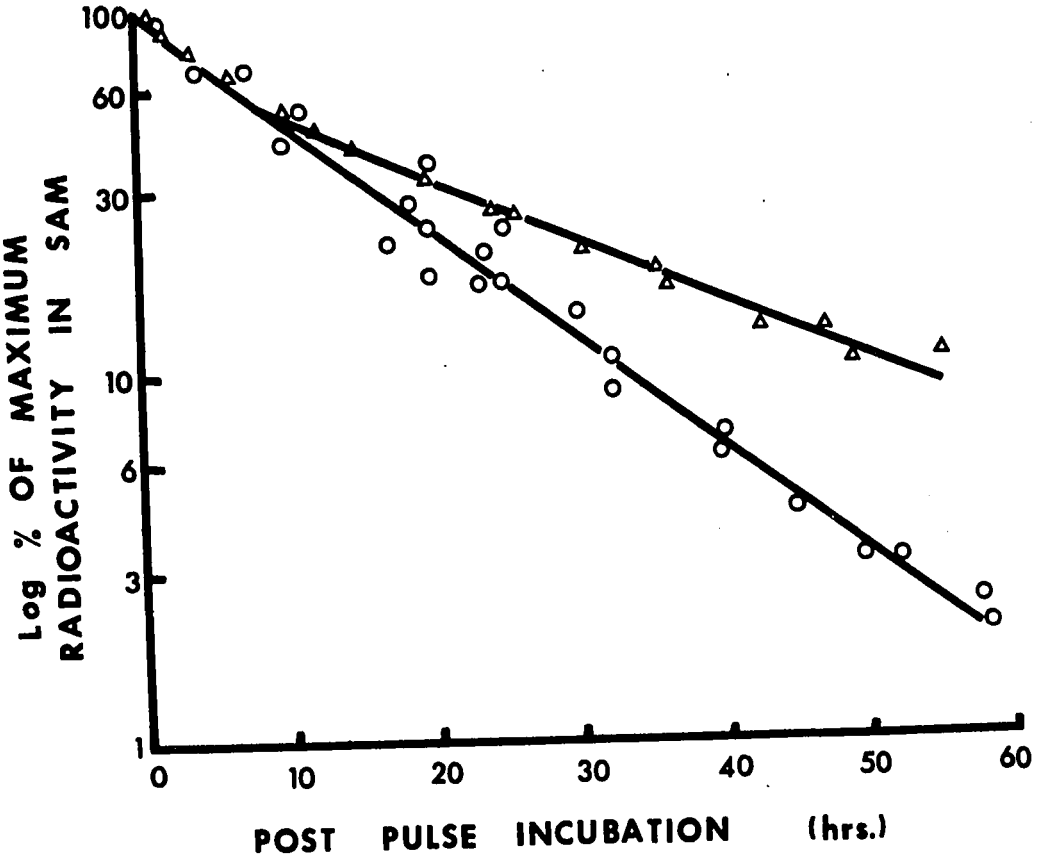
Data collated from ten separate pulse experiments using C^3H_3 or $^{14}CH_3$ or ^{35}S labelled SAM are shown in Figure 12, where the logarithm of the percentage of maximum radioactivity in SAM is plotted as a function of time. The data for C^3H_3 and $^{14}CH_3$ were statistically indistinguishable and were therefore pooled. The line due to loss of ^{35}S labelled SAM has two distinct slopes. The linear regression equation for the initial slope of ^{35}S loss was found to be virtually identical to the equation for the line due to loss of methyl labelled SAM.

The marked differences in the slopes of the two lines in the final phase of the experiment (Figure 12), suggest that ^{35}S from SAM is to some extent reincorporated into SAM. This reincorporation would conceivably involve the intermediary formation of SAHC which could act as a methyl acceptor, or alternatively SAHC might be cleaved (Duerre, 1968). In the latter case, sulphur from the cleavage product could reenter SAM via methionine. In order to

FIGURE 12

Turnover of labelled SAM in pea cotyledon slices.

S-adenosylmethionine was generated by pulse feeding ^{14}C and ^{3}H methionine and methionine-^{35}S for 7½ hours as in Figure 11. Data for the post pulse incubation were derived from ten separate experiments.


Log % OF MAXIMUM RADIOACTIVITY IN SAM
100
60
30
10
6
3
1
0
10
20
30
40
50
60
POST PULSE INCUBATION (hrs.)

examine these possibilities, cell-free extracts of pea cotyledons were prepared as described in the Methods.

Metabolism of SAHC in vitro

Experiments were conducted to examine the possible methylation of SAHC by potential methyl donors. S-adenosyl-^{14}C-8-L-homocysteine (specific activity 150,000 cpm/µmole) was incubated for 2 hours at 30°C with 50 µmole potassium phosphate buffer (pH 6.9) and 2 µmole 5-methyltetrahydrofolate or 2 µmole S-methyl-L-methionine together with cell-free extract (200 µgm protein) in a final volume of 1.0 ml. After termination of the reaction by addition of ethanol, protein was removed by centrifugation and the reaction mixtures were examined for SAM formation by thin layer chromatography and autoradiography. In no instance was SAM-^{14}C detected which suggests that the direct methylation of S-adenosylmethionine *in vitro* is of doubtful significance.

In order to examine the possible cleavage of SAHC, experiments were conducted by incubating cell-free extracts with S-adenosyl-^{14}C-8-L-homocysteine. The reaction mixtures (Table IV) were examined by thin layer chromatography and autoradiography for the presence of adenosine, homocysteine, adenine and ribosylhomocysteine. The only labelled product was adenosine, the formation of this product being dependent on the presence of the cell-free extract (Table IV).

In addition, unlabelled homocysteine was detected as the other cleavage product from SAHC. This cleavage was found to be markedly inhibited by additions of adenosine

TABLE IV

Production of adenosine from S-adenosylhomocysteine by cell-free extracts

Omission*	Adenosine formed (mμmoles)
None	360
Cell-free extract	nil
Potassium phosphate	40
Mercaptoethanol	90

* The complete system contained 50 μmole potassium phosphate buffer (pH 6.9), 5 μmole of 2-mercaptoethanol, 0.2 ml cell-free extract (containing 0.2 mgm protein), 2 μmole S-adenosyl-8-C^{14}-L-homocysteine (150,000 cpm/μmole), in a total volume of 1 ml. The reaction mixtures were incubated at 30°C for 2 hours.

and to some extent adenine, although other nucleosides had no such effect (Table V).

Transmethylation Reactions and Methionine Biosynthesis in vitro

Since homocysteine is the recognized acceptor of methyl groups for the formation of methionine, it is highly likely that sulphur may reenter SAM by being cycled via homocysteine. In order to examine the possible role of SAHC in methionine biosynthesis, this nucleoside was incubated with several potential methyl donors and samples of the cell-free extract. After incubation at 30°C for 2 hours, the reaction mixtures were examined for the presence of methionine (Table VI). It is clear that SAHC only acts as a precursor of methionine when a suitable methyl donor is added. The involvement of this compound in methionine biosynthesis probably occurs after cleavage, the resulting homocysteine then being methylated. Some support for this comes from the observation that homocysteine was also an active precursor of methionine in this system (Table VI).

A Consideration of Possible Methyl Donors in Pea Seeds

A number of methyl donors have been shown to be metabolically active in various organisms. These compounds have in common the ability to donate a methyl group to homocysteine to form methionine. Consequently this reaction provides a very convenient assay of transmethylase activity. SAM appears to be of universal occurrence (Mudd and Cantoni, 1964) and has been shown to be present in pea

TABLE V

The effect of various bases on the production of adenosine from S-adenosylhomocysteine

Additions	Adenosine formed (mμmoles)	Inhibition (%)
None	380	0
Adenine	280	26
Adenosine	10	97
Guanosine	400	0
Cytidine	390	0
Uridine	360	5
Thymidine	420	0

The complete system contained 50 μmoles of potassium phosphate buffer (pH 6.9), 5 μmole 2-mercaptoethanol, 0.2 ml of cell-free extract (containing 0.2 mgm protein) and 2 μmole S-adenosyl-8-^{14}C-L-homocysteine (150,000 cpm/μmole) in a total volume of 1 ml. Reaction mixtures were incubated for 2 hours at 30°C. Additions of 0.4 μmole of base or nucleoside were made as indicated.

TABLE VI

Synthesis of methionine from homocysteine and S-adenosyl-homocysteine in the presence of various methyl donors

Methyl donor added	Methionine formed (mμmoles)		
	Control	Homocysteine	SAHC
SAM	33	194	81
$5CH_3H_4PteGlu$	33	132	53
S-methylmethionine*	40	240	74
methyl cysteine	26	20	33
no methyl donor added	23	25	29

The assay system contained 50 μmole potassium phosphate buffer (pH 6.4), 5 μmole of 2-mercaptoethanol, 0.2 ml of cell-free extract (containing 0.2 mgm protein), 1 μmole of methyl donor and 1 μmole of methyl acceptor in a total volume of 1 ml. The controls contained no methyl acceptor. The reaction mixtures were incubated for 2 hours at 30°C. Methionine was assayed microbiologically with *S. faecalis*. *These data represent half the total methionine produced in each case.

seeds (Figure 8). On the other hand, SMM is widespread only in plant tissues (Cantoni, 1965) and it is clear from Table VII that pea tissues are able to synthesize SMM from methionine and SAM. The tetrahydrofolate pool would be expected to donate methyl groups from either $5CH_3H_4PteGlu$ or $5CH_3H_4PteGlu_3$. Such donation will clearly vary in different organisms (see introduction). Roos, *et al.* (1968) have recently shown the presence of $5CH_3H_4PteGlu$ in germinating peas. Thompson, (1967) states that S-methylcysteine is present in pea tissues and although not a sulphonium compound it has been suggested as a possible methyl donor. These four compounds (SAM, SMM, $5CH_3H_4PteGlu$ and S-methylcysteine), therefore, appear to be the most likely donors of methyl groups in pea seeds. Dimethylpropiothetin was not used in the present work because its distribution appears to be limited to the marine algae (Challenger, *et al.* 1957) and, furthermore, choline, although present in plant tissues, appears to be of limited importance as a methyl donor.

The results of experiments designed to test the ability of SAM, SMM, $5CH_3H_4PteGlu$ and S-methylcysteine to donate methyl groups to homocysteine are shown in Table VI. Of the four compounds used, all but S-methylcysteine were able to donate methyl groups to homocysteine. These results are in agreement with extensive studies with animal tissues which showed that methylcysteine was not an effective methyl donor (Thompson, 1967). Perhaps this result is not

TABLE VII

Synthesis of SMM by cell-free extracts

*Omission from reaction system	SMM formed (mμmoles)
None	320
cell-free extract	0
2-mercaptoethanol	290
methionine	40

*The complete assay system contained 8 μmole of L-methionine, 0.2 ml of cell-free extract (containing 200 μgm of protein), 1 μmole SAM-$^{14}CH_3$ (specific activity 5 μCi/μmole), 5 μmole 2-mercaptoethanol and 100 μmole of potassium phosphate buffer (pH 6.9) in a total volume of 2 ml. The reaction mixtures were incubated for 90 minutes at 30°C. Reaction products were identified by thin layer chromatography and autoradiography.

surprising when it is remembered that S-methylcysteine is the next lower homologue of methionine, and methionine *per se* is certainly unable to act as a source of methyl groups. The fact that the other three donors used (Table VI) were good sources of methyl groups, is consistent with earlier work (Abrahamson and Shapiro, 1965; Turner and Shapiro, 1961) which demonstrated that SMM is a better source of methyl groups than SAM.

The Homocysteine Dependent Transmethylases

It is apparent from the data presented in Table VI that SMM, SAM and $5CH_3H_4PteGlu$ transmethylases are present in pea seeds during germination and furthermore all three compounds are active as donors of methyl groups to homocysteine. There have been numerous studies on homocysteine dependent transmethylase systems isolated from many different organisms. However, not one of these studies has shown unequivocally whether the three methyl donors, SMM, SAM and $5CH_3H_4PteGlu$ are active with one transmethylase or several distinct transmethylase enzymes. Abrahamson and Shapiro (1965) showed that the enzyme isolated from jack bean meal was able to use both SMM and SAM as sources of methyl groups. Taylor and Weissbach (1967) isolated an enzyme from *E. coli* which was able to transfer methyl groups from methyl-B_{12}, $5CH_3H_4PteGlu$ and SAM to homocysteine. The presence of several methyl group precursors, presents an enigma which may be solved only when the ability of all the potential methyl donors to support methionine synthesis is

studied in a single tissue. With this in mind, the transfer of methyl groups from SMM, SAM and $5CH_3H_4PteGlu$ to homocysteine was studied using partially purified enzymes from germinating pea seeds.

Partial Purification of Methyltransferase Activities

In the first stage of purification, most of the enzyme activity was precipitated when the $(NH_4)_2SO_4$ concentration was raised to 20-60% of saturation. When a sample of this fraction was applied to a Sephadex G-50 column, all of the transmethylase activity added to the column was recovered in fractions 13-20. These fractions were pooled and the protein precipitated by adding solid $(NH_4)_2SO_4$ to give 60% of saturation. A sample of this precipitated protein was dissolved in buffer (see Materials and Methods) and applied to a Sephadex G-100 column and eluted with 0.05 M potassium phosphate buffer (pH 6.9). Each fraction was assayed for homocysteine transmethylase activity with SMM, SAM and $5CH_3H_4PteGlu$ serving as methyl donors. It is evident from Figure 13, that there are at least two distinct homocysteine dependent transmethylases in pea seeds. The activity of SMM and SAM-homocysteine transmethylase is greatest in fractions 26-35 which were pooled and used in subsequent enzyme studies. The activity of $5CH_3H_4PteGlu$-homocysteine transmethylase is greatest in fractions 19-25 which were pooled and used as the enzyme preparation for subsequent studies.

From Table VIII it is clear that salt fractionation

TABLE VIII

Fractionation of extracts catalyzing the synthesis of methionine from $5CH_3H_4PteGlu$

Fractionation Step	Total Protein (µg)	Total Activity (units$\times 10^{-6}$)	Specific Activity (units/mg$\times 10^{-6}$)	Purification factor	Recovery (%)
Crude extract	37,000	1,110	30	--	100
20-60% $(NH_4)_2SO_4$ fractionation	29,400	1,140	39	1.3	100
Sephadex G-50 chromatography	2,760	580	210	7.0	59
Sephadex G-100 chromatography (fraction 23)	45	36.4	810	27.0	3.3

and subsequent column chromatography on Sephadex resulted in a 27-fold purification of the 5-methyltetrahydrofolate-homocysteine transmethylase. The purification of SAM and SMM-homocysteine methyltransferases, using the same procedure results in approximately a 130-fold purification for both of these enzymes (Tables IX and X). From the observations that SMM and SAM-methyltransferase activities are recovered in the same fractions during column chromatography on Sephadex G-100, (Figure 13) and that the purification factors for the SMM and SAM methyltransferase activities are very similar for each purification step, suggests that a single methyl transferase accepts methyl groups from either SMM or SAM. Shapiro, *et al.*(1964) were able to show that a homocysteine dependent methyltransferase from *S. cerevisiae* was active with both SMM and SAM. Furthermore, the ratio of the activities with these two donors, remained unchanged during the purification procedures, suggesting that only one enzyme was responsible. In the present work it is therefore concluded, that a single methyltransferase is responsible for transfer of methyl groups from either SMM or SAM to form methionine. This enzyme will be referred to as SAM-transmethylase in subsequent experiments which were designed to examine its properties.

Properties of the Homocysteine Methyltransferases

1. Effect of enzyme concentration on reaction rate

The standard isotopic assay procedure was used to measure the effect of protein concentration on SAM-methyl-

TABLE IX

Fractionation of extracts catalyzing the synthesis of methionine from SMM

Fractionation Step	Total Protein (μg)	Total Activity (units$\times 10^{-6}$)	Specific Activity (units/mg$\times 10^{-6}$)	Purification factor	Recovery (%)
Crude extract	37,500	4,500	120	--	100
20-60% $(NH_4)_2SO_4$ fractionation	29,400	4,410	150	1.3	98
Sephadex G-50 chromatography	2,760	3,040	1,100	9.1	68
Sephadex G-100 chromatography (fraction 32)	15	251	16,700	139	5.6

TABLE X

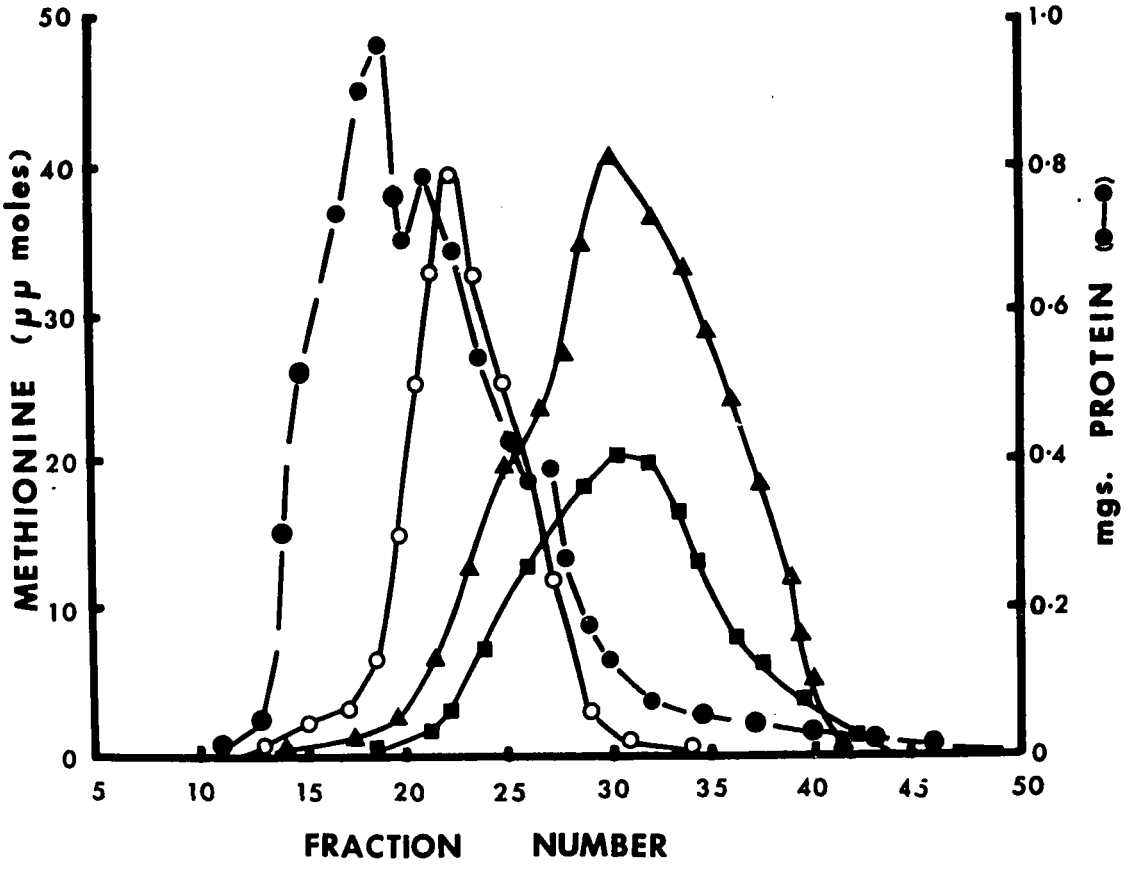
Fractionation of extracts catlyzing the synthesis of methionine from SAM

Fractionation Step	Total Protein (μg)	Total Activity (units$\times 10^{-6}$)	Specific Activity (units/mg$\times 10^{-6}$)	Purification factor	Recovery (%)
Crude extract	37,000	9,370	250	--	100
20-60% $(NH_4)_2SO_4$ fractionation	29,400	7,930	270	1.1	85
Sephadex G-50 chromatography	2,760	6,900	2,500	10.0	73
Sephadex G-100 chromatography (fraction 32)	15	482	32,100	132.2	5.2

FIGURE 13

Chromatography of homocysteine methyltransferases on Sephadex G-100

Protein (30 mgm in 1.5 ml) recovered from a Sephadex G-50 column was added to a column of Sephadex G-100 (50 x 2.8 cm). After elution with 0.05 M potassium phosphate buffer (pH 6.9), each tube was assayed for protein (● - ●) and for SMM-transmethylase (■ - ■), SAM-transmethylase (▲ - ▲) and $5CH_3H_4PteGlu$-transmethylase (○ - ○) using the standard assay procedures outlined in the Materials and Methods.


50
40
30
20
10
0
METHIONINE (μμ moles)
1·0
0·8
0·6
0·4
0·2
0
mgs. PROTEIN
5
10
15
20
25
30
35
40
45
50
FRACTION NUMBER

transferase and $5CH_3H_4$PteGlu-methyltransferase activities. The results (Figure 14) show that the reaction velocities for SAM-methyltransferase are limited by protein concentrations greater than approximately 100 µg. The velocities for the $5CH_3H_4$PteGlu-transmethylase reaction are linear when expressed as a function of protein concentrations up to 700 µg of protein. In subsequent work, samples of enzyme containing 100 µg of protein were routinely used for assay of SAM-transmethylase activity and 200 µg of protein were used for assay of $5CH_3H_4$PteGlu-transmethylase activity.

2. Effect of incubation time on reaction velocity

Samples taken after different periods of incubation (Figure 15) showed that the rate of product formation was linear for at least 60 minutes with both enzymes. In all subsequent enzyme assays an incubation time of 60 minutes at 30°C was therefore used.

3. Effects of storage on transmethylase activity

Both enzymes were freshly prepared and samples were stored at 4°C and -15°C. Aliquots were taken and assayed for transmethylase activity over a ten day period. The results, (Figure 16) show that $5CH_3H_4$PteGlu-transmethylase loses activity over a ten day period, but is more stable at -15°C than at 4°C. The SAM-transmethylase appears to be quite stable at -15°C but there is some loss in activity if stored at 4°C (Figure 17).

4. Effect of pH on transmethylase activity

The standard isotopic assay procedure was used to

FIGURE 14

The relationship between transmethylase activities and protein concentration

Reaction mixtures (total volume 0.5 ml) contained 1 mμmole of $5^{14}CH_3H_4PteGlu$ or SAM-$^{14}CH_3$, 50μmoles of potassium phosphate buffer (pH 6.9), 1 μmole of L-homocysteine and varying amounts of protein as indicated. The reactants were incubated for 60 minutes at 30°C. 5-methyltetrahydrofolate-homocysteine transmethylase activity (● - ●) and SAM-homocysteine transmethylase activity (▲ - ▲) were assayed by ion exchange column chromatography.

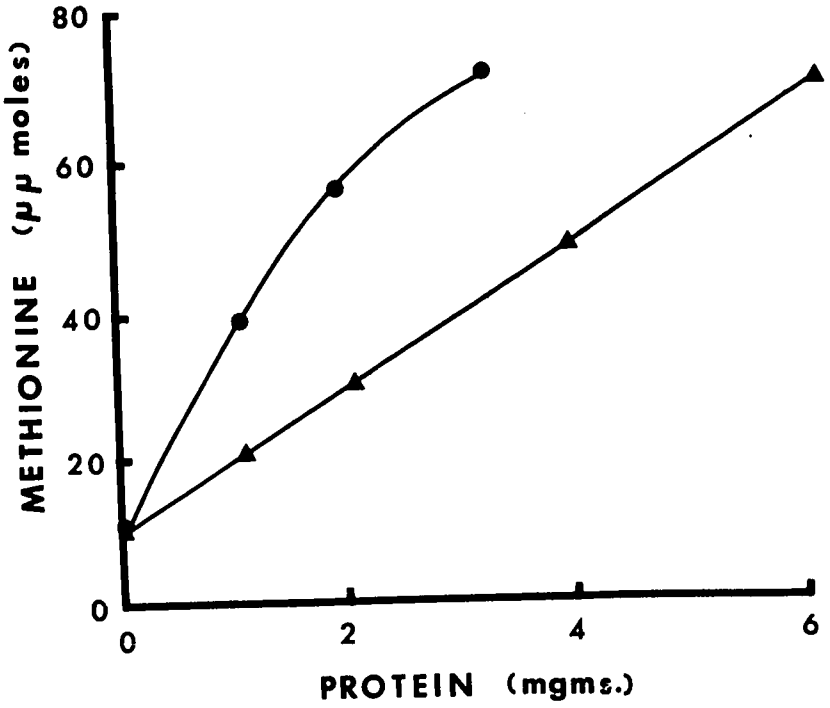

80
60
40
20
0
METHIONINE (μμ moles)
0
2
4
6
PROTEIN (mgms.)

FIGURE 15

Effect of incubation time on product formation

The standard microbiological assay was used to examine the effect of incubation time on product formation when SMM (o - o) was used as the methyl donor. When SAM (● - ●) and $5CH_3H_4PteGlu$ (Δ - Δ) were the methyl donors, the standard isotope assay was used. The reaction mixtures were incubated at 30°C and samples withdrawn at the indicated times, for assay.

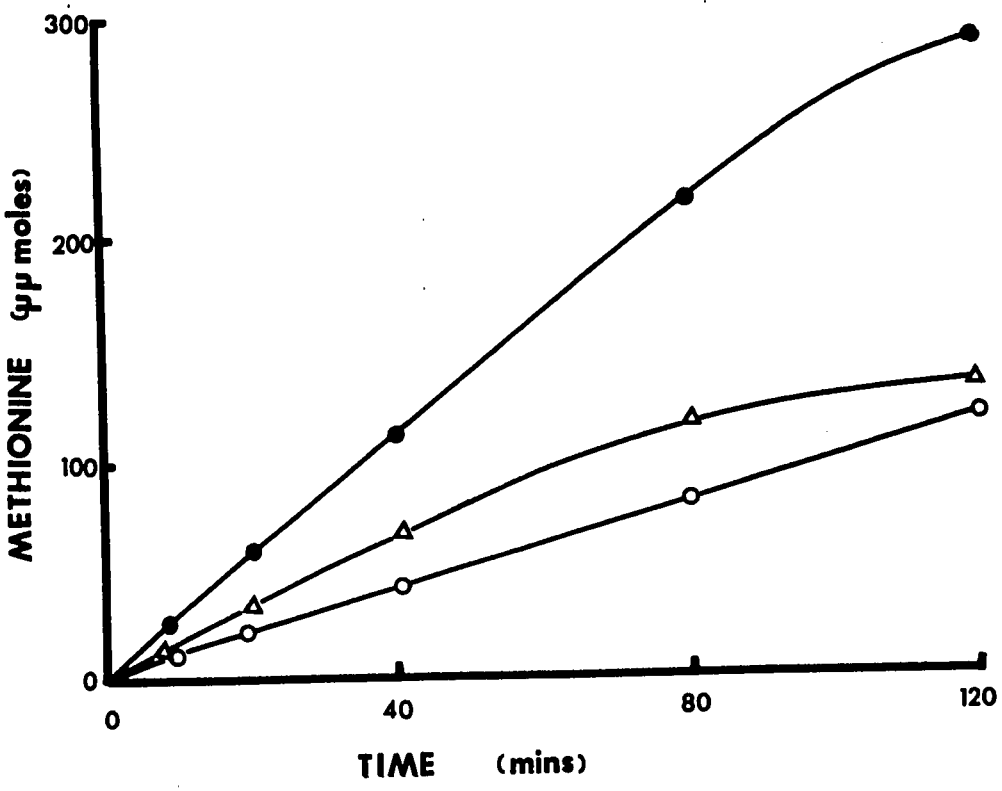

300
200
100
0
METHIONINE (μμ moles)
0
40
80
120
TIME (mins)

FIGURE 16

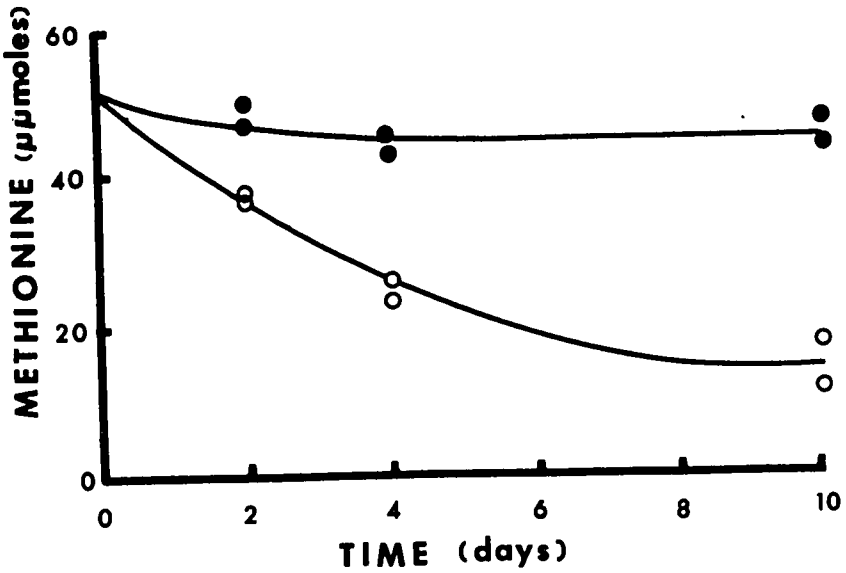
Effect of storage on SAM-homocysteine transmethylase activity

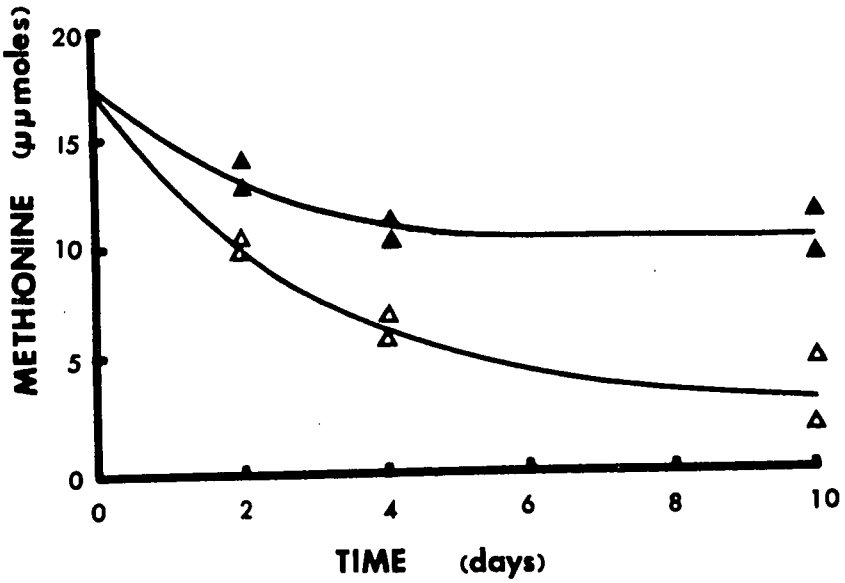
The partially purified enzyme was prepared by Sephadex G-100 column chromatography. Fractions 26-35 were pooled and stored at 4°C (o - o) and -15°C (● - ●) and then assayed using the standard isotopic assay.

FIGURE 17

Effect of storage on $5CH_3H_4$PteGlu-homocysteine transmethylase activity

After preparation by Sephadex G-100 column chromatography the partially purified enzyme (fractions 19-25) was stored at 4°C (Δ - Δ) and -15°C (▲ - ▲). Isotopic assays were then carried out over a period of ten days.


METHIONINE (μmoles)
60
40
20
0
0
2
4
6
8
10
TIME (days)


METHIONINE (μmoles)
20
15
10
5
0
0
2
4
6
8
10
TIME (days)

assess the effect of pH on transmethylase activity, using 0.1 M Tris-HCl buffers for pH values of 7.5 and higher and 0.1 M potassium phosphate buffers for pH values of 7 and lower. It is seen from the results in Figure 18 that SAM-transmethylase has a pH optimum of 7.1. This pH value is lower than the optimum of 7.5 to 8.0 reported by Shapiro, *et al.* (1964) for *S. cerevisiae*, and by Abrahamson and Shapiro (1965) for the enzyme from jack bean. The pH curve for $5CH_3H_4PteGlu$-transmethylase (Figure 18) does not show such a distinct optimum. However, the transmethylase from hog liver, requiring vitamin B_{12} as a prosthetic group, will transfer methyl groups from either $5CH_3H_4PteGlu$ or $5CH_3H_4PteGlu_3$ and has a sharp optimum at about pH 7.0.

5. Effect of homocysteine concentration on transmethylase activities

The relationship between methionine production and the levels of homocysteine present in the reaction mixtures is shown in Figure 19. In obtaining these curves, standard assay reaction mixtures were used but the concentration of homocysteine was varied. It can be seen (Figure 19) that SAM-transmethylase became saturated with homocysteine at a concentration of approximately 1 µmole/0.5 ml. $5CH_3H_4PteGlu$ transmethylase also became saturated with homocysteine at a concentration of approximately 1 µmole/0.5 ml.

6. Effect of EDTA on transmethylase activity

The chelating agent EDTA has been shown to inhibit the SAM-transmethylases of various microorganisms but not that

FIGURE 18

Effect of pH on homocysteine transmethylase activity

Standard isotope assay conditions were used with incubation being carried out at 30°C for 60 minutes. For pH values below 7.0, 50 µmoles of potassium phosphate buffer was used. For pH values of 7.5 and above, Tris-HCl (50 µmoles) was used. SAM-transmethylase (● - ●) and 5CH H PteGlu-transmethylase (▲ - ▲) activities were measured after column chromatography of an aliquot of the reaction mixtures.

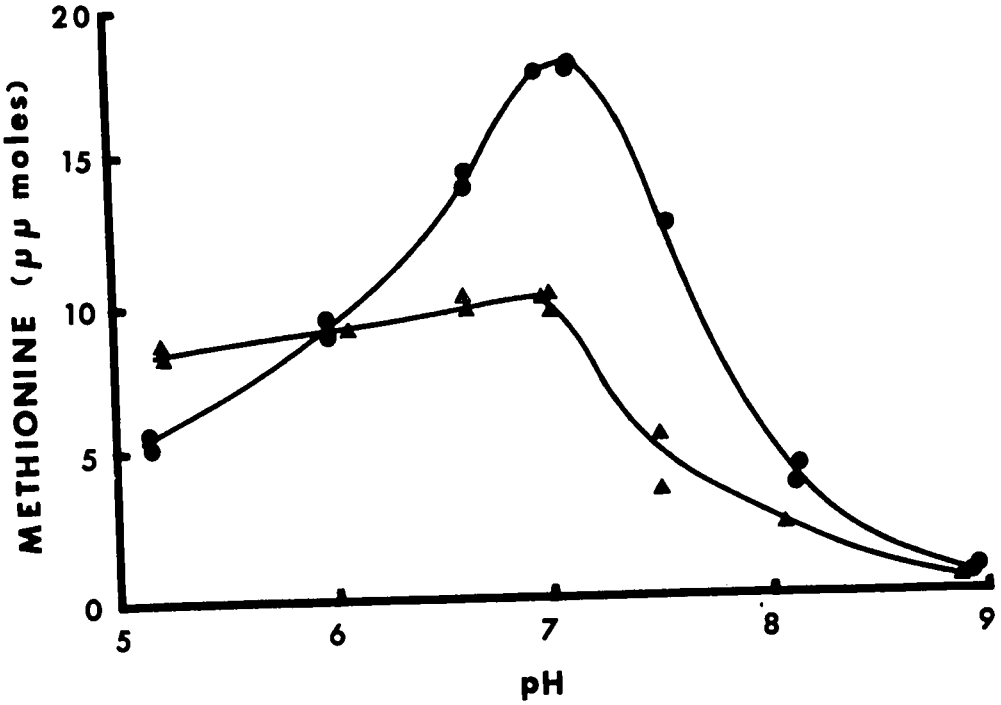
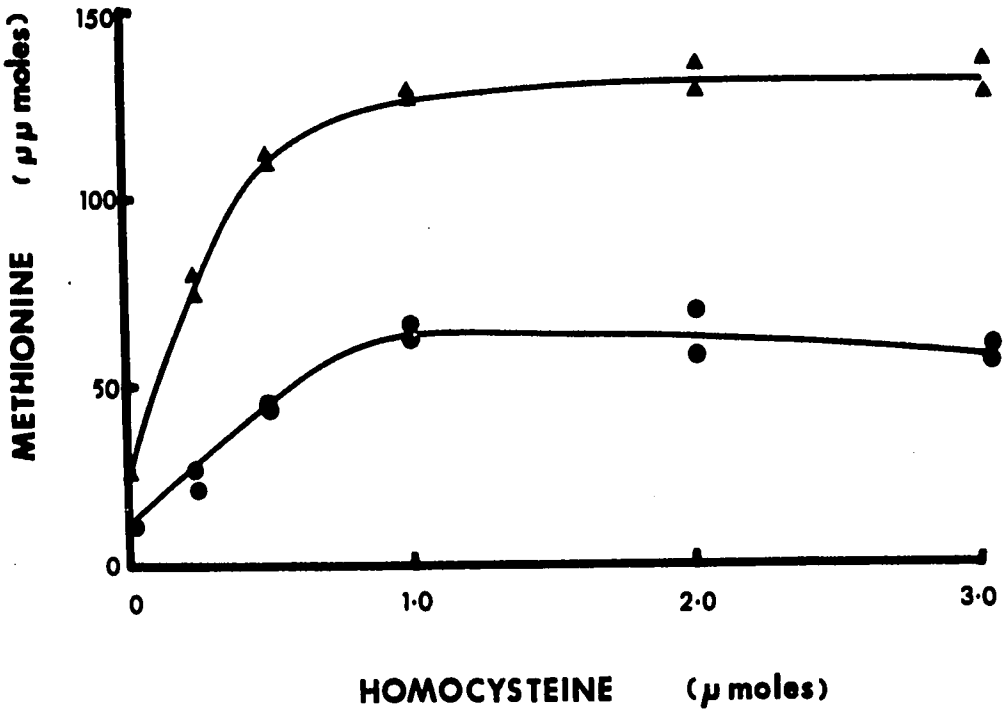

20
15
10
5
0
METHIONINE (μμ moles)
5
6
7
8
9
pH

FIGURE 19

Effect of homocysteine concentration on transmethylase activities

Isotopic assays were used to measure the effect of homocysteine concentration on SAM-transmethylase (▲ - ▲) and $5CH_3H_4PteGlu$-transmethylase (● - ●). Standard reaction mixtures contained variable concentrations of homocysteine as indicated. Reaction mixtures were incubated for 60 minutes at 30°C.


METHIONINE (μμ moles)
150
100
50
0
0
1·0
2·0
3·0
HOMOCYSTEINE (μ moles)

of rat liver (Shapiro and Yphantis, 1959). The SAM-transmethylases of higher plants appear to differ in their sensitivity to EDTA. For example, the jack bean enzyme (Abrahamson and Shapiro, 1965) was only 17% inhibited by a level of $5x10^{-2}$M EDTA while the enzyme in crude extracts of pea seeds (Turner and Shapiro, 1961) was 87% inhibited at a concentration of $8x10^{-3}$M EDTA. Where an inhibitory effect of EDTA has been reported, the inhibition is partially reversed by addition of certain divalent metals, especially Zn^{2+}, (Shapiro and Yphantis, 1959). EDTA inhibitory effects have not been reported for the $5CH_3H_4$PteGlu-transmethylase system from any organism.

In the present investigation the effects of various EDTA concentrations on transmethylase activities were studied. It is apparent from Figure 20 that both transmethylases are inhibited by very low concentrations of EDTA.

7. Effect of 2-mercaptoethanol on methyltransferase activity

Shapiro (1965) has reported that the presence of 2-mercaptoethanol in assay mixtures caused significant increases in methionine production. This effect was most pronounced at low levels of homocysteine. This effect was, therefore, studied with the two transmethylases over a range of 2-mercaptoethanol and homocysteine concentrations (Tables XI and XII). The results show that higher levels of 2-mercaptoethanol cause a depression of methionine formation with SAM-transmethylase (Table XI). This effect is even more

FIGURE 20

Effect of EDTA on transmethylase activities

Standard isotopic assays were used to measure the effect of EDTA concentration on SAM-transmethylase (o - o) and $5CH_3H_4PteGlu$-transmethylase (Δ - Δ). The reaction mixtures contained variable concentrations of EDTA as indicated and were incubated for 60 minutes at 30°C.

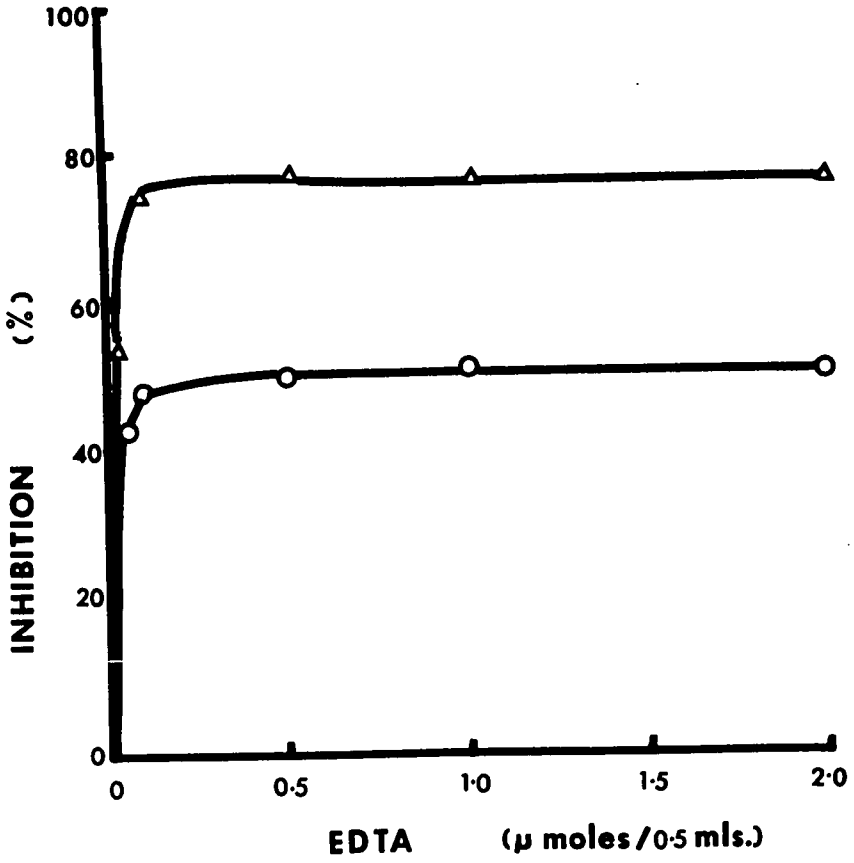

100
80
60
40
20
0
INHIBITION (%)
0
0·5
1·0
1·5
2·0
EDTA (μ moles / 0·5 mls.)

TABLE XI

Effect of 2-mercaptoethanol and homocysteine on SAM-homocysteine methyltransferase activity

2-mercaptoethanol (µmoles)	L-homocysteine (µmoles)		
	0	1.0	2.0
	Methionine formed (mµmoles)		
0	22	100	68
0.1	13	46	41
1.0	10	34	63
5.0	20	38	45

Apart from variations in the concentrations of homocysteine and 2-mercaptoethanol, the reaction mixtures were the same as in the standard isotope assay. After incubation for 60 minutes at 30°C the isotopic assay was used to detect SAM-transmethylase activity.

TABLE XII

Effect of 2-mercaptoethanol and homocysteine on $5CH_3H_4$PteGlu-homocysteine transmethylase activity

2-mercaptoethanol (µmoles)	L-homocysteine (µmoles)		
	0	1.0	2.0
	Methionine formed (mµmoles)		
0	2.3	9.5	8.2
0.1	2.2	7.5	9.6
1.0	1.9	8.4	4.1
5.0	0.5	3.7	3.1

Standard isotopic assay reaction mixtures were used except that concentrations of 2-mercaptoethanol and homocysteine were varied as indicated. After incubation for 60 minutes at 30°C transmethylase activity was detected by column chromatography.

pronounced with $5CH_3H_4$PteGlu-transmethylase (Table XII) where higher levels of 2-mercaptoethanol cause a 50% inhibition at both levels of homocysteine. The addition of 2-mercaptoethanol to the assay systems is thus unnecessary for maximum methionine formation. These results are consistent with findings of Rosenthal, *et al.* (1965) who showed that both SAM and $5CH_3H_4$PteGlu could transfer a methyl group enzymically to 2-mercaptoethanol to form S-methylmercaptoethanol. It is apparent that 2-mercaptoethanol may be acting as a competitive inhibitor in the enzyme catalysed reactions studied in the present work.

8. Inhibition of transmethylase activities by methionine

Product inhibition of homocysteine-transmethylase activity is well documented (Abrahamson and Shapiro, 1965; Shapiro, 1965). The results of experiments testing the effect of methionine concentration on the transmethylases from pea seeds are shown in Figure 21. It is evident that very low concentrations of methionine produced a significant inhibition of $5CH_3H_4$PteGlu-transmethylase activity. The effect with SAM-transmethylase, however, is to stimulate the reaction rate at low concentrations, before causing an inhibitory effect. Balish and Shapiro (1967) observed a similar effect by methionine on the SMM-transmethylase from *E. coli*.

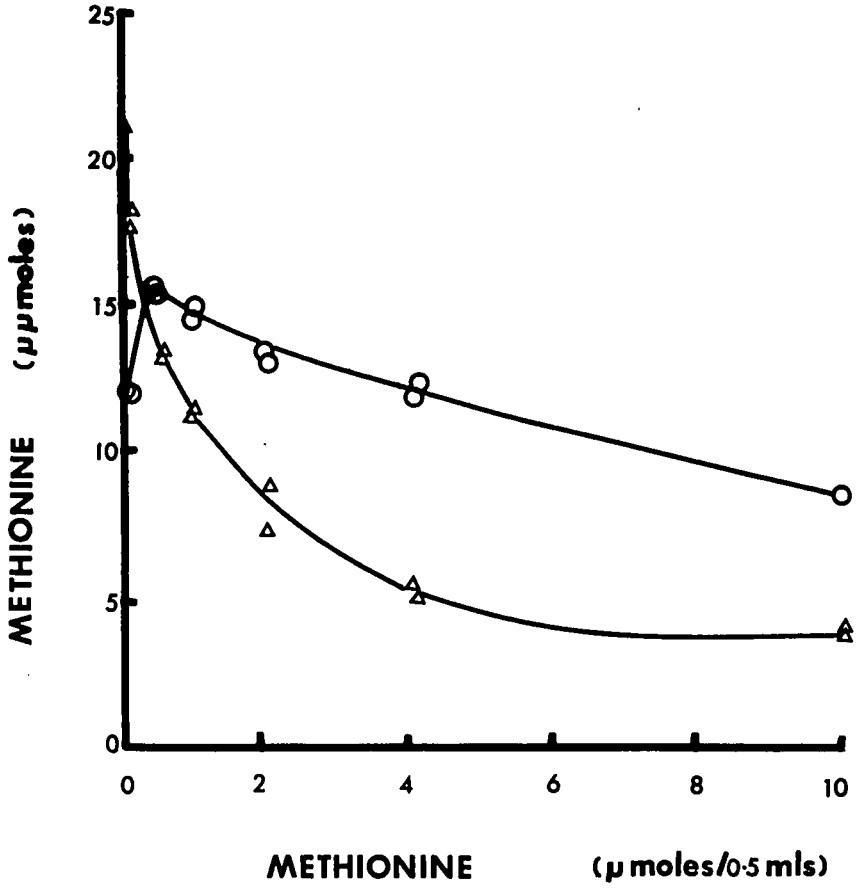
9. Michaelis constants

The apparent Michaelis constants for SMM, SAM and $5CH_3H_4$PteGlu were determined from Lineweaver-Burk plots

FIGURE 21

Product inhibition of transmethylase activities

Different concentrations of L-methionine (up to 10 µmoles) were added to standard isotope reaction mixtures in a total volume of 0.5 ml. After incubation for 60 minutes at 30°C, the methionine formed from SAM-transmethylase (o - o) and from $5CH_3H_4PteGlu$-transmethylase (Δ - Δ) was determined using the isotopic assay.


25
20
15
10
5
0
0
2
4
6
8
10
METHIONINE (μ μ moles)
METHIONINE (μ moles/0·5 mls)

(Lineweaver and Burk, 1934).

(a) The results obtained for SMM-transmethylase by microbiological assay are shown in Figure 22. The apparent value of K_m for SMM is 2.2×10^{-6}M. For the jack bean enzyme, Abrahamson and Shapiro (1965) obtained a K_m for SMM of 5.5×10^{-5}M. Thus the apparent affinity of substrate and enzyme is about 20 times greater in the present system, isolated from pea seeds.

(b) The isotopic assay was used to obtain kinetic data for SAM-transmethylase (Figure 23). From these data, the calculated apparent K_m for SAM is 5.0×10^{-6}M which is smaller than the figure of 8.6×10^{-4}M obtained for the corresponding enzyme from *S. cerevisiae* (Shapiro, *et al.* 1965).

(c) The apparent K_m for $5CH_3H_4PteGlu$-transmethylase was calculated from the data in Figure 24 and found to be 2.6×10^{-8}M. The only values of K_m available for this enzyme are from *N. crassa* ($K_m = 9.0 \times 10^{-4}$M) and from *S. cerevisiae* ($K_m = 4.0 \times 10^{-4}$M) (Burton, *et al.* 1969). These authors found that these enzymes were particularly active with the diglutamate and triglutamate forms of S-methyltetrahydrofolate but had only slight activity with $5CH_3H_4PteGlu$.

FIGURE 22

Double reciprocal plot of methionine synthesis versus concentration of S-methylmethionine

The reaction mixtures contained 100 µmoles potassium phosphate buffer (pH 6.9), 4.0 µmoles of L-homocysteine and 200 µg of protein in a total volume of 1 ml. The quantities of S-methylmethionine were varied as shown. After incubation at 30°C for 60 minutes, the reaction was terminated in an ice bath and assayed microbiologically for methionine content. Since two molecules of methionine are formed in this reaction, the values used in this figure represent half of the total methionine assayed.

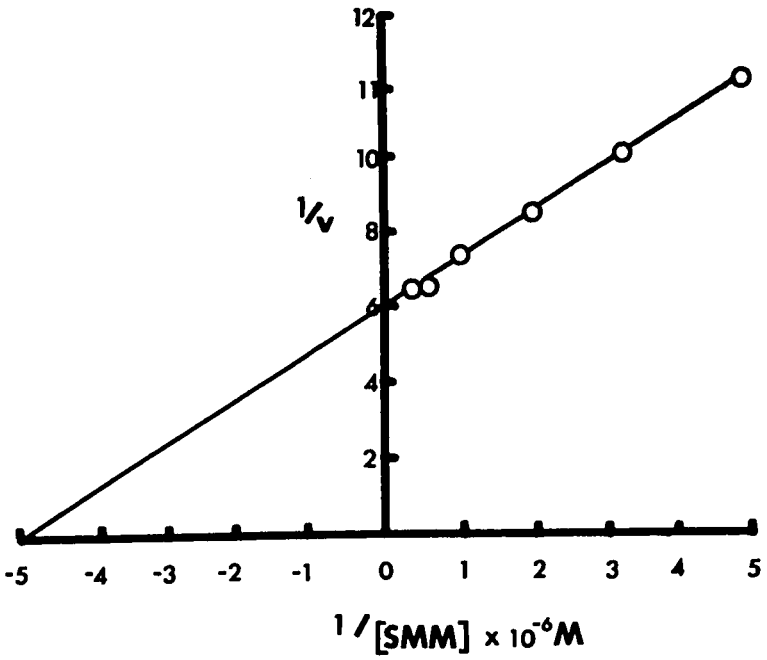

12
10
8
6
4
2
1/v
-5
-4
-3
-2
-1
0
1
2
3
4
5
1/[SMM] x 10⁻⁶M

FIGURE 23

Double reciprocal plot of methionine synthesis versus S-adenosylmethionine concentration

The reaction mixtures contained 50 µmoles of potassium phosphate buffer (pH 6.9), 1 µmole of L-homocysteine and 100 µg of protein in a total volume of 0.5 ml. Quantities of SAM-^{14}C were added over the range of 0.5 to 5.0 mµmole. After incubation at 30°C for 60 minutes, the reaction was terminated in an ice bath and assayed using the isotopic assay.

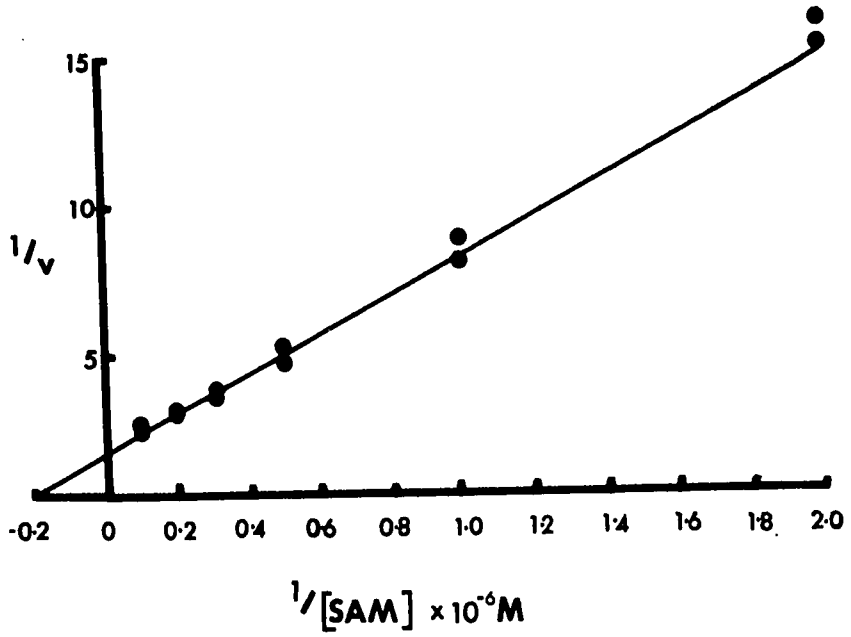
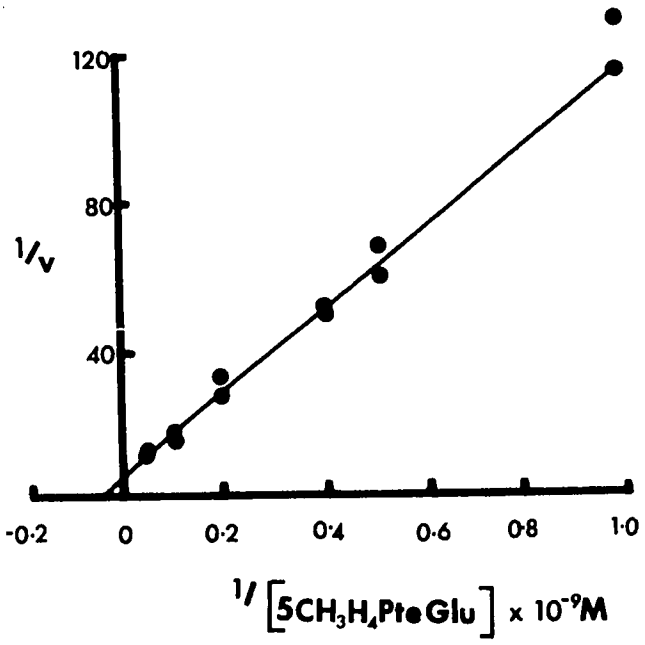

15
10
5
1/v
-0·2
0
0·2
0·4
0·6
0·8
1·0
1·2
1·4
1·6
1·8
2·0
1/[SAM] x 10⁻⁶M

FIGURE 24

Double reciprocal plot of methionine synthesis versus $5CH_3H_4PteGlu$ concentration

The reaction mixtures contained 50 µmoles of potassium phosphate buffer (pH 6.9), 1 µmole of L-homocysteine, 100 µg of protein in a total volume of 0.5 ml. $5^{14}CH_3H_4Pte$-Glu (specific radioactivity 5.0×10^5 dpm/mµmole) was added over a range of 0.5 to 5.0 mµmoles. After incubation at 30°C for 60 minutes, the reaction was terminated in an ice bath and methionine assayed using a standard isotope assay.


120
80
40
1/v
-0·2
0
0·2
0·4
0·6
0·8
1·0
1/ [5CH3H4PteGlu] x 10-9M

S-adenosylmethionine Synthesis and Turnover

The experimental results in Figure 8 show that after 24 hours the level of SAM in germinating pea seeds reaches a maximum. During this period there is net synthesis of both SAM and SAHC. It is of interest that the levels of SAM and SAHC in pea seeds is within the range reported for these compounds in other tissues (Table XIII).

The biosynthesis of SAM in pea seeds appears to be from ATP and methionine, catlaysed by the enzyme, methionine adenosyltransferase. Support for this, is the observation that SAM is rapidly labelled and after 7 hours has a specific activity approaching that of the methionine fed (Table II). These data clearly implicate methionine as a direct and efficient precursor of SAM. Methylthioadenosine does not appear to be a precursor of SAM in pea seeds, although it is involved in SAM synthesis in the yeast *Candida utilis*. (Schlenk and Ehninger, 1964).

Davies (1966) concluded that the pool of SAM present in turnip storage tissue was not metabolically active. It is clear that the pool of SAM present in pea seeds during germination, (Figure 8) is in a rapid state of turnover. Apart from a small loss of radioactivity to the external solution, the decline of radioactivity in SAM may be directly attributed to the involvement of this compound in transmethylation reactions. Although there is a possibility of the formation of methylthioadenosine from SAM, (Schlenk and Ehninger, 1964) this appears unlikely to account for the

TABLE XIII

Quantities of SAM and SAHC in different tissues

SOURCE	SAM (µmoles/g F.wt.)	SAHC (µmoles/g F.wt.)
Candida utilis[1]	0.9 - 1.5*	
S. cerevisiae	0.3 - 0.8*	
Rat tissues[2]		
liver	0.07	
heart	0.03	
kidney	0.16	
muscle	0.08	
Rat tissues[3]		
liver	0.06	
heart	0.06	
kidney	0.05	
brain	0.03	
Turnip storage tissue[4]	0.20	
Pea seeds (2-day-old)[5]	0.04	0.03
Vertebrate liver [6]		
rat		0.06
rabbit		0.07
chicken		0.05
elasmobranchs		0.01 - 0.05

[1]Data from Schlenk (1965)

[2]Data from Pansuwana (1961)

[3]Data from Baldessarini (1966)

[4]Data from Davies (1966)

[5]Data from this study

[6]Data from Salvatore, *et al.* (1968)

*cultures without methionine supplement

large losses of labelled SAM, as radioactivity was not observed in methylthioadenosine in any experiment. Reversal of methionine adenosyltransferase would also fail to account for losses of SAM, since this reaction is energetically unfavorable and essentially irreversible (Cantoni, 1965).

Involvement in transmethylation reactions would result in equal losses of both ^{35}S and methyl labelled SAM from the tissue. However if the resulting SAHC is utilized for the regeneration of SAM, the rate of loss of ^{35}S from SAM would be appreciably less. Such differences are illustrated by the data presented in Figures 11 and 12. It is clear from Figure 12 that initially the rate of loss of SAM, labelled with ^{35}S and labelled in the methyl group, is identical. However after approximately 10 hours of the post pulse incubation, the rate of ^{35}S labelled SAM loss becomes appreciably less than loss of ^{14}C or ^{3}H. It therefore appears that about 10 hours are required before sulphur is reincorporated into the SAM pool. This long apparent recycling time may be due to compartmentation of SAHC, which would result in dilution of the ^{35}S being recycled. Some evidence for this comes from Figure 8 which shows that appreciable levels of SAHC are present in pea cotyledons, however the levels of radioactivity detected in this compound were generally very low. These data could be explained by the presence of a small metabolically active pool of SAHC in equilibrium with a relatively large pool of SAHC which is not metabolically active. It is also possible

that the concentrations of adenine or adenosine in the cells may be high enough to effectively reduce the rate at which SAHC is cleaved enzymically (Table V).

Re-incorporation of sulphur into SAM could conceivably occur by direct methylation of SAHC or by recycling of sulphur through homocysteine and methionine, as occurs in yeast (Duerre, 1968). Evidence for the direct methylation of SAHC *in vitro* is still lacking (Duerre, 1968). Attempts to demonstrate such a reaction in the present work were unsuccessful, as SAHC labelled with ^{14}C was not incorporated into SAM. On the basis of these findings, it appears unlikely that this reaction has much importance in the regeneration of SAM. However as pointed out by Duerre (1968), direct methylation of SAHC need not occur to account for the incorporation of radioactive isotopes from labelled SAHC into SAM *in vivo*. In support of this Duerre has obtained evidence for a pathway whereby SAHC is cleaved enzymically forming adenosine and homocysteine. Homocysteine is then methylated to produce methionine which gives rise to SAM.

The Recycling of Sulphur

The present studies with cell-free extracts from pea tissues, known to be active in the biosynthesis of both SAM and SAHC (Figure 8), have indicated the existence of a similar pathway for the regeneration of SAM. It is clear that four basic enzymic reactions must be demonstrable for the operation of this pathway (Figure 25, I-IV). Reaction I is a homocysteine dependent transmethylation. Methyl

FIGURE 25

Metabolism of S-adenosylmethionine in germinating pea seeds

Turnover of SAM and possible relationships between recycling of sulphur and transmethylation reactions.

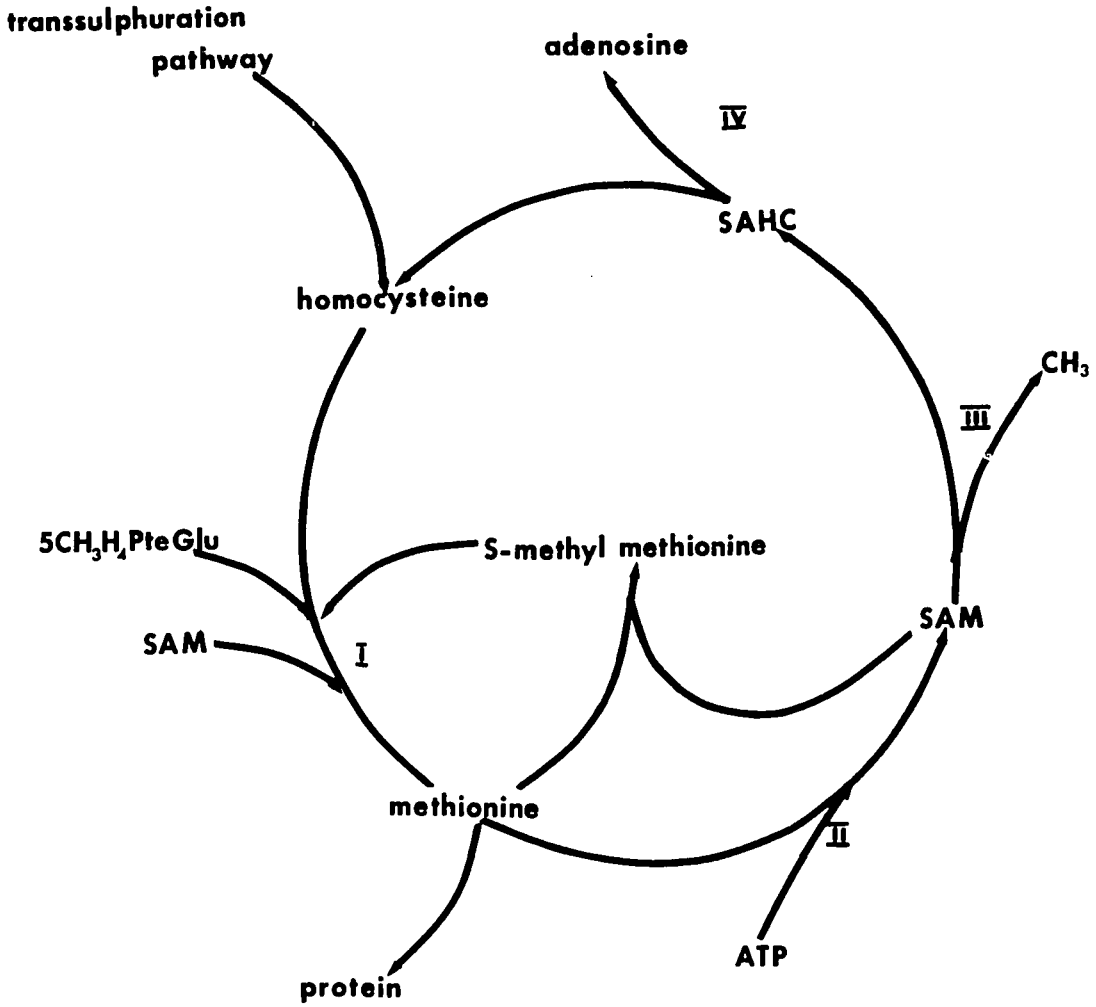
Enzymes catalysing the reactions are:

I homocysteine methyltransferases

II ATP: methionine adenosyltransferase

III methyltransferases (numerous)

IV S-adenosylhomocysteine hydrolase


transsulphuration
pathway
adenosine
IV
SAHC
homocysteine
CH3
III
5CH3H4PteGlu
S-methyl methionine
SAM
SAM
I
methionine
II
ATP
protein

groups for the synthesis of methionine in this tissue may be derived from 5-methyltetrahydrofolate, SMM or SAM, as these donors were effective *in vitro* (Table VI). The synthesis of SAM (reaction II) is catalysed by methionine adenosyltransferase. Although this enzyme has not been extensively studied in plants, there is good reason to believe that this enzyme is responsible for the synthesis of SAM in pea cotyledons. For example, methionine was found to be an excellent precursor of SAM, (Figure 11) and cell-free extracts were shown to catalyse the synthesis of this compound from methionine and ATP (Table III). While reaction III has not been examined *in vitro*, there is considerable evidence in the literature for such transmethylation reactions (Meister, 1965). In pea cotyledon tissues the rapid loss of methyl labelled SAM is undoubtedly related to such reactions. The SAHC formed in reaction III may be cleaved to either ribosylhomocysteine and adenine as occurs in yeast, (Duerre, 1962) or to adenosine and homocysteine (reaction IV). No evidence was obtained for the formation of ribosylhomocysteine in the present work but cell-free extracts readily formed labelled adenosine from S-adenosyl-8-^{14}C-L-homocysteine (Table IV). The studies with cell-free extracts have therefore shown that the enzymes necessary for the regeneration of SAM are present in germinating pea seeds.

For the scheme outlined in Figure 25 to operate, it is clear that a continuous input of methyl groups is required. The source of these methyl groups cannot include SAM if this

compound is to be active in diverse transmethylation reactions. Both 5-methyltetrahydrofolate and S-methylmethionine could be effective methyl donors *in vivo*, as both were utilized for the synthesis of methionine *in vitro* (Table VI). In agreement with earlier work with animal tissues (Thompson, 1967) methylcysteine did not serve as a donor of methyl groups for methionine biosynthesis (Table VI). Since S-methylmethionine is formed from methionine (Karr, *et al.* 1967; Green and Davis, 1960), it appears that *de novo* synthesis of methyl groups would most likely occur from the tetrahydrofolate pool, probably via 5-methyltetrahydrofolate. Roos, *et al.* (1968) have recently reported on the synthesis of 5-methyltetrahydrofolate in germinating pea seedlings. They found that 5-methyltetrahydrofolate monoglutamate was the major folate derivative present. It is significant that this monoglutamate derivative was utilized in the synthesis of methionine *in vitro* (Table VI). The enzyme from pea seeds appears to differ from that present in leaf tissues (Woods, *et al.* 1965), (Elford, *et al.* 1965), which requires the triglutamate form of 5-methyltetrahydrofolate for the homocysteine dependent formation of methionine. In both cases these statements are unsupported by experimental data. However Burton, *et al.* (1969) have recently shown that a transmethylase from *E. coli* would transfer methyl groups from $5CH_3H_4PteGlu_3$ and $5CH_3H_4PteGlu_2$ but the monoglutamate derivative had little activity.

Considering these publications, further work is clearly

required on the $5CH_3H_4PteGlu$-transmethylase from pea seeds to examine its degree of substrate specificity.

The presence of two homocysteine dependent transmethylases in the tissues of germinating pea seeds has been demonstrated (Figure 13). One of these, $5CH_3H_4PteGlu$-transmethylase will transfer methyl groups from $5CH_3H_4PteGlu$ to homocysteine and possibly accounts for *de novo* synthesis of methionine from the tetrahydrofolate pool. The other transmethylase will transfer methyl groups to homocysteine from SAM or SMM. The general properties of these enzymes have been discussed in the Results section and it is apparent that they possess several properties in common with other homocysteine dependent transmethylases.

Homocysteine Transmethylases and the Regulation of Methionine Synthesis

Since 1939 when du Vigneaud, *et al.* established the importance of methyl transfer reactions, the biosynthesis of methionine has been studied in considerable detail in many organisms. Despite these studies the mechanisms controlling methionine biosynthesis are not clear. However with the data available from this study and from evidence obtained in numerous other investigations (Shapiro, *et al.* 1965; Balish and Shapiro, 1967; Abrahamson and Shapiro, 1965; Karr, *et al.* 1967) it should be possible to develop an hypothesis to explain some of the factors contributing to the control of methionine biosynthesis in pea seeds.

Roos, *et al.* (1968) have shown that a major component of the folate pool in pea cotyledons is $5CH_3H_4PteGlu$. It has been demonstrated elsewhere in this thesis that $5CH_3H_4PteGlu$ is an active donor of methyl groups to homocysteine, forming methionine. This finding is consistent with previous work, (Woods, *et al.* 1965; Taylor and Weissbach, 1966; Taylor and Weissbach, 1967), which has shown that either $5CH_3H_4PteGlu$ or $5CH_3H_4PteGlu_3$ is the form in which methyl groups are donated from the folate pool. This reaction is of primary importance, as it probably is the only one contributing to *de novo* synthesis of methionine.

Once formed methionine may have one of three possible fates (Figure 25). It may be incorporated into protein, SAM, or SMM. The observation that methionine is preferentially used for methylation of nucleic acids in *E. coli* (Starr, 1965) and that methionine will inhibit respiration by combining with ATP to form SAM (Davies, 1966), is a consequence of a highly favoured reaction (Cantoni, 1965). If methionine adenosyltransferase continues to operate at this level, it is possible that supplies of methionine for protein synthesis may become limiting. There is no information regarding the intracellular site of SAM synthesis and while methionine adenosyltransferase could be compartmented the function of SAM argues against this. Induction and repression of methionine adenosyltransferase must therefore be a strong possibility.

Karr, *et al.* (1967) using wheat germ, studied SAM-

methionine methyltransferase, the enzyme responsible for formation of SMM. Although they provide no values for the Michaelis constants, a calculation from their data indicates a K_m for methionine of approximately 4×10^{-3}M. Thus it is probable that this enzyme, with an apparently low affinity for methionine, will be unable to compete with methionine adenosyltransferase for the methionine available unless factors other than methionine availability are important.

It is concluded by the writer that SMM in plant cells may have a very important role, not as a source of methyl groups as suggested by Karr, *et al.* (1967), but as a source of methionine residues for protein synthesis. The fact that SMM can only donate methyl groups to homocysteine, and that two molecules of methionine are formed for each methyl group transferred, supports this conclusion. There is a possibility that the SMM pool may be compartmented. Obviously, an inducible SMM-homocysteine methyltransferase would be a very efficient mechanism to produce methionine residues as required.

It is also suggested by the writer that the formation of methionine from SAM and homocysteine is a reaction that is of little physiological significance. This reaction is uneconomical and probably occurs *in vitro* because SMM and SAM are able to occupy the same site on the enzyme by virtue of their common active centre, the sulphonium group (Figure 1). As evidence for this, all reported studies (Abrahamson and Shapiro, 1965; Shapiro and Yphantis, 1959; Balish and

Shapiro, 1967) have shown that SAM is inferior to SMM as a methyl donor in the formation of methionine from homocysteine.

As with all biological systems, the interpretation of biochemical reactions is complicated by the complexities of compartmentation. In mature pea cotyledons, Bain and Mercer (1966) have demonstrated the existence of eleven morphologically different types of subcellular membranes, so there are good possibilities for compartmentation in this tissue, which might effectively contribute to the control of methionine biosynthesis.

Future Studies of Methionine Biosynthesis

At present, the lack of evidence makes many of the above statements speculative and much more work is required before the control of methionine biosynthesis is fully understood. Compared to other tissues, the situation in pea seeds may be anomalous, because methionine will be produced by extensive proteolysis of storage protein (Lawrence, *et al.* 1959). For this reason, further studies on the control of methionine biosynthesis could be more profitably carried out on tissues which do not have the complicating feature of methionine production by proteolysis.

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