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Methionine Metabolism in Mammals: Synthesis of S-Adenosylhomocysteine in Rat Tissues[1]

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We have developed a sensitive double-isotope assay to measure the enzymatic synthesis of *S*-adenosylhomocysteine from adenosine and L-homocysteine. The enzyme is present in all rat tissues except the small intestinal mucosa. In the liver, the enzyme is found in the postmicrosomal supernatant. We studied some of the kinetic properties of the liver enzyme. At high concentration, both substrates inhibit product formation. *S*-Adenosylmethionine also inhibits the reaction but other metabolites of methionine do not. The specific activity of the enzyme in liver increases in animals fed a high protein ration. Treatment with hydrocortisone and estradiol also resulted in an increase in the specific activity while administration of thyroxine led to a decrease.

In mammals, the regulation of methionine metabolism may depend on the disposition of homocysteine (1, 2). This compound can be converted irreversibly to cystathionine or may be remethylated, thereby conserving methionine. For this reason, we have focused our attention on the factors which regulate the activities of cystathionine synthase, betaine homocysteine methyltransferase (EC 2.1.1.5), and N^5-methyltetrahydrofolate homocysteine methyltransferase (3, 4). However, homocysteine may also react with adenosine to form *S*-adenosylhomocysteine (5). Since this reaction is reversible and the biological "flow" is from methionine to homocysteine, the enzyme involved has been designated *S*-adenosylhomocysteine hydrolase (EC 3.3.1.1).[3] In fact, the thermodynamic equilibrium strongly favors the synthesis of *S*-adenosylhomocysteine (5). There is also evidence that synthesis can be significant *in vivo*. *S*-Adenosylhomocysteine, but not homocysteine, accumulates in mammalian liver (6). This finding is magnified in patients with cystathionine synthase deficiency who excrete *S*-adenosylhomocysteine and its derivatives in the urine (7). It seems likely that the direction of the biological flow depends on the removal of homocysteine and adenosine. In turn this shifts the unfavorable equilibrium toward hydrolysis. Should adenosine and homocysteine accumulate, synthesis would occur.

An increase in tissue *S*-adenosylhomocysteine due to either decreased hydrolysis or augmented synthesis may effect significantly other reactions involved in the methionine pathway. *S*-Adenosylhomocysteine is a potent inhibitor of several transmethylation reactions (8–11). It may also facilitate the synthesis of N^5-methyltetrahydrofolate (12).

In the present study, we present a new technique for the assay of *S*-adenosylhomocysteine hydrolase in the direction of synthesis. Using this assay we have studied the

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[3] As noted, this enzyme has been named *S*-adenosylhomocysteine hydrolase (EC 3.3.1.1). In this paper, we use the term *S*-adenosylhomocysteine synthase since we are assaying in the direction of synthesis. In addition, the thermodynamics of the reaction favor synthesis of *S*-adenosylhomocysteine rather than hydrolysis.

distribution of the enzyme in rat tissue and the effect of diet and hormone treatments on the hepatic activity of the enzyme. In addition, we have investigated some of the kinetic properties of the enzyme.

EXPERIMENTAL PROCEDURE

Reagents. All chemicals were of reagent grade and were purchased from commerical sources. *S*-[8-^{3}H]Adenosylhomocysteine was synthesized enzymatically from [8-^{3}H]adenosine (Schwarz/Mann) using the method of Shapiro and Ehninger (13). We prepared L-homocysteine from L-homocysteine thiolactone hydrochloride (Calbiochem) according to the procedure of Duerre (14). Our partially purified enzyme preparation is the 0–80% ammonium sulfate fraction of de la Haba and Cantoni (5).

Animals and extracts. We routinely used male Sprague–Dawley rats weighing 150–200 g. The animals were stunned and exsanguinated by carotid transection. Tissue extracts were prepared immediately by homogenization in cold .01 M potassium phosphate buffer, pH 7.4, followed by centrifugation at 8000*g* for 15 min at 4°C. In the standard extract of liver, we employed 3 vol of buffer. Protein concentrations were determined by the Lowry method (15).

Diets and hormones. Wayne Lab Blox was the standard diet. General Biochemicals supplied the special diets including: protein-free (vitamin supplemented), low-protein (3.5% casein), normal-protein (26% casein), and high-protein (55% casein) rations. The animals were fed the diets for at least seven days before we tested the effect of diet on enzyme activity. The following hormones were injected subcutaneously: hydrocortisone acetate (Pfizer, 5 mg/day for 7 days); L-thyroxine (Nutritional Biochemicals, 0.5 mg/day for 7 days); estradiol (Schering, 0.2 mg/day for 7 days); growth hormone (Nutritional Biochemicals, 2 U/day for 7 days); and glucagon (Eli Lilly, 1.2 mg in six divided doses during the 24 hr prior to sacrifice). Control animals were injected with the appropriate solvent or vehicle. In the glucagon experiments, both the experimental and control animals received a low-protein diet for 7 days and were fasted during the period of hormone treatment. In all of the other studies of the effects of hormone treatments, the rats were fed the standard laboratory ration.

Expression of results. We define 1 U of enzyme activity as the quantity required for the synthesis of 1 nmole of *S*-adenosylhomocysteine during 10 min under standard assay conditions. Results appear as U/mg protein, U/g liver, or total hepatic U/g body weight. For statistical comparisons we employed Students "*t*" test for unpaired samples.

Routine assay procedure. The assay solution contained 0.2 M potassium phosphate, pH 7.3; 2 mM L-homocysteine; and 2.5 mM [8-^{14}C]adenosine, (10^5 dpm) in a final volume of 1.0 ml. We stopped the reaction with 0.1 ml 30% perchloric acid and then added 0.1 ml of *S*-[8-^{3}H]adenosylhomocysteine (1.5×10^6 dpm). After centrifugation, 1 ml of the supernatant was neutralized with 1 M $KHCO_3$. The precipitate was again removed by centrifugation and 1 ml of the supernatant was diluted with 4 ml of water. Four milliliters of this material was placed on a column of AG-50 (H^+) × 4 (100–200 mesh), 0.9 × 3.0 cm. The column was washed with 10 ml of 1% thiodiglycol, 30 ml of 1 N HCl, and 10 ml of 1% thiodiglycol. This wash eluted the unreacted adenosine while the *S*-adenosylhomocysteine is retained on the column. The product was eluted with 3 N NH_4OH. More than 90% appears in an 8-ml aliquot. Subsequently, we mixed 2 ml of the ammonium hydroxide eluate with 20 ml Scintisol (Isolab). ^{3}H and ^{14}C are determined directly in an Intertechnique Model 40 Liquid Scintillation System.

RESULTS

Validation of assay. In this assay we used the ^{3}H/^{14}C ratio in the NH_4OH eluate in order to calculate the total amount of ^{14}C-labeled product that was formed during the reaction. A significant contribution to the ratio by other ^{14}C compounds would invalidate the method. This possibility was tested in studies with both the partially-purified enzyme and the crude liver preparation. After the usual incubation we chromatographed the perchloric acid supernatant as well as the NH_4OH eluate from the column. Chromatography was performed on Whatman No. 1 paper in the following solvents: (1) *n*-butanol–acetic acid–water 4:1:5; (2) acetone–water, 4:1; (3) isopropanol–formic acid–water, 7:1:2; and (4) *t*-butanol–methyl ethyl ketone–ammonia–water, 4:3:1:2. The chromatograms were cut into 1-cm strips which were oxidized to allow determinations of ^{14}C and ^{3}H. We found that the ratio of isotopes that co-chromatographed with authentic *S*-adenosylhomocysteine was equivalent in perchloric acid supernatant and NH_4OH eluate. Both values were also in good agreement with the ratio obtained when we counted the NH_4OH eluate directly.

Conditions of assay. In a series of studies with 0.05 M potassium phosphate buffers, we found that pH 7.3 was optimal. Increasing

the buffer concentration to 0.2 M did not effect the reaction rate; therefore, we employed this higher concentration routinely. Equivalent activity was observed with other buffer ions (HEPES, Tricine, and Tris).

As shown in Table I, product formation was proportional to the concentration of the substrates. With 2.0 mM L-homocysteine the maximum velocity of the reaction was observed at adenosine = 1.0 mM. Concentrations of adenosine in excess of 2.5 mM inhibited product formation. At 10 mM adenosine, *S*-adenosylhomocysteine synthesis was 61% of the maximum value. Similarly, product formation was directly proportional to the concentration of L-homocysteine at the lower concentrations of this substrate (Table I, Experiment B). With 2.5 mM adenosine, the maximum velocity corresponded to 1.0 mM L-homocysteine. Concentrations of L-homocysteine greater than 2.5 mM inhibited the reaction with total inhibition at 10.0 mM. This inhibition was specific for homocysteine (Table II).

TABLE I

EFFECT OF SUBSTRATE CONCENTRATION ON PRODUCT FORMATION[a]

	nmoles/10 min
Experiment A (L-homocysteine = 2.0 mM)	
Adenosine (mM)	
0.1	69.8
0.25	157.0
0.5	263.7
0.75	283.8
1.0	327.2
2.5	295.2
5.0	262.1
Experiment B (adenosine = 2.5 mM)	
L-Homocysteine (mM)	
0	0
0.1	50.3
0.25	118.4
0.5	235.9
1.0	282.2
2.5	268.0
5.0	139.3
10.0	0

[a] We employed 0.1 mg of partially-purified enzyme in these assays.

TABLE II

EFFECT OF SULFHYDRYL COMPOUNDS ON *S*-ADENOSYLHOMOCYSTEINE SYNTHESIS[a]

Compound added (μmoles)	Product formed (nmoles)
None	554
L-Homocysteine, 5.0	52.5
L-Cysteine, 5.0	585
L-Glutathione (reduced), 8.0	436
Dithiothreitol, 5.0	558

[a] 0.1 mg of partially-purified enzyme was assayed under standard conditions. The unsupplemented reaction medium contained 2 mM L-homocysteine.

L-Cysteine and dithiothreitol did not alter the reaction rate while reduced glutathione inhibited it to a minimal degree.

We tested the effect of several intermediates of sulfur amino acid metabolism. The addition of L-methionine, 10 mM, resulted in a 20% reduction in product formation. L-Cystathionine, 6.0 mM, and *S*-adenosylhomocysteine, 5.2 mM, had no effect. *S*-Adenosylmethionine strongly inhibited the synthesis of *S*-adenosylhomocysteine. Product formation in the presence of 3.5 mM *S*-adenosylmethionine was 6% of the control value.

The synthesis of *S*-adenosylhomocysteine depended on the duration of incubation up to 30 min (Table III). Product formation was proportional to the amount of added protein from 0.1 mg to 0.4 mg with the partially-purified preparation and from 1 mg to 5 mg with a crude extract of liver (Table IV). We performed all routine assays within these ranges.

Tissue distribution of enzymes. We found enzyme activity in ten rat tissues but not in extracts prepared from small intestinal mucosa (Table V). Product formation depended on the presence of homocysteine in the reaction media. *S*-Adenosylhomocysteine was identified specifically in the studies of liver, kidney, pancreas, and brain. We considered the possibility that the low level of activity in some tissues was due to the presence of inhibitors or of competing enzymes in the crude extracts. However, in coincubation studies, preparations of brain,

TABLE III

EFFECT OF DURATION OF INCUBATION ON PRODUCT FORMATION[a]

Incubation time (min)	Product formation (nmoles)
10	166.1
20	300.2
30	443.0
60	648.1

[a] 0.1 mg partially-purified enzyme was assayed under standard conditions.

TABLE IV

EFFECT OF ENZYME CONCENTRATION ON PRODUCT FORMATION[a]

Preparation	Concentration	Product formation (nmoles/ 10 min)
Crude extract	1 mg	284.3
	2 mg	515.6
	5 mg	1196.3
	10 mg	1235.2
Partially-purified	0.04 mg	10.5
	0.10 mg	150.4
	0.20 mg	346.8
	0.30 mg	496.3
	0.40 mg	640.7

[a] We employed the standard assay technique. The concentration of enzyme preparation is expressed as mg of protein added to the incubation media.

heart, and lung failed to inhibit liver enzyme activity. Extracts of small intestine and spleen contained a heat-labile factor which inhibited the liver enzyme. We found nothing to suggest that the inhibition by the small intestine preparation resulted from tryptic digestion of the liver enzyme. The intestinal extract is rich in adenosine deaminase. Preparations of this enzyme competed effectively for substrate with hepatic *S*-adenosylhomocysteine synthase. Possibly adenosine deaminase obscured the presence of *S*-adenosylhomocysteine synthase in crude extracts of small intestine. Based on studies in which we co-incubated varying amounts of intestinal extracts with liver enzyme, we can state that intestinal *S*-adenosylhomocysteine synthase would be detected in our assay system if the specific activity of the enzyme was approximately 100 nmoles/mg protein/10 min.

TABLE V

SPECIFIC ACTIVITY OF *S*-ADENOSYLHOMOCYSTEINE SYNTHASE IN RAT TISSUES[a]

Tissue	Specific activity (nmoles/mg protein/10 min)
Liver	387
Kidney	144
Pancreas	519
Brain	81
Adrenal	126
Spleen	21
Heart	10
Lung	29
Testes	59
Adipose	29
Small intestine	0

[a] Values represent means of triplicate determinations on pooled extracts prepared from groups of 5 animals.

Subcellular location of enzyme. The postmicrosomal supernatant fraction of liver contains almost all of the *S*-adenosylhomocysteine synthase found in the crude homogenate. Specific activity in the postmicrosomal fraction is 3.4 times that of the homogenate. The "nuclear" fraction had a specific activity equal to 30% of the crude preparation. We found no enzyme in the mitochondrial or microsomal fractions.

Effect of diet. Enzyme specific activity is highest in the livers of animals fed the high-protein diet (Table VI). This increase is significant ($P < .01$) when compared to each of the other diet groups. The significance of the differences in enzyme activity between the high-protein and low-protein fed animals is independent of the means employed to express the results. In contrast, the specific activity of the enzyme does not differ significantly between the low-protein and normal-protein groups. In this case, there is a significant difference when we employ U/g liver or U/liver/g body wt. This suggests that the increase in enzyme activity in the livers of animals fed the normal ration is relatively nonspecific and is associated with an increase in the content of other soluble proteins. Similarly, the increase in enzyme

TABLE VI

EFFECT OF DIET ON *S*-ADENOSYLHOMOCYSTEINE SYNTHESIS IN RAT LIVER[a]

Diet	U/mg protein	U/g liver	U/liver/g BW
Protein-free	137[b]	104	93
Low-Protein	100	100	100
Normal-Protein	120	185[c]	195[c]
High-Protein	206[c]	211[d]	226[d]

[a] Each diet group contained five animals fed the specified diet for 7 days. For each mode of expressing enzyme activity we list the mean value as a percentage of the mean value in the low-protein diet group.

[b–d] Statistical significance relative to low protein diet group: [b] $P = 0.05$–$.01$. [c] $P = 0.01$–$.001$. [d] $P < 0.001$.

TABLE VII

EFFECT OF HORMONES ON *S*-ADENOSYLHOMOCYSTEINE SYNTHASE IN RAT LIVER[a]

Treatment	U/mg protein	U/g liver	Total U/g body wt
Hydrocortisone (5)	124[b]	111	122[b]
Thyroxine (5)	84[b]	95	101
Estradiol (6)	120[c]	116[c]	124[c]
Growth hormone (5)	99	94	94
Glucagon (5)	95	82	96

[a] Results for the treated animals are expressed as the percentage of the value found simultaneously in an appropriate control group. The number of animals in each experimental group appears in parenthesis. We describe hormone preparations, dosages, and routes of administration in the text.

[b, c] Statistical significance relative to control value: [b] $P < 0.02$. [c] $P < 0.05$.

specific activity with total protein restriction (when compared to low-protein feeding) seems to reflect a loss of other soluble proteins rather than a true increase in *S*-adenosylhomocysteine synthase.

Effects of hormones on enzyme activity. None of the hormone treatments caused a marked change in hepatic activity of *S*-adenosylhomocysteine synthase (Table VII). Hydrocortisone and estradiol injections resulted in increased enzyme activity which was significant whether we expressed the results as specific activity (U/mg protein), as hepatic concentration (U/g liver), or as total hepatic content (U/liver/g body wt). This suggests a true increase in hepatic enzyme activity. In contrast, thyroxine treatment is associated with a statistically significant decrease in specific activity without a change in either the hepatic concentration or content of the enzyme. Thus, the decrease in specific activity might be the result of an increase in extractable protein. Indeed, the protein concentration in the crude extracts prepared from the livers of the thyroid-treated animals was 14% greater than that in the control preparations.

DISCUSSION

The reversible reaction between L-homocysteine and adenosine that yields *S*-adenosylhomocysteine is an integral part of the pathway for methionine metabolism in mammals. In the past, this enzyme has been assayed in the direction of hydrolysis, measuring the appearance of either free —SH groups or of adenosine. The effect of added homocysteine and adenosine could note be determined. In their original paper, de la Haba and Cantoni detailed several methods which measured enzyme activity in the direction of *S*-adenosylhomocysteine synthesis (5). In two of these techniques they measured the disappearance of substrate. These methods could not be used to assess accurately the effects of increasing substrate on product formation. Their third method employed paper chromatography in order to separate radioactive product from radioactive substrate ([8-^{14}C]adenosine). The appropriate spots were eluted and the radioactivity was measured. The ratio of ^{14}C in product and substrate was the basis for the quantitation of enzyme activity. Knudsen and Yall employed this technique in their study of the yeast enzyme (16). This method is too time-consuming for routine use and cannot be employed easily in the presence of enzymes competing for the substrate adenosine.

The assay which we developed avoided some of these problems. In our method, we also employed [8-^{14}C]adenosine as a substrate. After the reaction is terminated, we added *S*-[8-^{3}H]adenosylhomocysteine before we processed the reaction mixture through a cation exchange column. Since *S*-adenosylhomocysteine is relatively unstable during

exposure to acid, the addition of the second isotope allowed us to correct for the recovery of the ^{14}C-labeled product.

The significance of the *S*-adenosylhomocysteine hydrolase (synthase) reaction in normal methionine metabolism needs definition. As noted earlier, this enzyme can regulate both the availability of homocysteine and the tissue concentration of *S*-adenosylhomocysteine. In this study, we have obtained evidence that the activity of this enzyme is adaptable. Hepatic levels of *S*-adenosylhomocysteine synthase increase in animals fed a high-protein diet. Injections of estradiol and hydrocortisone also cause an increase in enzyme levels. All three of these treatments result in an increase in hepatic levels of *S*-adenosylmethionine synthetase (3). It is unlikely that the increase in the latter enzyme is linked directly to the increase in *S*-adenosylhomocysteine synthase. Growth Hormone treatment also increases *S*-adenosylmethionine synthetase (3) but has no effect on the *S*-adenosylhomocysteine enzyme.

In addition to regulation by control of the tissue levels of the enzyme, some degree of regulation of the *S*-adenosylhomocysteine hydrolase (synthase) reaction may be effected by the tissue levels of the reactants. The finding that *S*-adenosylhomocysteine synthesis can be inhibited by excess homocysteine or adenosine complements the observation by de la Haba and Cantoni that the hydrolytic reaction is inhibited by these compounds (5). The mechanism and the significance of the inhibition of *S*-adenosylhomocysteine synthase by *S*-adenosylmethionine needs clarification.

Similarly there is a need for additional information before we interpret the meaning of the distribution of *S*-adenosylhomocysteine synthase in rat tissues. In one regard, the present studies have extended our prevous observations based on a study of five other enzymes of methionine metabolism (4). It is still possible to conclude that the specific activities of the six enzymes are independent variables in each tissue. The specific activity of *S*-adenosylhomocysteine synthase does not correlate significantly with that of *S*-adenosylmethionine synthetase, cystathionine synthase, betaine homocysteine methyltransferase, N^5-methyltetrahydrofolate homocysteine methyltransferase, or cystathionase. Despite this marked variability, every tissue had been shown to possess the capacity to synthesize *S*-adenosylmethionine and to metabolize homocysteine by conversion to cystathionine and/or by remethylation to methionine. The absence of *S*-adenosylhomocysteine synthase in small intestine therefore represents the first suggestion of a block in methionine metabolism in any organ. If confirmed, it would raise several significant questions about the mechanism for metabolism of *S*-adenosylhomocysteine in small intestine as well as the role of cystathionine synthase in an organ which cannot generate homocysteine.

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