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CONFIRMATION OF A PING-PONG MECHANISM FOR S-ADENOSYL-L-METHIONINE:MAGNESIUM PROTOPORPHYRIN METHYLTRANSFERASE OF ETIOLATED WHEAT BY AN EXCHANGE REACTION

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Summary. An exchange reaction between unlabeled S-adenosyl-L-methionine and radiolabeled S-adenosyl-L-homocysteine has been used to confirm the occurrence of a ping-pong mechanism in S-adenosyl-L-methionine:magnesium protoporphyrin methyltransferase of etiolated wheat. The enzyme, S-adenosyl-L-homocysteine hydrolase, has been used to prepare radiolabeled S-adenosyl-L-homocysteine from labeled adenosine and DL-homocysteine. The exchange reaction was accomplished with a methyltransferase preparation purified by affinity chromatography on hemin-linked Sepharose 4B, and radioactivity was exchanged into unlabeled S-adenosyl-L-methionine to an extent of 70% of the theoretical maximum value.

S-Adenosyl-L-methionine:magnesium protoporphyrin methyltransferase (EC 2.1.1.11) catalyzes a step in the biosynthesis of chlorophylls and has been isolated from several different types of photosynthetic tissue, including a photosynthetic bacterium (1), a single-celled alga (2), and higher plants (3-5). Kinetic studies carried out in this laboratory have established that the methyltransferase from the alga, *Euglena gracilis*, exhibited a random Bi-Bi kinetic reaction mechanism (6), while that from the photosynthetic bacterium, *Rhodobacter sphaeroides*[1], exhibited an equilibrium ordered Bi-Bi mechanism with MgP as the first substrate (7). Ellsworth *et al.* (8) have carried out a kinetic study on the methyltransferase from etiolated wheat and determined that both its kinetic behavior and the pattern of inhibition by the two products, SAH and MgPME, was consistent with a ping-pong reaction mechanism:

$$E + SAM \rightleftharpoons [E{\cdot}SAM] \rightleftharpoons [E\text{-}CH_3{\cdot}SAH] \rightleftharpoons E\text{-}CH_3 + SAH$$
$$E\text{-}CH_3 + MgP \rightleftharpoons [E\text{-}CH_3{\cdot}MgP] \rightleftharpoons [E{\cdot}MgPME] \rightleftharpoons E + MgPME$$

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Abbreviations used: E, enzyme; E-CH_3, methylated enzyme; MgP, magnesium protoporphyrin; MgPME, magnesium protoporphyrin monomethyl ester; SAH (and SAH*), unlabeled (and labeled) S-adenosyl-L-homocysteine; SAM (and SAM*), unlabeled (and labeled) S-adenosyl-L-methionine.

[1]Previously known as *Rhodopseudomonas sphaeroides*.

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This kinetic mechanism is very difficult to prove conclusively because one seldom obtains exactly parallel lines predicted for it on "double reciprocal" (Lineweaver-Burke) plots. Work from this laboratory (9) has determined that the behavior of the wheat methyltransferase during affinity chromatographic purification was also consistent with its possessing a ping-pong mechanism; however, Hinchigeri *et al.* (9) were unable to demonstrate the formation of a methylated enzyme during its incubation with [*methyl*-^{14}C]SAM, and were thus unable to directly confirm such a mechanism.

Hinchigeri (10) proposed an exchange reaction which was designed to provide direct experimental evidence for the presence of a ping-pong mechanism in the methyltransferase. Incubation of the enzyme with SAM would result in the transfer of the S-methyl group of SAM to the enzyme in the first step of a ping-pong mechanism. If the incubation mixture contained radiolabeled SAH in place of MgP, however, exchange of it for unlabeled SAH in the enzyme's active site (followed by reversal of the first step) would yield labeled SAM:

$$E + SAM \rightleftharpoons [E{\cdot}SAM] \rightleftharpoons [E\text{-}CH_3{\cdot}SAH] \rightleftharpoons E\text{-}CH_3 + SAH$$
$$E\text{-}CH_3 + SAH^* \rightleftharpoons [E\text{-}CH_3{\cdot}SAH^*] \rightleftharpoons [E{\cdot}SAM^*] \rightleftharpoons E + SAM^*$$

We have carried out such an exchange study and have thus been able to provide direct experimental confirmation for the presence of a ping-pong mechanism for the methyltransferase of etiolated wheat.

METHODS AND MATERIALS

Preparation of labeled S-adenosyl-L-homocysteine. The method was a modification of that of de la Haba and Cantoni (11). SAH hydrolase (EC 3.3.1.1; Sigma; 1.2 units) was incubated in 1.0 mL of 5 mM potassium phosphate buffer, pH 7.2, at 37°C for 1 h with 50 mM DL-homocysteine (Sigma) and 10 mM [2,8-^{3}H]adenosine (ICN; 50 Ci mol^{-1}), or for 23 h with 60 mM DL-homocysteine and 10 mM [8-^{14}C]adenosine (ICN; 0.33 Ci mol^{-1}). The extent of reaction was assayed during the incubation by determining the decrease in the amount of homocysteine thiol groups by a modification of the nitroprusside reaction of Grunert and Phillips (12). Aliquots of 0.01 mL were equilibrated for 5 min in 8.0 mL saturated NaCl, followed by the addition of 1.0 mL of 0.067 M sodium nitroprusside and 1.0 mL of 1.5 M Na_2CO_4 plus 0.067 M NaCN; A_{540} was read after 1 min on a Coleman Junior II colorimeter. For preparation of [^{3}H]SAH, the reaction mixture was boiled for 2 min and added to a 1 x 13 cm column of Amberlite IRA-400 anion exchange resin (Sigma) equilibrated with 0.5 M $NaHCO_3$ by the method of Shapiro and Ehninger (13). Eluates of this and all other chromatographic procedures were monitored by an LKB Uvicord II flow monitor. Unreacted [2,8-^{3}H]adenosine was washed from the column with 600 mL water and the labeled SAH was eluted with 0.1 M NaCl and lyophilized. Labeled SAH fractions from two preparations were combined, diluted by the addition of 5 μmol of unlabeled SAH (Sigma), and desalted on a 1.7 x 88 cm column of Sephadex G-10 (Pharmacia). Following lyophilization, the SAH was found to have a specific activity of 11.6 Ci mol^{-1}. For preparation of [^{14}C]SAH, the small molecular weight products and excess reactants were separated from the enzyme by passage through a column of Sephadex G-75 (Pharmacia), eluted with 2 mM thiodiglycol (Sigma), and added to a 1 x 20 cm column of Dowex 1 anion exchange resin (Sigma) equilibrated with 0.5 M $NaHCO_3$ (13). The column was developed as described above for [^{3}H]SAH, except that the solvents all contained 2 mM thiodiglycol. The eluted [^{14}C]SAH fraction was found to have a specific activity of 0.27 Ci mol^{-1}.

Exchange reaction with a crude methyltransferase preparation. The methyltransferase was prepared from etiolated wheat (*Triticum aestivum*) in the isolation buffer of Hinchigeri *et al.* (9).

The extract contained ca. 15 mg mL^{-1} protein (determined by the method of Lowry *et al.* [14]) and was stored frozen until used. Methyltransferase activity in this crude extract was assayed by incubation of aliquots (containing 2.5, 5.0, and 10.0 mg protein) of the thawed supernatant with 0.035 mM MgP (synthesized by the method of Hinchigeri *et al.* [9]) and 0.219 mM [*methyl*-^{14}C]SAM (ICN; 1 Ci mol^{-1}) in 1 mL of the isolation buffer. A control containing 5.0 mg protein but no MgP was also run. Incubation was for 1 h at 37°C. The reaction was terminated by the addition of 4 mL acetone-0.1 M NH_4OH (9:1, v/v), and the metalloporphyrins extracted into diethyl ether by the method of Ellsworth and Murphy (15). The activities of Fig. 2 were calculated from the total nCi of radioactivity incorporated into the ether extracts, after subtraction of the control value. For exchange reactions with the crude extract, aliquots of the thawed supernatant containing 2.5 mg protein were incubated with 0.2 mM SAM and 0.2 mM [^{3}H]SAH (5.8 Ci mol^{-1}) brought to a final volume of 1.0 mL with 0.2 M Tris-Cl, pH 7.8. Incubations were carried out at 37°C for 10, 20, 30, 45, and 60 min. The reactions were terminated by the addition of 3.0 mL acetone. After 5 min, the acetone was evaporated, 1.0 mL of water was added, and denatured protein was removed by centrifugation for 10 min at 5000*g*. A "0 min" control was carried out by an immediate denaturation of the enzyme with acetone, followed by incubation of the sample for 60 min at 37°C to detect any chemical exchange. The solutions were then applied to columns containing 3.8 mL of Dowex 50W cation exchange resin (Sigma) equilibrated with 0.1 M NaCl by the method of Shapiro and Ehninger (13). The [^{3}H]SAH was eluted with 200 mL of 0.1 M NaCl, during which time the radioactivity in the eluate was reduced to a background level. The SAM fraction was then eluted with 3 M H_2SO_4; fractions of 10 mL were collected and values obtained for their absorbance at 260 nm (measured with a Varian model 634 spectrophotometer) and radioactive content (measured in 5 mL Scinti-Verse II [Fischer] on an LKB-Wallac model 1217 Rackbeta liquid scintillation counter).

Exchange reaction with purified methyltransferase. The crude extract was purified by affinity chromatography on hemin-linked Sepharose 4B. CNBr-activated Sepharose 4B (Sigma) was first reacted with 1.0 M 1,6-diaminohexane (Sigma) in 0.4 M $NaHCO_3$, pH 10.0, before being coupled to hemin by the method of Suttnar *et al.* (16). The crude extract (in 10.0 mL containing 0.23 mM SAM) (Sigma) was added to 16 mL of the affinity gel in a Sephadex K9/15 column, and developed at 4°C by the method of Hinchigeri *et al.* (9). The column was washed with 200 mL of the isolation buffer containing 0.23 mM SAM, and the methyltransferase was eluted following a change in the pH of the buffer from 7.8 to 9.0, and omission of SAM. The column eluate fraction containing the highest methyltransferase activity (15 nmol product h^{-1} [mg protein]$^{-1}$) and 0.02 mg protein mL^{-1}, as determined by the method of Bradford (17), was used for the exchange reaction *without prior freezing*. The activity of this fraction represented a 44-fold purification over that of the crude extract (0.34 nmol product h^{-1} [mg protein]$^{-1}$); 0.6 mL was combined with 1.0 mM SAM and 1.0 mM [^{14}C]SAH (15.3 mCi mol^{-1}) to a final volume of 1.0 mL of the isolation buffer, pH 7.8. Incubation was for 3 h at 37°C. The reaction was terminated by the addition of 3.0 mL of acetone and the SAM fraction was isolated as described above for the crude extract except that 3.8 mL of Amberlite CG-120 (Sigma) equilibrated with 0.1 M NaCl was used as the cation exchange resin.

RESULTS AND DISCUSSION

Radioactively labeled SAH is not available commercially; hence, it must be generated either from unlabeled SAH by specifically tritiating the 5'-position (18), synthesized chemically from labeled adenosine *via* 5'-chloro-5'-deoxyadenosine and L-homocysteine (19), or generated enzymatically. Using the latter approach, an attempt was first made to form labeled SAH from [*carboxy*-^{14}C]SAM using the enzyme, catechol O-methyl transferase (EC 2.1.1.6), and protocatechuic acid (20). This procedure, however, led to yields of only about 0.7% after 4 h of incubation (S.J. Eglsaer, unpublished observations). Next, the enzyme, SAH hydrolase (EC 3.3.1.1), was used (11,21). Although, metabolically, this enzyme catalyzes the hydrolysis of SAH, the equilibrium constant of this reaction (K_{eq} = 1.4 x 10^{-6}) greatly favors the direction of the *synthesis* of SAH (11). Fig. 1 shows two time courses for the synthesis of SAH from

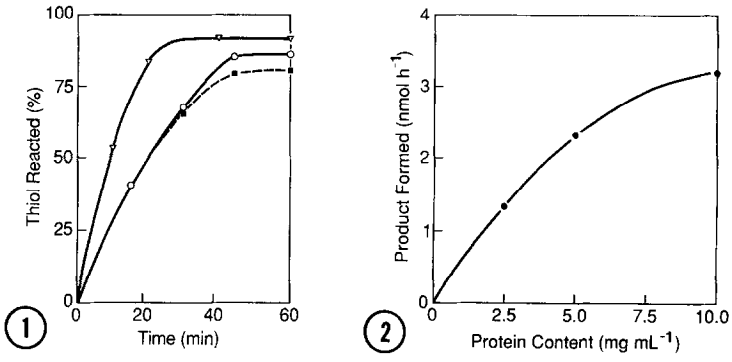


Fig. 1. Synthesis of SAH from adenosine and DL-homocysteine by the enzyme, SAH hydrolase. The disappearance of homocysteine thiol was measured by a nitroprusside colorimetric assay (12). Synthesis with fresh enzyme, using unlabeled adenosine (▽), or after storage of the enzyme for 45 days at 4°C, using unlabeled adenosine (O) or [3,8-^{3}H]adenosine (■).

Fig.2. Assay for SAM:MgP methyltransferase activity in a crude extract of etiolated wheat. The presence of the product, MgPME, was inferred from the incorporation of radioactivity into extracts.

adenosine and DL-homocysteine. When the enzyme was fresh, 1.2 units[2] brought the reaction 91% to completion in 25 min; however, it required 45 min to go 80-85% to completion when the enzyme was older. The older enzyme was used to synthesize [2,8-^{3}H]SAH fron [2,8-^{3}H]-adenosine and DL-homocysteine (Fig. 1). The labeled SAH was separated from residual labeled adenosine by ion exchange on Amberlite IRA-400/HCO_3^- (results not shown).

A crude extract of etiolated wheat was analyzed for methyltransferase activity as shown in Fig. 2. The product, MgPME, was not isolated and identified specifically; however, based on the radioactivity recovered in the assay, the maximum yield of MgPME (at 10 mg protein mL^{-1}) represented a 9.2% conversion of added MgP in 1 h. The actual conversion was probably higher as porphyrins become irreversibly absorbed onto the denatured proteins. Aliquots of the crude extract were incubated with equimolar amounts of [^{3}H]SAH and SAM for 10, 20, 30, 45, and 60 min. The initial specific activity of the [^{3}H]SAH was estimated to be 5.8 Ci mol^{-1}, based on its absorbance at 260 nm. Hence, the maximum specific activity expected to be incorporated into SAM during the exchange would be one-half that value, or 2.9 Ci mol^{-1}. Following the exchange, the residual [^{3}H]SAH was separated from the SAM pool by ion exchange on Dowex 50W/Na$^+$. The purified SAM fraction (Fig. 3) was found to be labeled after 10 min of incubation and its specific activity increased in a linear manner for the first 30 min of incubation, reaching a maximum of 0.14 Ci mol^{-1} after 45 min (Fig. 4). This specific activity is far less than the maximum expected, and no doubt reflects the fact that the [^{3}H]SAH had extensively decomposed. It is likely that tritium atoms on the 3- and 8- positions of the

[2]A unit is defined as 1 nmol SAH *hydrolyzed* min^{-1}; however, our results have demonstrated that 1 unit of the enzyme could *synthesize* 500 nmol of SAH min^{-1} (*cf.* Fig. 1). It is assumed that the enzyme selects the L-isomer of homocysteine from the racemic DL-mixture used for the synthesis.

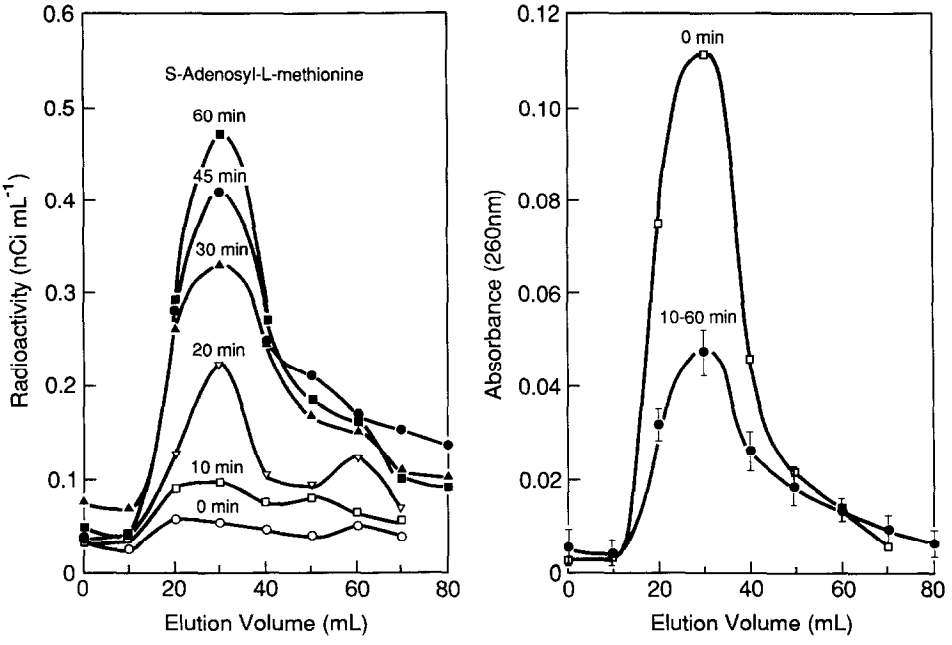
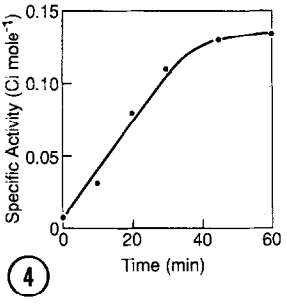


Fig. 3. Elution of labeled SAM fractions from a Dowex 50W/Na^+ cation exchange resin with 3 M H_2SO_4, following exchange between [^{3}H]SAH and unlabeled SAM by the methyltransferase preparation of Fig. 2. Labeled SAH was first eluted with 600 mL of 0.1 M NaCl (not shown). Fractions were assayed for radioactivity (left side) after 0 (○), 10 (□), 20 (▽), 30 (▲), 45 (●), and 60 (■) min of exchange, and A_{260} (right side) after 0 (□) and 10-60 (●) min of exchange; values for the latter all fell within the experimental limits indicated.

adenine ring exchanged with protons during prolonged exposure to water. Also, SAH is chemically unstable and subject to hydrolysis of the glycosidic bond to S-ribosylhomocysteine (22) or to spontaneous oxidation during exposure to air (23). The recovery of SAM after the chemical exchange control was 88.2%, but varried between 41.5% and 50.9% (average 47.4%) for the enzymic exchanges (Fig. 3). The recovery of SAM was not related to the time of incubation; hence it may not have been due to a biochemical decomposition by some other enzyme in the crude extract, but to a loss or decomposition during the extraction procedures.

The results of the exchange did, however, provide conclusive evidence for the occurrence of a ping-pong mechanism in an unspecified methyltransferase contained in the extract. It remained only to show that the *desired* SAM:MgP methyltransferase was responsible for the exchange. This question was addressed after first preparing fresh labeled SAH, taking the following precautions during either its synthesis or its purification: adenosine labeled with ^{14}C instead of ^{3}H was used, a longer reaction time was employed, the SAH hydrolase was removed from the reaction mixture by gel filtration on Sephadex G-75 prior to purification, and all solvents during gel filtration and ion exchange chromatography contained the antoxidant thiodiglycol. Fig. 5 shows the results of an ion exchange separation of the reaction mixture on Dowex 1/HCO_3^-. The [^{14}C]SAH was again eluted with 0.1 M NaCl; a yield of 28.4% was obtained, and its specific activity was found to be 0.27 Ci mol^{-1}. There was a significant amount of radioactivity and material absorbing at 260 nm which was eluted in the water wash; it was not, however, unreacted adenosine. Fig. 5 indicates where adenosine would have been



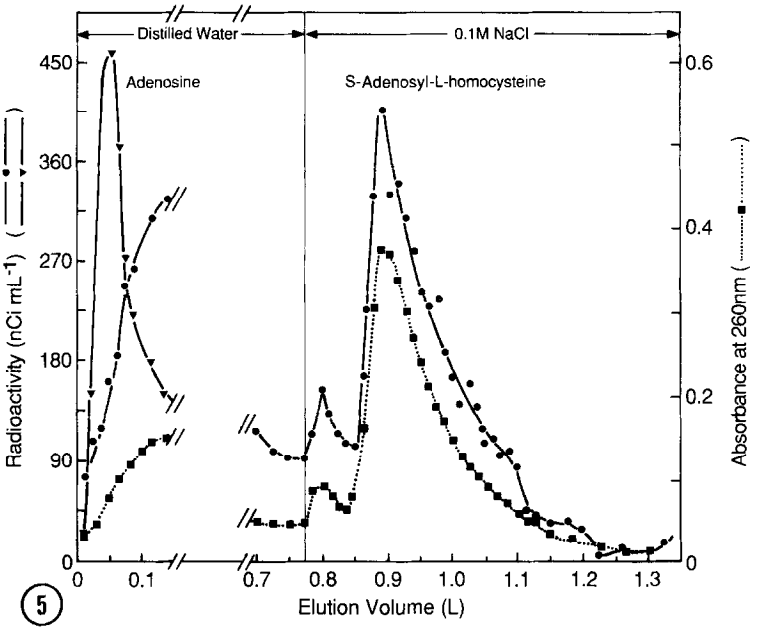


Fig. 4. Specific activities of labeled SAM fractions following the separations of Fig.3.

Fig. 5. Separation of labeled SAH on Dowex 1/HCO_3^- anion exchange resin, following its synthesis from [8-^{14}C]adenosine and DL-homocysteine. Fractions were assayed for radioactivity (●) and A_{260} (■). The figure also shows the elution of radioactivity due to [8-^{14}C]adenosine (▼) during a separate run.

eluted if it were present. It may, therefore, have been inosine, adenine, or other decomposition product(s) of adenosine and/or SAH.

Equimolar amounts of the freshly prepared [^{14}C]SAH and SAM were then incubated for 3 h with a freshly prepared methyltransferase fraction purified by affinity chromatography. The method of Hinchigeri *et al.* (9) was used for the affinity chromatography which employs hemin, a competitive inhibitor of MgP, as the affinity ligand. The enzyme is bound by the gel only in the presence of SAM (9). This method thus assures that any methyltransferase in the eluate has affinity for a metalloporphyrin, virtually limiting it to the desired methyltransferase. The specific activity of the [^{14}C]SAH was estimated to be 15.3 mCi mol^{-1}, based on its absorbance at 260 nm. Hence, the maximum specific activity expected to be incorporated into SAM during the exchange would be 7.65 mCi mol^{-1}. Following the exchange, the residual [^{14}C]SAH was separated from the SAM pool by ion exchange on Amberlite CG-120/Na^+ (Fig. 6). A recovery of 67.7% of the added SAM was obtained, and its specific activity was found to be 5.4 mCi mol^{-1}, or 70.5% of the theoretical maximum value[3]. A ping-pong mechanism for S-

[3]Exchange to this extent indicates that the first step of the ping-pong mechanism (the formation of the methylated enzyme) and its reversal (the transfer of the methyl group back to SAH) must be in a very rapid equilibrium compared with the second step (the transfer of the methyl group to MgP).

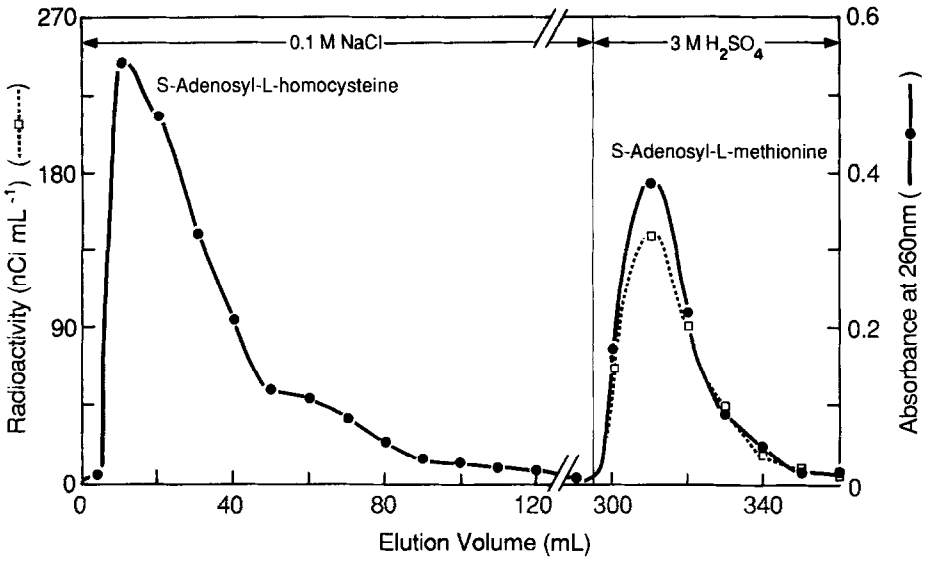


Fig. 6. Separation of labeled SAM from labeled SAH on Amberlite CG-120/Na+ cation exchange resin, following exchange between [^{14}C]SAH and unlabeled SAM by a methyltransferase purified by affinity chromatography on hemin-linked Sepharose 4B. Fractions were analyzed for radioactivity (□) and A_{260} (●).

adenosyl-L-methionine:magnesium protoporphyrin methyltransferase (EC 2.1.1.11) from etiolated wheat has, therefore, been firmly established.

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