Biochimica et Biophysica Acta, 438 (1976) 522—531

BBA 67860

A SENSITIVE ISOTOPIC ASSAY METHOD FOR *S*-ADENOSYLHOMOCYSTEINE HYDROLASE

SOME PROPERTIES OF THE ENZYME FROM RAT LIVER

OLAVI KAJANDER, TERHO ELORANTA and AARNE RAINA

Department of Biochemistry, University of Kuopio, Kuopio (Finland)

(Received February 16th, 1976)

Summary

A rapid and sensitive isotopic method is presented for the assay of *S*-adenosylhomocysteine hydrolase (EC 3.3.1.1.) activity, based on the formation of radioactive *S*-adenosylhomocysteine labelled in the adenosine portion. The radioactive product is separated either by low-voltage paper electrophoresis or by using phosphocellulose ion-exchange paper. Some kinetic properties of the enzyme from rat liver have shown to be clearly different from those reported earlier for this enzyme. The use of erythro-9-(2-hydroxy-3-nonyl)adenine, a potent inhibitor of adenosine deaminase, makes it possible to measure the *S*-adenosylhomocysteine hydrolase activity in tissues with a high adenosine deaminase activity, e.g. in intestinal mucosa.

Introduction

S-adenosylhomocysteine is formed from *S*-adenosylmethionine in enzymic transmethylation reactions, as first demonstrated by Cantoni and Scarano [1]. Several recent observations (referred to in ref. 2) demonstrating that *S*-adenosylhomocysteine is a potent inhibitor of a number of methylating enzymes utilising *S*-adenosylmethionine as the methyl donor, has renewed interest in this compound. As the concentration of *S*-adenosylhomocysteine in several vertebrate tissues is similar in magnitude to the level of *S*-adenosylmethionine [3], it is possible that *S*-adenosylhomocysteine may have some regulatory functions in transmethylation reactions in vivo [2,4—7]. The observation that *S*-

Please address correspondence to: Dr. Aarne Raina, Dept. of Biochemistry, University of Kuopio, P.O. Box 138, SF-70101 Kuopio 10, Finland.

adenosylhomocysteine is able in vitro to inhibit *S*-adenosylmethionine decarboxylase, an enzyme involved in the synthesis of polyamines, may suggest another points of regulation (Raina, A., unpublished results).

Another route leading to the formation of *S*-adenosylhomocysteine was described by the la Haba and Contoni [8] who found an enzyme in rat liver catalysing the following reaction:

Adenosine + L-homocysteine $\rightleftharpoons$ S-adenosylhomocysteine + H_2O

Although the equilibrium lies far in the direction of condensation, *S*-adenosylhomocysteine is hydrolysed by this enzyme, *S*-adenosylhomocysteine hydrolase (EC 3.3.1.1), if the products of the reaction, adenosine and L-homocysteine, are removed enzymically [8]. *S*-adenosylhomocysteine hydrolase activity has been detected in a number of mammalian tissues [6,9] with the exception of small intestine which was reported to be devoid of hydrolase activity [9]. The hydrolase activity of rat liver appears to be under nutritional and hormonal control [9].

The results quoted above prompted us to explore the relationship between polyamine and methionine metabolism in greater detail. *S*-adenosylhomocysteine hydrolase activity may be one controlling element in this system. The methods previously used for the assay of the hydrolase activity are mostly based on the disappearance of free sulphhydryl groups of L-homocysteine [8, 10] or on column-chromoatographic separation of the formed *S*-adenosylhomocysteine labelled in the adenosine portion [9,11]. In the present paper we describe an assay method for *S*-adenosylhomocysteine hydrolase activity which combines sensitivity and specificity and is fairly rapid. Our results show that some kinetic properties of *S*-adenosylhomocysteine hydrolase from rat liver are different from those previously published for this enzyme [6,9].

Materials and Methods

Chemicals

[8-^{14}C]adenosine (specific activity 59 mCi/mmol) was purchased from the Radiochemical Centre, Amersham, U.K. Adenosine, adenine, hypoxanthine and uric acid were products of E. Merck, Darmstadt. Inosine and xanthine were supplied by Fluka AG and dithiothreitol by Calbiochem. A sample of erythro-9-(2-hydroxy-3-nonyl)adenine, prepared by Dr. H.J. Schaeffer (Wellcome Research Laboratories, Research Triangle Park, N.C., U.S.A.) [12], was obtained through the generosity of Dr. Seymour S. Cohen, Denver, Colo., U.S.A.

S-adenosyl-L-homocysteine and L-homocysteine thiolactone were obtained from Sigma. L-homocysteine was prepared anaerobically from L-homocysteine thiolactone with 5 M NaOH according to the method of Duerre and Miller [13]. When stored under nitrogen at −20°C, L-homocysteine was found to be stable for at least one month. Sulphhydryl groups were determined with the nitroprusside reagent as described by Grunert and Phillips [14].

Enzyme preparations

Female Wistar rats were used at the age of 2—3 months. The animals were de-

capitated under ether anaesthesia, the livers were rapidly removed and homogenized with 3 vol. of cold 0.01 M potassium phosphate buffer (pH 7.3) in a Potter-Elvehjem homogenizer. For preparation of the enzyme from intestinal mucosa, the intestine was carefully rinsed with the homogenization medium. The homogenate was centrifuged for 45 min at 100 000 × *g* and the supernatant fraction was used for enzyme assays or stored at −80° C. Under these conditions the enzyme activity remained unchanged for at least one week. *S*-adenosylhomocysteine hydrolase from rat liver was partially purified to the ammonium sulphate step (0—80%) described by de la Haba and Cantoni [8].

Protein was measured by the method of Lowry et al. [15].

Assay of S-adenosylhomocysteine hydrolase activity

Incubation conditions. The enzyme activity was measured in the direction of *S*-adenosylhomocysteine synthesis. The standard incubation mixture contained, in a final volume of 0.25 ml, 3 mM L-homocysteine, 0.2 mM [8-^{14}C]adenosine (specific activity 2.0 mCi/mmol), 2 mM dithiothreitol, 100 mM potassium phosphate buffer (pH 7.3) and 20—60 μg of crude enzyme protein. Where indicated, an inhibitor of adenosine deaminase (erythro-9-(2-hydroxy-3-nonyl)adenine) was included at a concentration of $1—10 \cdot 10^{-6}$ M. After incubating the tubes for 5 min at 37° C the reaction was stopped by adding 25 μl of 30% perchloric acid. The precipitate was removed by centrifugation and the supernatant stored in ice until analyzed for radioactive *S*-adenosylhomocysteine as described below.

Under these conditions the reaction rate was linear with time up to at least 10 min and with the amount of the enzyme protein in the range indicated above. The reaction was completely dependent on the presence of L-homocysteine in the incubation mixture. The rate of *S*-adenosylhomocysteine synthesis was the same whether the incubation was carried out under nitrogen atmosphere or in the presence of air.

Paper electrophoretic separation of S-adenosylhomocysteine. A 50 μl-aliquot of the perchloric acid supernatant was neutralized with 3 μl of 3 M KOH in a small test tube. An appropriate amount of a mixture of marker substances (adenosine, *S*-adenosylhomocysteine, inosine and hypoxanthine) in 50 μl of water was added, the precipitate removed by centrifugation and an aliquot of the supernatant subjected to paper electrophoresis (cf. ref. 16). An aliquot of 10 or 20 μl of the mixture was applied to a paper strip (Whatman No. 1, 3 × 40 cm) previously wetted in buffer solution (0.05 M glycine, adjusted to pH 11.0 with sodium hydroxide). A constant potential of 300 V (8 V/cm) was applied, which produced sufficient resolution of the above compounds in 120—150 min. The paper strips were dried and the fractions marked under ultraviolet light. The fractions were cut out and counted for radioactivity in a toluene-based liquid scintillator (4 g of PPO, 50 mg of dimethyl-POPOP/l of toluene) with an efficiency of 65%.

Separation of S-adenosylhomocysteine by phosphocellulose ion-exchange paper. The method used for separation of *S*-adenosylmethionine by McKenzie and Gholson [17] was modified as follows. An aliquot (20—40 μl) of the non-neutralized perchloric acid supernatant was applied to a double layer of phosphocellulose ion-exchange paper (Whatman P 81, diameter 24 mm). The papers

were wetted thoroughly with water, washed with 300 ml of 0.001 M HCl, dried and counted for radioactivity as described above. Under these conditions *S*-adenosylhomocysteine but not adenosine, inosine nor hypoxanthine were retained by the ion-exchange paper. The use of 0.005 M HCl for washing resulted in some loss (about 10%) of *S*-adenosylhomocysteine. On the other hand, washing with 0.1 M acetic acid did not effectively remove radioactive adenosine nor its degradation products from the paper.

The results obtained by these two methods generally agreed within 5%. The enzyme activity is expressed as nmol of *S*-adenosylhomocysteine formed/min per mg of protein if not otherwise indicated. The recovery of *S*-adenosylhomocysteine was checked by adding radioactive *S*-adenosylhomocysteine to the system after the addition of perchloric acid and was found to be 98—100%. Therefore, no corrections for losses of the product during the isolation procedure are needed under standard assay conditions (c.f. ref. 9).

Results

Paper electrophoretic separation of S-adenosylhomocysteine

Several acid and alkaline buffer systems including citric acid, barbituric acid, boric acid and glycine buffers were tested for paper electrophoretic separation of radioactive *S*-adenosylhomocysteine from adenosine and various metabolites, e.g. inosine and hypoxanthine originating from adenosine as a result of various competing enzyme activities present in crude enzyme preparations [18]. With the 0.05 M glycine buffer (pH 11.0) which was finally adopted, a distinct separation of *S*-adenosylhomocysteine from adenosine and other adenosine metabolites was achieved (Fig. 1). Some overlapping occasionally found between *S*-adenosylhomocysteine and adenine did not interfere, because the latter was not found among adenosine metabolites in this system. Analysis

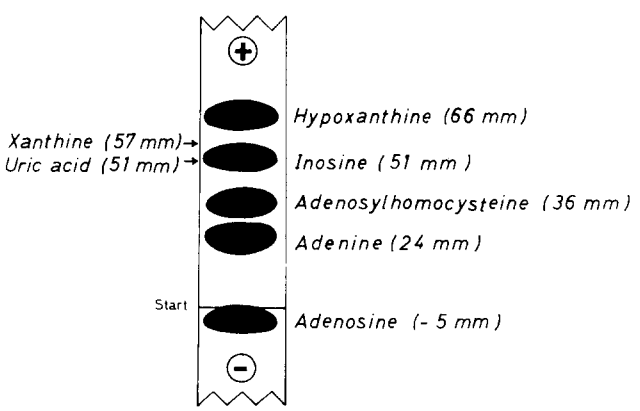

Fig. 1. Paper electrophoretic separation of *S*-adenosylhomocysteine, adenosine and some of its degradation products. A mixture of adenosine, adenine, adenosylhomocysteine, inosine and hypoxanthine (25 nmol of each in 20 μl) was applied on paper. Running conditions: 0.05 M glycine buffer, pH 11.0, 300 V (8 V/cm), running time 2.5 h. The positions of xanthine and uric acid run on a duplicate strip are shown by arrows. The numbers in parentheses refer to migration distances in mm from the starting line. All compounds except adenosine migrated towards anode (+). The bands are drawn as seen under ultraviolet light.

of the reaction products obtained with crude liver enzymes revealed two major radioactive fractions corresponding to adenosine and *S*-adenosylhomocysteine. Some radioactivity was also found in fractions corresponding to inosine and hypoxanthine, which probably were overlapped by small amounts of radioactive xanthine and uric acid, all degradation products of adenosine [18]. An exact identification of these metabolites was not considered to be essential for the purpose of this work. When the inhibitor of adenosine deaminase was present in the incubation mixture (cf. below), adenosine and *S*-adenosylhomocysteine were the only radioactive fractions detected on paper electrophoretograms. The identity of radioactive *S*-adenosylhomocysteine as the reaction product was further confirmed by ascending paper chromatography on Whatman No. 1 paper with two solvent systems, *n*-butanol/acetic acid/water (2 : 1 : 1) or the upper phase of the mixture of 2-butanol and water, respectively. The radioactive spots completely matched with those of authentic marker compounds. Furthermore, the distribution of radioactivity between adenosine and *S*-adenosylhomocysteine was the same on paper chromatography and paper electrophoresis.

Effect of inhibition of adenosine deaminase

Recently, a potent inhibitor of intestinal adenosine deaminase, erythro-9-(2-hydroxy-3-nonyl)adenine, has been synthesized by Schaeffer and Schwender [12]. As seen in Table I, at concentrations as low as $1 \cdot 10^{-6}$ M this compound almost completely prevented the formation of radioactive products, which moved in the inosine-hypoxanthine area. With the crude liver enzyme the formation of *S*-adenosylhomocysteine was not significantly affected by the inhibitor, if all adenosine was not consumed in the reaction (Table I). When incubation time was prolonged, less adenosylhomocysteine was formed in the absence of the inhibitor (Fig. 2). The decline of the reaction rate was obviously due to consumption of all adenosine in the reaction mixture.

TABLE I

EFFECT OF *ERYTHRO*-9-(2-HYDROXY-3-NONYL)ADENINE ON THE FORMATION OF RADIOACTIVE METABOLITES FROM [8-^{14}C]ADENOSINE BY CRUDE LIVER ENZYME

Incubations were carried out in triplicate in the presence of the inhibitor. The incubation mixture contained 25 nmol of [8-^{14}C]adenosine and 58 μg of enzyme protein. For other details see Materials and Methods. "Other metabolites" refers to radioactive products which moved in the area of inosine and hypoxanthine on paper electrophoresis. Inhibition of the formation of metabolites other than *S*-adenosylhomocysteine is expressed as per cent of uninhibited incubation.

Inhibitor concentration (M)	Radioactive products (nmol/5 min)		
	S-adenosyl-homocysteine	Other metabolites	Inhibition %
None	14.01	3.55	0
$1 \cdot 10^{-9}$	13.82	3.25	8
$1 \cdot 10^{-8}$	13.82	1.66	53
$1 \cdot 10^{-7}$	14.16	0.46	87
$1 \cdot 10^{-6}$	14.21	0.18	95
$1 \cdot 10^{-5}$	14.21	0.13	97
$1 \cdot 10^{-4}$	14.49	0	100

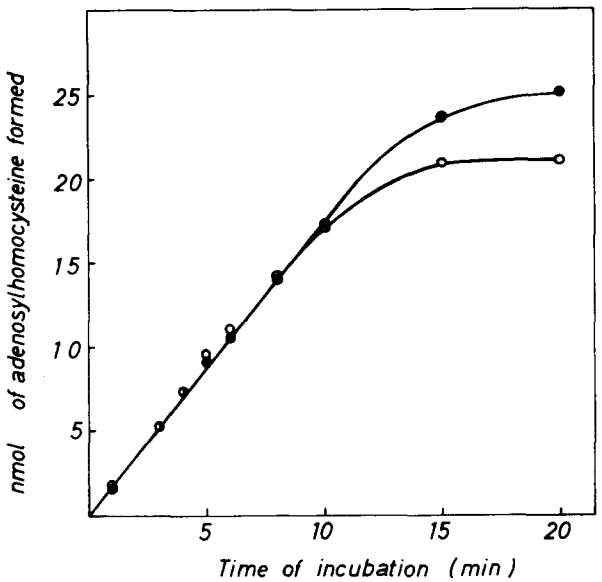

Fig. 2. Linearity with time of the synthesis of *S*-adenosylhomocysteine in the presence and absence of adenosine deaminase inhibitor. Incubations were carried out under standard incubation conditions (except the concentration of adenosine was 0.1 mM) with (•) or without (○) $1 \cdot 10^{-6}$ M erythro-9-(2-hydroxy-3-nonyl)adenine. Crude supernatant fraction of rat liver (37 μg of protein) was used as the source of the enzyme. Each point represents the mean of two analyses.

It has been reported that *S*-adenosylhomocysteine synthase (hydrolase) activity could not be detected in extracts of small intestine of the rat, possibly because the high adenosine deaminase activity present in crude intestinal extracts might effectively compete for the adenosine substrate [9]. As shown in Table II, intestinal mucosa does contain *S*-adenosylhomocysteine hydrolase activity although it is much lower than the hydrolase activity in the liver. Table II also demonstrates the rapid degradation of adenosine by intestinal extracts in the absence of the deaminase inhibitor.

TABLE II

EFFECT OF THE INHIBITOR OF ADENOSINE DEAMINASE ON THE FORMATION OF METABOLITES OF ADENOSINE BY CRUDE LIVER AND INTESTINAL EXTRACTS IN *S*-ADENOSYLHOMOCYSTEINE HYDROLASE ASSAY SYSTEM

Incubations were carried out for 5 min under standard incubation conditions in the presence or absence of $1 \cdot 10^{-5}$ M erythro-9-(2-hydroxy-3-nonyl)adenine. Crude supernatant fractions from rat liver (41 μg protein) or intestinal mucosa (116 μg) were used as enzyme source. The concentration of adenosine was 0.1 mM.

Enzyme source	Inhibitor	Radioactive products formed (nmol/min per mg protein)	
		S-adenosyl-homocysteine	Other metabolites
Liver	absent	84.2	13.5
	present	86.1	0.6
Small intestine	absent	0.4	37.5
	present	5.2	1.5

Some properties of S-adenosylhomocysteine hydrolase of rat liver

S-adenosylhomocysteine hydrolase activity of rat liver had a broad pH optimum with little change in activity between pH 7.0—7.5 (data not shown). At saturating concentrations of the substrates (adenosine and L-homocysteine) the formation of *S*-adenosylhomocysteine by crude liver extracts was linear with time (cf. Fig. 1) and the amount of the enzyme protein (the results not shown).

The results depicted in Fig. 1 indicated that *S*-adenosylhomocysteine hydrolase apparently had a high affinity for adenosine. As seen in Fig. 3, the reaction showed Michaelis-Menten type kinetics. An apparent K_m value of $7 \cdot 10^{-7}$ M for adenosine was obtained from the usual double reciprocal plot. Even this value might be too high because a large portion of the substrate is being consumed during the reaction at lower substrate concentrations. Accordingly, a somewhat lower value was obtained when the integrated Michaelis-Menten equation [19] was applied (see the insert in Fig. 3). No substrate inhibition was observed with the adenosine concentrations tested (up to 5 mM).

The dependence of the rate of formation of *S*-adenosylhomocysteine on L-homocysteine concentration is shown in Fig. 4. An apparent K_m value of $8 \cdot 10^{-5}$ M was obtained for L-homocysteine. The result was practically the same if the concentration of adenosine in the reaction mixture was 1 mM instead of 0.1 mM (the data not shown). As seen in Fig. 4, high concentrations of L-homocysteine (10—15 mM) slightly inhibited the formation of *S*-adenosylhomocysteine.

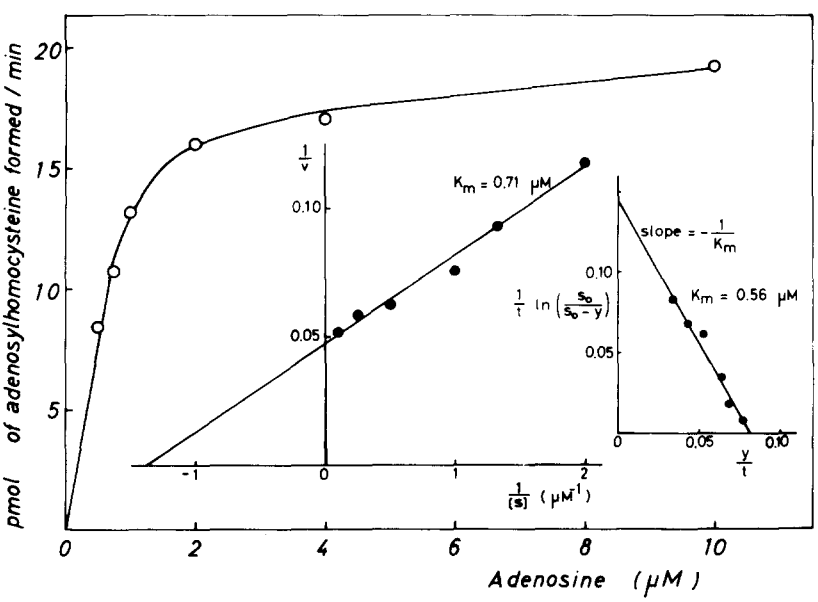

Fig. 3. Effect of the concentration of adenosine on the synthesis of *S*-adenosylhomocysteine. The incubation mixture contained 3 mM L-homocysteine, $1 \cdot 10^{-5}$ M adenosine deaminase inhibitor, 0.5—10 · 10^{-6} M adenosine (specific activity 59 mCi/mmol), 2 mM dithiothreitol and 0.5 μg of crude supernatant protein of rat liver. Incubation time was 5 min. Each point represents the mean of two incubations. The insert in the figure shows the apparent K_m value for adenosine using the integrated Michaelis-Menten equation. S_o, the concentration of adenosine at the start of the incubation; y, the concentration of *S*-adenosylhomocysteine at the end of the incubation.

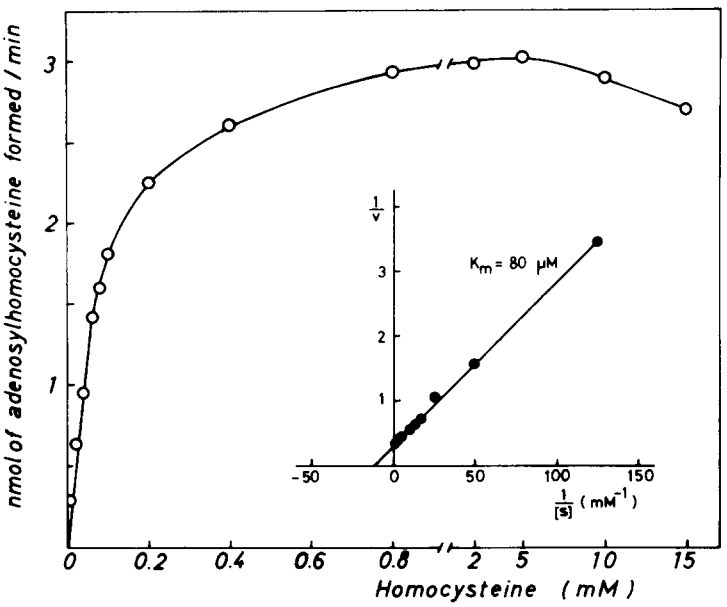

Fig. 4. Effect of the concentration of L-homocysteine on the synthesis of *S*-adenosylhomocysteine. The incubation mixture contained 0.1 mM adenosine (specific activity 2.0 mCi/mmol), $1 \cdot 10^{-5}$ M adenosine deaminase inhibitor, $8 \cdot 10^{-6}$—$15 \cdot 10^{-3}$ M L-homocysteine and 58 µg of crude rat liver enzyme. Incubation time was 5 min. Each point represent the mean of two incubations.

The results presented above considerably differ from those published recently from other laboratories [6,9]. Using partially purified *S*-adenosylhomocysteine hydrolase from rat liver Walker and Duerre [6] obtained an apparent K_m value of 1.5 mM for adenosine and 4.5 mM for L-homocysteine. Furthermore, a marked substrate inhibition was observed with L-homocysteine. Finkelstein and Harris [9] observed a half-maximal reaction rate at about 0.25 mM adenosine and 0.3 mM L-homocysteine. Also these authors found a strong substrate inhibition with L-homocysteine. We tried to reproduce the results of Walker and Duerre [6] by determining the disappearance of free SH groups of L-homocysteine during the reaction. Although the sensitivity of this method is very limited, we observed that the reaction was fully saturated at adenosine concentrations of 0.1—10 mM. An apparent K_m value of about $15 \cdot 10^{-5}$ M was obtained for L-homocysteine (the results not shown). Again, no inhibition was observed at higher concentrations of L-homocysteine (up to 10 mM). We also checked our results by using partially purified *S*-adenosylhomocysteine hydrolase from rat liver. The specific activity of these enzyme preparations was 2—3-fold higher than those used by Finkelstein and Harris [9]. The results were in complete agreement with those obtained with the crude supernatant fraction.

Discussion

The assay method for *S*-adenosylhomocysteine hydrolase activity described in this report has some significant advantages over methods based on the measurement of the disappearance of free sulphhydryl groups of L-homocysteine [8,10,20]. It combines specificity and great sensitivity. It was possible to deter-

mine *S*-adenosylhomocysteine hydrolase activity with as little as 0.5 μg of protein of crude rat liver extract (cf. Fig. 3). Furthermore, the assay method is not hampered by side reactions catalyzed by crude enzyme preparations, such as oxidation of L-homocysteine [20] or the metabolic pathway leading to the formation of uric acid from adenosine [18]. The addition of protective thiol compounds to the homogenization medium, e.g. for the assay of polyamine synthesizing enzymes in the same enzyme preparations (cf. ref. 21), does not interfere with the present method.

A sensitive double-isotope assay for *S*-adenosylhomocysteine hydrolase activity was recently developed by Finkelstein and Harris [9]. This method involved column-chromatographic separation of *S*-adenosylhomocysteine labelled in the adenosine portion. This method compares with the present method in sensitivity but evidently is more laborious, especially when compared with the use of ion-exchange paper as described in the present paper. Although separation of *S*-adenosylhomocysteine by paper electrophoresis is somewhat more tedious than by using ion-exchange paper, it has certain advantages: the main metabolites of adenosine, as well as the amount of adenosine remaining after the incubation can be assayed at the same time. Both separation methods produced very reproducible results: the duplicate determinations usually did not deviate by more than 2—3%.

An apparent K_m value of $7 \cdot 10^{-7}$ M for adenosine and $8 \cdot 10^{-5}$ M for L-homocysteine were obtained in the present work. Little or no substrate inhibition was observed with L-homocysteine and adenosine at concentrations up to 10—15 mM. These results differ considerably from those recently reported by other authors [6,9]. With regard to the substrate inhibition, the reason for this discrepancy is not obvious at the present time. In one case, looking at the data [9] it seems possible that consumption of all the substrate (adenosine) during the reaction has led to erroneous kinetic values.

The regulation of liver *S*-adenosylhomocysteine hydrolase activity in vivo appears to be under nutritional and hormonal control [9]. Its activity was increased in animals fed with a high-protein diet. Treatment with hydrocortisone or oestradiol also resulted in a stimulation of the hydrolase activity [9]. An increase in the *S*-adenosylhomocysteine hydrolase was also observed in the livers of B_6-deficient rats (Eloranta and Raina, unpublished results). The possible role of this enzyme in the regulation of methionine metabolism remains to be determined. Although it catalyses the condensation of adenosine and L-homocysteine, i.e. the synthesis of *S*-adenosylhomocysteine, it appears likely that physiological conditions favor the reverse reaction, the hydrolysis of *S*-adenosylhomocysteine [8,18]. Modulation of this activity might play a role in the regulation of biological methylation [2,4—6,22] as well as methionine metabolism in general [7].

After submission of this work the presence of *S*-adenosylhomocysteine hydrolase in rat intestinal mucosa was also reported by Finkelstein and Harris [23].

Acknowledgements

This work was supported by grants from the National Research Council for Medical Sciences, Finland, and from the Sigrid Jusélius Foundation.

References

1 Cantoni, G.L. and Scarano, E. (1954) J. Am. Chem. Soc. 76, 4744
2 Borchardt, R.T. and Wu, Y.S. (1974) J. Med. Chem. 17, 862—868
3 Salvatore, F., Utili, R., Zappia, V. and Shapiro, S.K. (1971) Anal. Biochem. 41, 16—28
4 Zappia, V., Zydek-Cwick, C.R. and Schlenk, F. (1969) J. Biol. Chem. 244, 4499—4509
5 Deguchi, T. and Barchas, J. (1971) J. Biol. Chem. 246, 3175—3181
6 Walker, R.D. and Duerre, J.A. (1975) Can. J. Biochem. 53, 312—319
7 Finkelstein, J.D., Kyle, W.E. and Harris, B.J. (1974) Arch. Biochem. Biophys. 165, 774—779
8 de la Haba, G. and Cantoni, G.L. (1959) J. Biol. Chem. 234, 603—608
9 Finkelstein, J.D. and Harris, B. (1973) Arch. Biochem. Biophys. 159, 160—165
10 Knudsen, R.C. and Yall, I. (1972) J. Bacteriol. 112, 569—575
11 Duerre, J.A. (1968) Arch. Biochem. Biophys. 124, 422—430
12 Schaeffer, H.J. and Schwender, C.F. (1974) J. Med. Chem. 17, 6—8
13 Duerre, J.A. and Miller, C.H. (1966) Anal. Biochem. 17, 310—315
14 Grunert, R.R. and Phillips, P.H. (1951) Arch. Biochem. Biophys. 30, 217—225
15 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
16 Raina, A. (1963) Acta Physiol. Scand. 60, suppl. 218, 1—81
17 McKenzie, R.M. and Gholson, R.K. (1973) Anal. Biochem. 53, 384—391
18 Cortese, R., Perfetto, E., Arcari, P., Prota, G. and Salvatore, F. (1974) Int. J. Biochem. 5, 535—545
19 Dixon, M. and Webb, E.C. (1964) Enzymes, 2nd edn., pp. 114—116, Longmans Green, London
20 de la Haba, G. (1962) in Methods in Enzymology (Colowick, S.P. and Kaplan, N.O., eds.), Vol. 5, pp. 752—755, Academic Press, New York
21 Raina, A. and Jänne, J. (1975) Med. Biol. 53, 121—147
22 Swiatek, K.R., Simon, L.N. and Chao, K.-L. (1973) Biochemistry 12, 4670—4674
23 Finkelstein, J.D. and Harris, B. (1975) Arch. Biochem. Biophys. 171, 282—286