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Compartmentalization of *S*-Adenosylhomocysteine in Rat Liver

DETERMINATION AND CHARACTERIZATION OF THE *IN VIVO* PROTEIN BINDING*

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A method for the determination of *S*-adenosylhomocysteine (AdoHcy), which is associated with proteins *in vivo*, was developed. This method involves homogenization of tissue or cells in saturated, ice-cold solution of ammonium sulfate containing adenosine and *S*-adenosylmethionine to suppress unspecific binding of AdoHcy during sample processing. The homogenate was then extensively diluted, filtered, the precipitated protein washed, and AdoHcy extracted with perchloric acid. With this method it could be demonstrated that 30–50% of AdoHcy in rat liver and isolated rat hepatocytes is associated with proteins. Under physiological conditions, the major fraction of protein-bound AdoHcy resides in the microsomal fraction, whereas most free AdoHcy was recovered in the cytosol. The total AdoHcy content in hepatocytes could be markedly elevated by addition of adenosine or methionine. Under both conditions, protein-bound AdoHcy increased 2-fold and then leveled off, whereas free AdoHcy accounts for a further, massive, rise in intracellular AdoHcy. This suggests that AdoHcy is binding to saturable sites *in vivo*. AdoHcy in hepatocytes was labeled by incubating the cells with L-[^{35}S]methionine, and within the first 60 min, free AdoHcy attained a significantly higher specific activity than protein-bound AdoHcy. Furthermore, chase with excess unlabeled methionine or with cycloleucine, revealed a shorter half-life of radioactive sulfur in free AdoHcy than in protein-bound AdoHcy. This shows that protein-bound and free AdoHcy represent kinetically distinct AdoHcy pools.

S-Adenosylhomocysteine (AdoHcy)[1] is a product of AdoMet-dependent transmethylation reactions. The compound is catabolized to adenosine and Hcy. This reaction is catalyzed by the ubiquitous enzyme, AdoHcy hydrolase. Since AdoHcy is a potent negative feedback inhibitor of most transmethylases, this compound has been assigned an essential role in the control of the overall transmethylation rate (1).

AdoHcy in tissues and cells under physiological conditions and following pharmacological perturbation of AdoHcy catabolism can be determined by convenient high performance liquid chromatography techniques (2–6). Procedures have been developed which prevent *post mortem* changes in cellular AdoHcy content (7, 8). These methods form the basis for numerous studies pointing to the metabolism of AdoHcy as a potential target for drug design (9, 10). In addition, it has been reported that the AdoHcy metabolism is decreased following virus infection (11, 12) and increased in malignant cells (13).

A large fraction of intracellular adenosine and Hcy, which are products of the AdoHcy hydrolase reaction, exists as a complex with cellular proteins (14–16). AdoHcy forms a stable complex with hydroxyindole *O*-methyltransferase (17) and with isolated membranes from rat brain (18) and liver (19, 20). Compartmentalization of AdoMet in liver and yeast has recently been established (21). These findings suggest that AdoMet and products of transmethylation may exist as complexes with proteins *in vivo*. However, no study has been devoted to the possible compartmentalization of intracellular AdoHcy.

The present paper describes a method for the determination of protein-bound AdoHcy in whole liver and hepatocytes from the rat. With this method it is demonstrated that bound AdoHcy exists as a distinct AdoHcy pool *in vivo*. Such knowledge may be critical for the interpretation of data on the relation between cellular AdoHcy and biological events.

EXPERIMENTAL PROCEDURES AND RESULTS

DISCUSSION[2]

We used a procedure involving homogenization of tissue or cells in ammonium sulfate followed by retention of precipitated protein on nitrocellulose filters to demonstrate that AdoHcy is partly protein-bound *in vivo*. The AdoHcy acceptors responsible for the *in vivo* protein binding of AdoHcy are probably identical to the catalytic or regulatory sites of various methyltransferases and AdoHcy hydrolase. Most protein-bound AdoHcy is localized to the microsomal fraction (Table II) which is rich in phospholipid methyltransferase (20, 32), but other AdoHcy binding sites residing in the microsomes may exist as well (20). Protein-bound AdoHcy in the soluble fraction is in accordance with tight binding of AdoHcy to soluble enzymes like hydroxyindole *O*-methyltransferase (17, 33) and AdoHcy hydrolase (14). In addition, some methyltransferases are bound to AdoHcy immobilized to Sepharose

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[1] The abbreviations used are: AdoHcy, *S*-adenosylhomocysteine; AdoMet, *S*-adenosylmethionine; Hcy, homocysteine; HPLC, high performance liquid chromatography.

[2] Portions of this paper (including "Experimental Procedures," "Results," part of "Discussion," Figs. 1–6, and Tables I and II) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 86M-3991, cite the authors, and include a check or money order for $6.80 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

beads (34–36), suggesting tight binding of AdoHcy to these enzymes.

We have recently demonstrated that a large portion of intracellular Hcy (16) and adenosine (15) in liver is complexed with proteins. In the present paper we demonstrate that a substantial portion of AdoHcy is complexed with proteins, probably enzymes participating in AdoHcy metabolism. Notably, the amount of protein-bound AdoHcy in whole liver, 7–18 nmol/g (Fig. 2), is of the same order of magnitude as the concentration of AdoHcy hydrolase in mouse and rat liver, *i.e.* about 10 μM (14, 37). Thus, these products of transmethylation probably offer examples of a general phenomenon addressed by Srere (38). He points out that enzyme concentrations in cells are usually in the order 10^{-6}–10^{-5} nmol/g, and significant fractions of some substrates may be protein-bound. On this basis he (38) and others (39, 40) add a cautionary note concerning the translation of *in vitro* data into metabolic terms.

The fact that different AdoHcy compartments exist should be taken into account when estimations of metabolic flux through AdoHcy and Hcy are made, which are based on concentrations of metabolites and enzyme activities (41, 42). Compartmentalization of AdoHcy may modulate the response of an AdoMet-dependent methylation reaction to changes in the AdoMet/AdoHcy ratio.

Careful inspection of published data on turnover rates of sulfur in AdoHcy and related compound (16) shows that isotope equilibrium exists between homocysteine and AdoMet, whereas total AdoHcy shows a higher specific activity during chase with methionine. In the present report it is demonstrated that free AdoHcy represents a pool kinetically distinct from protein-bound AdoHcy. Before (Fig. 4) and immediately after chase with either methionine (Fig. 5) or cycloleucine (Fig. 6) free AdoHcy has a higher specific activity than bound AdoHcy, which in turn is nearly equal to that of AdoMet.

AdoMet is the immediate metabolic precursor of AdoHcy. This implies that when AdoHcy is efficiently removed from the intracellular compartment and the turnover rate of this metabolite is production rate limited, AdoHcy must have a specific activity equal to that of AdoMet. Alternatively, if AdoHcy tends to accumulate, this metabolite could attain a higher specific activity than AdoMet. The theoretical basis of this general statement has been outlined (43).

We observed that free AdoHcy has a higher specific activity than AdoMet (Figs. 4, 5, and 6) and ^{35}S in free AdoHcy declined at a rate higher than or equal to that of ^{35}S in AdoMet. The latter observation shows that no accumulation of AdoHcy occurs. To reconcile these findings one must postulate the existence of different AdoMet pools. One AdoMet pool rapidly incorporates ^{35}S from exogenous methionine, and this pool gives rise to most free AdoHcy. This AdoMet pool must be small relative to the total AdoMet content, because a biphasic decay curve for AdoMet could not clearly be distinguished (Figs. 5 and 6).

The existence of a "labile" and a "stable" AdoMet pool in rat hepatocytes has been suggested by Farooqui *et al.* (21) using a double labeling technique. A long labeling period of 2 h revealed the stable pool whereas the labile pool was selectively labeled during a short period of 30 min. We observed that the differential labeling of free and bound AdoHcy was prominent during the first 60 min (Fig. 4).

The experiment depicted in Fig. 6 resembles those published by Farooqui *et al.* (21) in that AdoMet synthesis was inhibited with 25 mM cycloleucine. We observed that the inhibitor does not completely block AdoMet synthesis in rat hepatocytes, since the amount of AdoMet and the co-eluting radioactivity showed a transient increase following addition of inhibitor. Furthermore, total inhibition of AdoMet synthesis implies that the specific activities of the AdoMet pools remain constant, whereas the amount of labeled AdoMet declines. This contrasts to the result obtained with rat hepatocytes (Fig. 6; Ref. 21). Thus, such experiments involving incomplete inhibition of AdoMet synthesis do not provide data on the turnover rates of different AdoMet pools, but merely suggest the existence of such pools. However, data presented in Fig. 6 show that free AdoHcy is not in isotope equilibrium with AdoMet and bound AdoHcy; also under conditions of inhibited AdoMet synthesis.

The existence of different compartments for AdoHcy and AdoMet in rat liver strongly suggests that only particular intracellular pools are available for some methyltransferases. This possibility should be considered in relation to the remarkable finding of Vance and Vance (44), showing that the methylation of phosphatidylethanolamine involved in lipoprotein secretion is insensitive to inhibitors of AdoHcy catabolism.

In the experiments depicted in Fig. 3, *A* and *B*, intracellular AdoHcy in liver cells was elevated 10–40-fold by addition of either methionine or adenosine. The former procedure probably causes increased flux through AdoHcy (16), whereas addition of adenosine elevates AdoHcy by increasing its formation from adenosine and homocysteine and inhibition of degradation (1). Under both conditions, there was only a 2-fold increase in protein-bound AdoHcy, from 50–70 pmol/10^6 cells to 100–150 pmol/10^6 cells, and at this level a plateau was observed (Fig. 3, *A* and *B*). This suggests the presence of saturable AdoHcy binding sites in the hepatocytes. These data also allow an intracellular titration of the amounts of AdoHcy binding sites in liver cells (100–150 pmol/10^6 cells), and suggest that under physiological conditions these sites are half-saturated.

In conclusion, about one-half of the intracellular AdoHcy in rat liver forms a complex with saturable sites under physiological conditions, and free and bound AdoHcy represent distinct kinetic compartments. These two pools may have different metabolic origin and regulatory function. Compartmentalization of AdoHcy should be taken into account when evaluating the biological effects of AdoHcy under physiological conditions, following pharmacological intervention (9) and in disease states (11, 13).

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SUPPLEMENTARY MATERIAL TO

Compartmentalization of S-Adenosylhomocysteine in Rat Liver

DETERMINATION AND CHARACTERIZATION OF THE *IN VIVO* PROTEIN BINDING

Asbjørn M. Svardal and Per Magne Ueland

EXPERIMENTAL PROCEDURES

Materials - L-Homocysteine, DL-homocysteine, adenosine, AdoHcy, DL-dithioerythritol were purchased from Sigma Chemical Co. St. Louis, Mo. AdoMet was obtained from Koch-Light Laboratories Ltd, Colnbrook Bucks, England, and was purified on a Partisil 10 SCX column eluted with 1.2 M ammonium formate, pH 3.8 containing 20 % methanol, to remove material which co-chromatographed with AdoHcy. Ammonium sulfate, pro analysis, was purchased from Merck, Darmstadt, FRG. Other reagents were obtained from sources given previously (16).

Preparation of Subcellular Fractions of Rat Liver and Assay for Marker Enzymes - Animals were stunned and exsanguinated and the livers were immediately removed and chilled in 0.25 M sucrose containing 10 mM Hepes (pH 7.4), 1 mM EDTA and 100 µM 9-ß-D-arabinofuranosyladenine. The nuclear fraction (N), the mitochondrial fraction (M), the lysosomal fraction (L), the microsomal fraction (P) and the soluble fraction (L) were prepared by a slight modification (16) of the method of de Duve (22).

Glutamate dehydrogenase (EC 1.4.1.3.) (23), succinate phenazine methosulphate oxidoreductase (EC 1.3.99.1.) (24), acid phosphatase (EC 3.1.3.2.) (25), rotenone-insensitive NADPH cytochrome c oxidoreductase (EC 1.6.2.4.) (26) and lactate dehydrogenase (EC 1.1.1.27.) (27) were assayed as described in the references. All spectrophotometric measurements were performed with a Kontron recording spectrophotometer (Uvicon 810).

Preparation and Incubation of Isolated Rat Hepatocytes - The isolated hepatocytes were prepared by a collagenase perfusion method (28) and were incubated in an isotonic salt solution containing Hepes buffer, pH 7.4, bovine serum albumin, glucose, salts, and antibiotics, as described elsewhere (29). The temperature was 37 °C .

Pulse-Chase Experiments with L-[^{35}S] Methionine - Hepatocytes (5.0×10^6 cells/ml) were incubated for 30 minutes with 1µM [35S]methionine (16 Ci/mmol). The radioactive methionine was chased by addition of 2mM cold methionine. Immediately before the chase and during the chase period, samples of 1 ml from the cell suspension were layered onto a mixture (500 µl) of dinonylphthalate and dibutylphthalate (1:3), placed in 1.5 ml polyethylene tubes. The cells were separated from the medium by centrifugation at 7000 rpm for 5 seconds in a Beckman type B microfuge. Both the oil and the tubes were cooled to about -12 °C prior to use, to prevent metabolic alteration after centrifugation and before extraction of the cells. The cell pellet was homogenized in perchloric acid or ammonium sulfate, as described below.

Determination of Protein-Bound AdoHcy - When not otherwise stated, the liver or cell pellet was homogenized at a dilution of 1/30 (w/v) in an ice-cold solution of saturated ammonium sulfate containing 1 mM adenosine plus 1 mM AdoMet, using an Ultra-Turrax tissue homogenizer. The homogenate was then immediately diluted in ice-cold ammonium sulfate containing adenosine to a final dilution of about 1/240. Then the whole sample (about 10 ml) was applied to and the precipitated protein collected on Millipore filters (HAWP, 0.45-µm pore size), using a Millipore filter manifold. The filters were washed three times with 3 ml of ice-cold ammonium sulfate containing 1 mM adenosine plus 1 mM AdoMet, and were then extracted in 500 µl 0.8 N perchloric acid. AdoHcy was determined in the acid extract, using an HPLC method (16).

The possible binding of AdoHcy to the proteins during tissue processing was examined by homogenizing tissues or cells in the presence of increasing amounts of AdoHcy. The amount of added (free) AdoHcy was plotted versus protein-bound AdoHcy , and the small amount of AdoHcy artificially bound could be corrected for, as described in detail in the result section.

Determination of Total and Free AdoHcy - Tissue or cell pellet was homogenized (1/10,w,v) in 0.8 N perchloric acid, using an Ultra-Turrax homogenizer. Protein was removed from the extract by centrifugation, and total AdoHcy was determined in the acid extract by HPLC.

AdoHcy (10-100 µM) was added to crude liver homogenate. Protein was then precipitated with perchloric acid, and the added AdoHcy was virtually completely recovered in the supernatant. This result, which is in accordance with a similar experiment with cultured cells (30), suggested that the method involving acid precipitation determines total AdoHcy, and AdoHcy is not bound to the protein.

Free AdoHcy refers to total AdoHcy minus protein-bound AdoHcy.

Determination of Specific Radioactivity of AdoHcy and AdoMet by HPLC - Cells were extracted with perchloric acid as described in the preceding paragraph. AdoHcy and AdoMet were assayed in the same sample on a cation exchange column (Partisil 10 SCX, 0.46x25 cm), which was equilibrated and eluted with a gradient system described in detail previously (30). Alternatively, AdoHcy was determined by reversed-phase liquid chromatography (16). The AdoHcy and AdoMet peaks were collected, using a programmable fraction collector, model Foxy from ISCO, and the radioactivity was determined by liquid scintillation counting.

Determination of Protein - Protein was determined by the method of Bradford (31), using the Protein Assay Kit from Bio-Rad. Bovine γ-globulin was used as standard.

RESULTS

Preliminary Studies on AdoHcy Binding to Proteins Precipitated with Ammonium Sulfate - Free and protein-bound AdoHcy were separated by homogenization and precipitation of tissue protein in saturated ammonium sulfate, as described in the Experimental section. Addition of AdoHcy to the homogenization medium increased the amount of protein-bound AdoHcy several-fold. The same results were obtained when AdoHcy was applied to the proteins retained on the filters. Separation of the protein precipitate from the filters followed by extraction of AdoHcy showed that most AdoHcy was associated with the proteins, and not bound to the filters (data not shown).

TABLE I

Suppression of unspecific binding of AdoHcy to proteins present in liver homogenate by adenosine and AdoMet

Liver (17 mg) was homogenized in saturated ammonium sulfate (500 µl) which was supplemented with either no or 100 µM AdoHcy. In some samples the homogenization medium also contained 1 mM adenosine and/or 1 mM AdoMet. The amount of protein-bound AdoHcy was otherwise determined as described in the Experimental section.

Present during homogenization	Protein-bound AdoHcy
	(nmol/g liver)
Added AdoHcy	
Control	418
Adenosine (1 mM)	43
AdoMet (1 mM)	27
Adenosine (1 mM) + AdoMet (1 mM)	23
No exogenous AdoHcy	
Control	13
Adenosine (1 mM) + AdoMet (1 mM)	8

The binding of AdoHcy to proteins during and after homogenization was determined as the difference between protein-bound AdoHcy in the absence and presence of high concentration (100 µM) of AdoHcy added to the homogenization medium. Inclusion of adenosine or AdoMet in the medium markedly decreased the influence from added AdoHcy, as shown in table I. Artificial binding was also decreased as a function of dilution prior to filtration. In the absence of added AdoHcy, the amount of AdoHcy bound decreased upon dilution up to about 1/60 and then levelled off. In the presence of high concentration of AdoHcy in the homogenization medium, the bound fraction decreased as a function of extensive dilution and approached a level which was about two fold higher than that obtained in the absence of added AdoHcy at a dilution of 1/240 (Fig. 1).

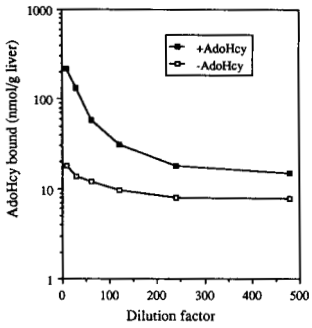

FIG. 1. **Effect of dilution of tissue homogenate on protein-bound AdoHcy.** Liver (55 mg) was homogenized in 500 µl of ice-cold saturated ammonium sulfate containing adenosine and AdoMet, as described in the Experimental section. In one series, the homogenization medium was also supplemented with 100 µM AdoHcy. The homogenates were then diluted (1/10 to 1/480, w/v) in a solution of ammonium sulfate (containing adenosine) to the final concentrations indicated on the figure. The amount of protein-bound AdoHcy was determined as a function of dilution.

Determination of In Vivo Protein-Binding of AdoHcy - An assay for protein-bound AdoHcy was developed on the basis of the preliminary experiments described in the preceding paragraph. Ideally, there should be no dissociation or binding of AdoHcy during tissue processing.

Tissue samples (or cells) were routinely homogenized in the presence of adenosine (1 mM) and AdoMet (1 mM). The homogenization (in 500 µl) was performed in the absence or presence of increasing concentrations of AdoHcy. The homogenate was subjected to extensive dilution prior to filtration.

Protein-bound AdoHcy increased in a concentration dependent manner upon addition of AdoHcy to the homogenization medium. Endogenous AdoHcy in the hepatocytes was labeled by incubating the cells with [^{35}S]methionine. Experiments with these cells showed that unlabelled AdoHcy did not affect the amount of protein-bound [^{35}S]AdoHcy, but increased the total amount of bound AdoHcy (Fig. 2). This finding suggests that there is no dissociation or exchange of AdoHcy bound *in vivo*, but the interference from free AdoHcy in the medium could be attributed to association of AdoHcy with proteins.

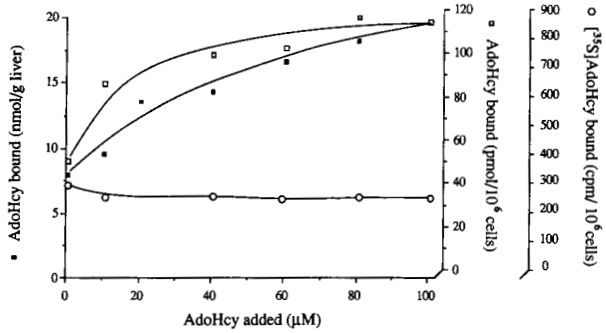

FIG. 2. **Protein-bound AdoHcy in hepatocytes and in whole liver homogenized in the presence of increasing concentration of added AdoHcy.** Hepatocytes (5.6x10^6 cells/ml) were incubated in the presence of [^{35}S]methionine (1 µM for 30 minutes) to label endogenous AdoHcy. Cells (5.6x10^6) or liver (17mg) were homogenized in a solution of ammonium sulfate (500 µl) supplemented with increasing amounts of AdoHcy, and then diluted as described in the Experimental section. The amount of protein-bound AdoHcy in cells (□) or liver (■), and the amount of endogenous AdoHcy complexed with proteins from liver cells (○), were determined as a function of added AdoHcy.

The assumption is made that the artificial increase in bound AdoHcy shows the same dependence on endogenous, free AdoHcy, as on AdoHcy added to the medium. To prevent interference from AdoHcy binding during tissue processing the samples were extensively diluted after the homogenization until the protein-bound fraction was essentially independent on dilution. The possibility of interference was evaluated by homogenization in the presence of increasing concentration of added AdoHcy, and then subjected to dilution, and a curve similar to figure 2 was obtained. However, in the experiments reported in the present paper (Figs. 3A,B), total AdoHcy was less than the concentration of added AdoHcy which increased bound AdoHcy by 30 % (Fig. 2).

Relation Between Free and Protein-Bound AdoHcy - AdoHcy in both whole liver and isolated hepatocytes under physiological conditions was distributed between a bound and a free fraction. The amount of free AdoHcy was about 20 nmol/g in whole liver and 50-100 pmol/10^6 cells in hepatocytes, whereas the values for protein-bound AdoHcy were 8 nmol/g and 50 pmol/10^6 cells for whole liver and isolated hepatocytes, respectively. Free AdoHcy in hepatocytes varied somewhat from one cell preparation to another.

Adenosine is the natural substrate and inhibitor of AdoHcy hydrolase and functions as an inhibitor of AdoHcy catabolism and precursor of AdoHcy *in vivo* (1). Addition of adenosine (0.5 mM or 1 mM) to hepatocytes caused a transient increase in total, intracellular AdoHcy. Free AdoHcy showed a pronounced (about 10 fold) increase, and thereby accounted for most AdoHcy under these conditions. In contrast, protein-bound AdoHcy increased only 2 fold and then levelled off. The protein-bound AdoHcy decreased to pretreatment values upon normalization of the AdoHcy content (Fig. 3 A).

Supplementing the medium with a high concentration of methionine has a similar effect on cellular AdoHcy as that observed with adenosine. The protein-bound fraction increased only about two fold and then reached a plateau whereas a further increase in total AdoHcy was due to increase in free AdoHcy (Fig. 3B).

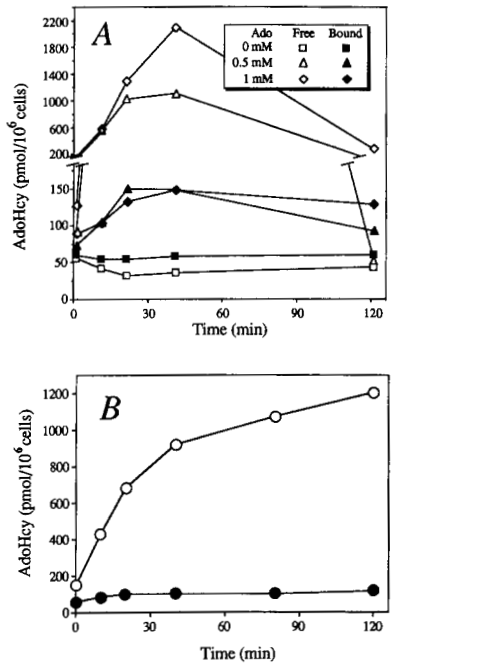

FIG. 3. **Free and protein-bound AdoHcy in hepatocytes exposed to A, adenosine or to B, high concentration of methionine.** *A*, Hepatocytes (5.2x10^6 cells/ml) were incubated in the presence of either 0.5 mM adenosine, 1 mM adenosine or no addition, and the amount of free (open symbols) and protein-bound AdoHcy (closed symbols) was determined as a function of time of incubation. *B*. This panel shows a similar experiment where adenosine was replaced with 2 mM methionine. Protein-bound AdoHcy (●) and free AdoHcy (○) were determined as a function of time after addition of methionine to the cell suspension.

Subcellular Distribution - The amount of free and protein-bound AdoHcy was determined in the subcellular fractions of rat liver. The fractions were prepared at about 2°C and in the presence of 100 µM 9-ß-D-arabinofuranosyladenine (9,10) to prevent metabolic degradation of AdoHcy.

Protein-bound AdoHcy (44%) is largely associated with the microsomal fraction, whereas most free AdoHcy (54%) was recovered in the cytosol. The recoveries of protein-bound and free AdoHcy relative to whole homogenate were 85 % and 90 %, respectively (Table II).

There was a reproducible loss of most free AdoHcy in the S+P fraction during the last centrifugation step giving the separate P and S fractions, and only 46% of the amount of AdoHcy in the crude homogenate was recovered. AdoHcy is tightly bound to isolated microsomes since it could not be removed by treatment of the fraction with char-coal for 10 minutes at 0°C , whereas all AdoHcy in the cytosol (S) fraction was removed following such treatment. This suggests that AdoHcy is selectively lost from the S-fraction. Therefore, the amount of free AdoHcy in the S-fraction was determined as the difference between the amount of free AdoHcy in the S+P fraction (23,000x g supernatant (16) and the P-fraction.

We found no binding of AdoHcy to the nitrocellulose tubes used for the ultracentrifugation step. It is possible that loss of AdoHcy in this step is related to conditions favoring enzymatic degradation, i.e. dilution of the extract and increase in temperature to 2-6 °C for 60 minutes.

Table II

Subcellular distribution of AdoHcy and marker enzymes in rat liver

The enzymes, free and protein-bound AdoHcy, and protein were assayed as described in the "Experimental Procedures". The absolute values for the enzyme activities, AdoHcy binding and protein in total homogenate (i.e. cytoplasmic extract + nuclear fraction) are given in the left column of the table. The enzyme activities, the amounts of free and protein-bound AdoHcy and protein in the separate fractions are expressed as percentages of the total homogenate. The relative specific activity of the enzymes, given in parentheses,represents the percentage recovered in the fraction divided by the percentage of protein in the fraction, taking the cumulative values of fractions N, M, L, P, and S as 100%.

	Absolute values	Distribution (%) in fraction and (in parentheses) relative specific content					Recovery
		N	M	L	P	S	
	nmol/min/g liver						%
Acid phosphatase	6100	4.7 (0.6)	10.9 (0.7)	48.2 (7.7)	23.1 (1.6)	7.8 (0.2)	94.7
Lactase dehydrogenase	510000	4.5 (0.6)	1.1 (0.1)	0.7 (0.1)	3.5 (0.3)	77.6 (1.7)	87.4
Succinate: phenazine methosulfate oxidoreductase	6300	16.3 (1.7)	74.9 (4.6)	9.2 (1.4)	2.9 (0.2)	0.1 -	103.4
Glutamate dehydrogenase	148000	20.0 (2.0)	70.4 (4.0)	13.1 (1.8)	4.5 (0.3)	4.0 (0.1)	112.0
Rotenone-insensitive NADPH-cytochrome c oxidoreductase	3300	4.9 (0.6)	5.6 (0.4)	7.9 (1.3)	64.6 (4.6)	8.5 (0.2)	91.5
	nmol/g liver						
Bound AdoHcy	6.9	10.1	7.0	11.9	44.3	12.0	85.3
Free AdoHcy	20.8	11.5	6.3	5.1	12.7	54.2	89.8
	mg/g liver						
Protein	204	9.7	17.0	7.1	16.4	56.7	106.9

N, Nuclear fraction
M, Mitochondrial fraction
L, Lysosomal fraction
P, Microsomal fraction
S, Soluble fraction

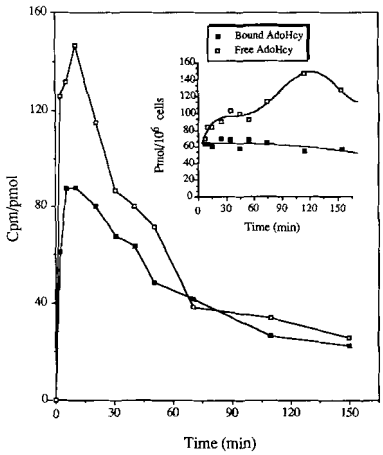

FIG. 4. **Differential labelling of free and protein-bound AdoHcy in hepatocytes.** The specific activities of free (□) and protein-bound (■) AdoHcy in hepatocytes (3.9×10^6 cells) were determined as a function of time of incubating the cells with 1 μM [^{35}S]methionine. *Inset* shows the amount of free and protein-bound AdoHcy during the experimental period.

Incorporation and Chase of Radiolabelled Sulfur in AdoHcy - Sulfur in AdoHcy and related compounds in isolated hepatocytes were labelled by incubating the cells with [^{35}S]methionine (1μM). During the first 60 minutes of incubation, the specific activity of free AdoHcy was significantly higher than the specific activity of protein-bound AdoHcy. After prolonged incubation, both AdoHcy fractions showed the same specific activity (Fig. 4).

The decay curves for labelled sulfur in free and protein-bound AdoHcy and in AdoMet were determined following chase with excess unlabelled methionine. The specific activity of free AdoHcy was higher than that of bound AdoHcy (and AdoMet) during the first part of the chase period, but the curve for free AdoHcy gradually approached those of bound AdoHcy and AdoMet. Accordingly, free AdoHcy had a somewhat shorter half-life (Fig. 5). This was observed in 6 separate experiments. The half-lives varied in the range 30-60 seconds from one cell preparation to another, and showed a considerable overlap, but non-parametric statistical analysis (Wilcoxon match paired signed rank test) showed that the kinetics of free AdoHcy was significant different from the kinetics of bound AdoHcy and AdoMet (p=0.3).

Since there is essentially no formation of radioactive AdoMet during the chase period, this half-life corresponds to the turnover rate of AdoMet under this condition.

In another experiment, the AdoMet synthetase inhibitor, cycloleucine, was used to chase radioactive sulfur in AdoMet and AdoHcy (Fig. 6). Under these conditions, there was a slight increase in total AdoMet and radioactivity associated with AdoMet immediately after the chase had started (data not shown) suggesting that AdoMet synthesis was not totally inhibited. The apparent half-lives were orders of magnitude higher, but the results were similar to those presented in figure 5. Free AdoHcy showed a much higher specific activity than bound AdoHcy and AdoMet in the first part of the chase period. The decay curve for ^{35}S in free AdoHcy was biphasic, showing a rapid initial phase followed by a slow phase. In some experiments, there was a slight tendency towards biphasic decay curves also for bound AdoHcy and AdoMet, but for these parameters monoexponential curves gave a good curve fit. The terminal phases for free AdoHcy, bound AdoHcy and AdoMet showed nearly the same slope. (Fig. 6).

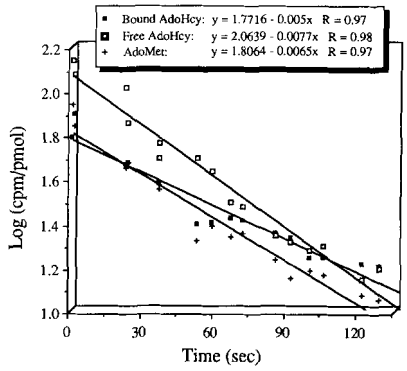

FIG. 5. **Specific activity and turnover of sulfur in free and protein-bound AdoHcy and in AdoMet following chase with methionine.** Hepatocytes (5.4×10^6 cells/ml) were incubated in the presence of [^{35}S]methionine (1 μM) for 30 minutes. Then the radioactive methionine was chased by addition of unlabelled methionine (2 mM) to the cell suspension. The figure shows semilogarithmic plots of the specific activities of free AdoHcy, protein-bound AdoHcy and AdoMet versus time after start of the chase. The straight lines are obtained by log-linear least square regression analysis.

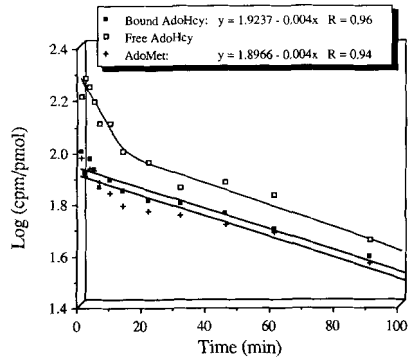

FIG. 6. **Specific activity and turnover of sulfur in free and protein-bound AdoHcy and in AdoMet following chase with cycloleucine.** The experimental design was exactly as described in the legend to figure 5, except that unlabelled methionine was replaced with cycloleucine (25 mM).

The observation that free AdoHcy attained a higher specific activity than bound AdoHcy (and AdoMet) was unexpected. Therefore, the interference from radioactive compound(s) which co-chromatographed with AdoHcy, or the presence of an UV absorbing material leading to overestimation of protein-bound AdoHcy were considered as sources for erratic results.

The specific activities of both free and protein-bound AdoHcy were determined under various chromatographic conditions. These include a strong cation-exchange column (Partisil 10 SCX) eluted with mobile phases of different ionic strengths (20-80 mM ammonium formate buffer, pH 3.5) and reversed-phase chromatography (ODS Hypersil) with various concentrations of methanol (5-15 % methanol in 15 mM acetate buffer, pH 4.5). The reversed-phase system was routinely used, since it gave the sharpest UV-peak eluting where the base-line absorption was stable.

Most of the sulfur containing methionine metabolites were accounted for in these systems. In a given experiment the specific radioactivities were 75.9 cpm/pmol (free AdoHcy) and 50.1 cpm/pmol (bound AdoHcy) when determined with the cation exchange column and the corresponding values were 76.9 cpm/pmol and 52.2 cpm/pmol in the reversed-phase system. Thus the specific activities were independent on the chromatographic system used. Furthermore, essentially no radioactivity eluted close to the AdoHcy peak in either system. Thus, there is no interference from base-line radioactivity.

The results reported above strongly indicate that the differential labelling of free and protein-bound AdoHcy is not an artifact. It is concluded that protein-bound and free AdoHcy are not in isotope equilibrium, and therefore represent kinetically distinct AdoHcy pools.

DISCUSSION

This paper describes a method for the determination of AdoHcy bound to proteins. Protein-bound AdoHcy is separated from the free fraction by precipitation of proteins with ammonium sulfate followed by retention of the proteins on nitrocellulose filters. Somewhat unexpectedly, we observed that AdoHcy in solution became associated with the proteins during or after tissue processing. This could be prevented by homogenization in the presence of adenosine and AdoMet, followed by extensive dilution of the homogenate. However, it is conceivable that protein-bound AdoHcy may be underestimated by this method, and that a fraction of loosely bound AdoHcy may dissociate and thereby escape detection.

We here report, for the first time, that a large fraction of AdoHcy in liver forms a stable complex with protein *in vivo*. The possibility that this protein binding of AdoHcy is an artifact related to association of free AdoHcy with proteins during tissue processing is unlikely. This statement is based on the following observations.

a) Interference from AdoHcy added to the homogenization medium was progressively decreased upon dilution, but a portion of the endogenous AdoHcy complexed with proteins was independent on dilution (Fig. 1).

b) A pronounced elevation of total AdoHcy content of hepatocytes was associated with only a two fold increase in protein-bound AdoHcy, suggesting that the AdoHcy binding sites are saturable (Figs. 3A,B). This contrasts to the unspecific binding of AdoHcy which increases several fold in the presence of high concentration of AdoHcy (Table I).

c) Differential labelling of protein-bound AdoHcy and free AdoHcy could be demonstrated in experiments presented in figures 4, 5 and 6. This finding is not compatible with artificial formation of the protein-AdoHcy complex from free AdoHcy in solution during tissue processing.

The specific activity of bound AdoHcy (SA_b) is determined from the equation

$$SA_b = \frac{RA_b}{M_b} \qquad (1)$$

and the specific activity of free AdoHcy (SA_f) from the equation

$$SA_f = \frac{RA_t - RA_b}{M_t - M_b} \qquad (2)$$

where RA_b and RA_t are radioactivity associated with bound and total fraction respectively, and M_t the amount of total AdoHcy and M_b the amount of bound AdoHcy.

It is important to note that quantitation of AdoHcy and determination of radioactivity is done in a single (and reproducible) operation (collection of HPLC peak), and partly loss of AdoHcy without corresponding loss of radioactivity associated with this fraction is unlikely. Two types of error of analysis may affect the results. AdoHcy in one compartment is transferred to the other compartment during tissue processing, i.e. free AdoHcy is associated with protein or protein-bound AdoHcy is dissociated into the free fraction. Alternatively, a portion of AdoHcy in one or both fractions is lost prior to analysis by HPLC.

The first type of experimental error implies that SA_b or SA_f approaches the specific activity of total AdoHcy (SA_t), and the differential labelling of free and bound AdoHcy (Figs. 5,6) would partly be obscured. Besides, the quantitative relations between these AdoHcy pools (Figs 2, 3 and 4) should also be distorted. The second type of error, in principle, should not affect SA_f or SA_b since a parallel loss of AdoHcy and the associated radioactivity would occur. Such an error would result in a false relation between M_f and M_b (Figs 2, 3 and 4). Thus, the major conclusion in the present paper that different AdoHcy pools exist, cannot be attributed to experimental errors. However, such errors may lead to an underestimation of the difference in the kinetic characteristics of these pools.