Homocysteine in Tissues of the Mouse and Rat*

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A method for the determination of L-homocysteine (Hcy) in tissues is described, which involves adsorption of adenosine and S-adenosyl-L-homocysteine (AdoHcy) in the tissue extract to dextran-coated charcoal, while leaving Hcy in solution. Sufficient dilution of the tissue homogenates and the presence of a reducing agent during the adsorption step are required to obtain high recovery of Hcy. Hcy is condensed with radioactive adenosine, and labeled AdoHcy is quantified by high performance liquid chromatography on a 3-μm reversed phase column.

The amount of Hcy was determined in several tissues (liver, kidney, brain, heart, lung, and spleen) of mice and rats, and the concentrations of Hcy were in the range 0.5-6 nmol/g, wet weight. Hcy concentration was about 1 μM in mouse plasma. In mice, liver contained the highest amount of Hcy, and kidneys were also rich in Hcy. Similar concentrations were found in rat tissues.

S-Adenosylhomocysteine (AdoHcy) hydrolase (EC 3.3.1.1), the enzyme which is believed to catalyze the only pathway leading to Hcy formation in vertebrates, was nearly completely inactivated in mice injected with the drug combination 9-β-D-arabinofuranosyladenine plus 2′-deoxycoformycin. This treatment induced a massive accumulation of AdoHcy in all tissues (Helland, S., and Ueland, P. M. (1983) Cancer Res. 43, 1847-1850). The amount of Hcy increased severalfold in kidney, whereas no change was observed in liver, heart, brain, lung, and spleen.

Hcy is formed from the endogenous transmethylase inhibitor, AdoHcy. The reaction is catalyzed by the enzyme AdoHcy hydrolase (EC 3.3.1.1) (1, 2) and represents the only known pathway leading to Hcy in vertebrates (1). Hydrolysis of AdoHcy catalyzed by this enzyme is a reversible reaction, which favors synthesis of AdoHcy from adenosine and Hcy. These two metabolites are continuously trapped in the intact cell, and the enzymatic reaction is, therefore, directed toward hydrolysis of AdoHcy (2). Adenosine is either phosphorylated to AMP or deaminated to inosine, while Hcy is metabolized to cystathionine or remethylated to methionine. The latter reaction is catalyzed by two enzymes, one of which is widely distributed in mammalian tissues and requires 5′-methyltetrahydrofolate as methyl donor (2, 3). These metabolic reactions are depicted in Fig. 1.

Interest in the role of Hcy in cellular functions has been stimulated by the discovery that the genetically determined disease, homocystinuria, is associated with a defect in the conversion of Hcy to cystathionine (4). Some patients afflicted with this disease suffer from thromboembolism, and Hcy is a possible atherogenic agent (5). Furthermore, numerous malignant cell lines do not grow on Hcy in the absence of methionine, whereas for some non-transformed cells, methionine can be replaced by Hcy (6, 7). Finally, Hcy induces cellular build-up of AdoHcy, which may in turn inhibit AdoMet-dependent transmethylation reactions (1, 2, 8, 9).

No data exist on the amount of Hcy in tissues under physiological conditions. There are several reports concluding that the Hcy content in animal and human tissues is below the detection limit (10-14). For example, no Hcy was detected in extracts from erythrocytes (10), hepatocytes, and lymphoma cells (12). Small amounts of Hcy (in the micromolar range) have been demonstrated in human plasma (15, 16). A sensitive radioenzymatic method for the measurement of Hcy has recently been developed by Kredich et al. (17) who showed that the plasma level of Hcy was elevated in patients with acute lymphatic leukemia. The amount of Hcy in plasma was reduced following treatment of these patients with the adenosine deaminase inhibitor, dCF (18).

The present article describes a sensitive method for the determination of Hcy in tissues. With this method we have measured the amount of Hcy in several tissues of mice and rats. The method allows the determination of Hcy in tissues containing high levels of AdoHcy resulting from treatment of the animals with an inactivator of AdoHcy hydrolase.

EXPERIMENTAL PROCEDURES

Materials—L-Homocysteine thiolactone, DL-homocysteine, L-homocystine, adenosine, ara-A, AdoHcy, DL-dithiothreitol, and DTE were purchased from Sigma. Charcoal (Norit) was from Norsk Medisinaldepot, Oslo, Norway, and dextran, grade C (clinical grade) from Mann (8-14C)Adenosine (0.59 Ci/mmol) and [2,5,8-3H]adenosine (45 Ci/mmol) were obtained from the Radiochemical Centre, Amersham. dCF was a gift from Parke-Davis. AdoHcy hydrolase (0.8 unit/mg) was purified to apparent homogeneity according to a published procedure (19). ODS Hypersil (3 μm) was obtained from Shandon Southern Ltd., Cheshire, UK. Columns for reversed phase liquid chromatography (3-μm Hypersil, 0.5 × 10 cm) were slurry packed at 9000 psi, using a Shandon column packer. The columns were equipped with a guard column (0.5 × 2.5 cm) which was dry-packed with 40-μm Pallguard LC-18 from Supelco, Inc., Bellefonte, PA.

Treatment of Animals—Mice were given repetitive intraperitoneal injections with the drug combination ara-A plus dCF. The first injection was 50 mg of ara-A and 0.16 mg of dCF/kg, followed by ara-A (25 mg/kg) plus dCF (0.16 mg/kg) each hour. The drugs were dissolved in 0.9% sodium chloride. The animals were killed 1 h after the last injection.
Isolation of Organs—Two procedures were used. 1) The animals were killed by decapitation, and the liver, kidney, brain, heart, lung, and spleen were immediately removed and placed in liquid nitrogen. The liver was placed in liquid nitrogen within 15 s, the brain within 25 s, and the other organs within 45 s. 2) The animals were anesthetized with ether, and the abdominal cavity opened to expose the liver, kidney, and spleen. Skin and tissue were removed from the skull bone. The animals were then allowed to breathe fresh air until just before they recovered and were then submerged into liquid nitrogen. The organs were isolated while still frozen and kept at −80°C until use.

Preparation of Tissue Extracts—The tissues were homogenized (1:4–1:32, w/v) in 0.8 N perchloric acid containing 10 mM EDTA at 0°C using a Ultra-Turrax homogenizer. The precipitated proteins were immediately removed by centrifugation, and the perchloric acid neutralized to pH 7.0 by the addition of 1.2 N KHCO₃/1.44 N KOH. After 15 min at 0°C the insoluble potassium perchlorate was removed by centrifugation. The neutralized extracts were immediately analyzed.

Processing of Plasma—Whole blood from mice was supplemented with EDTA and the plasma fraction immediately prepared and deproteinized with perchloric acid (1:4, v/v), which was neutralized to pH 7.0, as described above. The temperature was kept at 0°C.

Determination of L-Homocysteine—Samples (100 μl) of the neutralized tissue extracts (or plasma) were treated with DTE (10 mM) for 5 min at 37°C. The samples were then cooled (0°C), and 25 μl of dextran-coated charcoal in 100 mM potassium phosphate buffer, pH 7.4, containing DTE was added. The final concentrations were 0.5 mg/ml of dextran, 5 mg/ml of Norit, and 10 mM DTE. The extracts were treated with charcoal at 10 min at 0-4°C, and the charcoal was then removed by centrifugation. The following reagents were added to the supernatant (final concentrations in parenthesis): [3H]adenosine (20 μM), dCF (1 μM) and AdoHcy hydrolase (9 × 10⁻³ units/ml). The incubation was routinely carried out for 30 min at 37°C and was stopped by heating (100°C) for 5 min. Samples were analyzed by HPLC.

Reversed Phase Liquid Chromatography—Samples of 90 μl were analyzed on a 5-μm ODS column (10 × 0.5 cm) equipped with a guard column (2.5 × 0.5 cm) and equilibrated with 15 mM potassium acetate, pH 4.5, containing no methanol. Some compounds, including adenosine and AdoHcy were thereby somewhat concentrated on the top of the column and then eluted with a upward concave gradient with methanol from 0.3 to 8%, at a flow rate of 3 ml/min, corresponding to a back pressure of about 4000 p.s.i. The HPLC apparatus consisted of a SpectraPhysics SP 8700 solvent delivery system, a Perkin-Elmer ISS 100 autosampler, and a Kratos model Spectroflow 773 variable wavelength detector monitoring the absorbance at 260 nm. The autosampler was interfaced with a programmable fraction collector, model Fosxy from ISCO, equipped with a peak separator. The AdoHcy and adenosine peaks were collected into separate scintillation vials. The retention times for AdoHcy and adenosine were 7.23 and 9.63 min, respectively.

The column was washed with 10 ml of 80% acetonitrile before the injections. Samples were injected once every 16 min.

Determination of AdoHcy—This was performed by isotopic HPLC analysis of tissue extracts either on a cation exchange column or reversed phase column, as described previously (20).

RESULTS

Theoretical Basis—Trewyn and Kerr (21) reported that various nucleosides, but not Hcy, were adsorbed to charcoal. The assay procedure described in the present report combines this observation with the finding of Kreidich et al. (17) showing that Hcy in perchloric acid extracts from cells and plasma was condensed with radioactive adenosine in the presence of AdoHcy hydrolase.

Adenosine and AdoHcy in the extract were removed by adsorption to dextran-coated charcoal, while leaving Hcy in solution. Hcy was then condensed with radioactive adenosine (20 μM) to form AdoHcy. This reaction was catalyzed by AdoHcy hydrolase added to the neutralized tissue extract.

The AdoHcy hydrolase reaction favors synthesis of AdoHcy, and the equilibrium constant of the reaction (Keq) is about 1 μM.

\[
K_{eq} = \frac{[\text{Ado}] \times [\text{Hcy}]}{[\text{AdoHcy}]} = 1 \text{ μM} \quad (1, 2)
\]

When [Hcy] << [adenosine], the fraction (F) of Hcy converted to AdoHcy is

\[
F = \frac{[\text{Ado}]}{[\text{Ado}] + 1},
\]

F = 95.24% when [Ado] = 20 μM.

Thus, Hcy is almost quantitatively converted to AdoHcy.

Extraction of Hcy from Tissues—The amount of Hcy extracted from tissues as well as the recovery of exogenous Hcy added to the extraction medium (perchloric acid) were critically dependent on the amount of tissue homogenized per ml of medium. When the amount of tissue per ml was reduced, (from 1:4 to 1:16, w/v), the recovery of exogenous Hcy approached 100%, and at this point linearity of endogenous Hcy extracted versus the amount of tissue was obtained (Fig. 2). Essentially the same results were obtained with mouse liver (Fig. 2) and several other tissues (listed in Table 1) from mouse and rat (data not shown). In the light of these data, tissues were routinely homogenized at a concentration of 1:16, w/v.

Inclusion of DTE in the homogenization medium did not prevent trapping of exogenous or endogenous Hcy in the protein pellet (data not shown).

Attempts were made to extract Hcy from the neutralized pellet by incubation with 5 mM DTE for up to 40 min. Less than 20% of exogenous Hcy trapped in the pellet was recovered (data not shown).

Optimization of the Assay Procedure—Treatment of the neutralized extract with a reducing agent (DTE) and the presence of reducing agent in the charcoal suspension (see under "Experimental Procedures") were required to obtain high recovery (>50%) of endogenous and exogenous Hcy (data not shown). Furthermore, homocysteine was not assayed with the present method in the absence of DTE during these steps. These findings could be explained by adsorption of homocysteine, but not Hcy, to dextran-coated charcoal.

DTE was preferred as a reducing agent, because in the presence of dithiothreitol or 2-mercaptoethanol, radioactive side product(s) of the AdoHcy hydrolase reaction (2, 22-24) were formed which interfered with the determination of radioactive AdoHcy (data not shown). Furthermore, homocysteine was not assayed with the present method in the absence of DTE during these steps.

These findings could be explained by adsorption of homocysteine, but not Hcy, to dextran-coated charcoal.

The theoretical basis is as follows:

\[
\text{Hcy} + \text{Ado} \rightarrow \text{AdoHcy} \rightarrow \text{AdoMet} + \text{Met}
\]

Adenosine and AdoHcy (100 μM of each) were added to mouse liver extract, and more than 90% of these compounds were removed after 1 min of treatment with charcoal. Neither

Fig. 1. Enzymes involved in the metabolism of L-homocysteine. 1, AdoHcy hydrolase; 2, S-methyltetrahydrofolate-L-homocysteine methyltransferase; 3, betaine-L-homocysteine S-methyltransferase; 4, cystathionine-β-synthase; 5, adenosine kinase; 6, adenosine deaminase; 7, ATP-L-methionine S-adenosyltransferase; 8, AdoMet-dependent methyltransferase.
Homocysteine in Tissues

The concentration of Hcy in mouse and rat tissues frozen in vivo and mouse tissues frozen after death of the animal

Table I

| Species | Isolation of organs | Liver | Kidney | Brain | Heart | Lung | Spleen |
|---------|---------------------|-------|--------|-------|-------|------|--------|
| Mouse   |                     |       |        |       |       |      |        |
|         |                     | 3.63 ± 0.89 | 1.29 ± 0.11 | 0.89 ± 0.09 | 1.12 ± 0.10 | 1.02 ± 0.12 | 0.89 ± 0.09 |
| Rat     |                     | 3.79 ± 0.55 | 1.21 ± 0.12 | 0.76 ± 0.07 | 0.97 ± 0.08 | 1.13 ± 0.08 | 1.65 ± 0.07 |

*Mean of 9 determinations ± S.E.
* Organs were frozen in vivo using liquid nitrogen, as described in the text.
* Organs were frozen after death of the animal.

FIG. 2. Recovery of exogenous Hcy and extraction of endogenous Hcy from mouse liver at various dilutions of the extract. Hcy (final concentration of 2 μM) was added to the perchloric acid prior to homogenization of mouse liver, which was homogenized at concentrations indicated on the abscissa. The recovery of exogenous Hcy was determined as percent of the amount of Hcy added to the extract (C). In a parallel run, mouse liver was homogenized at various dilutions in perchloric acid containing no Hcy, and the amount of endogenous Hcy extracted was determined at various dilutions (O, □). The data are presented both as total amount of Hcy extracted (□) and as the amount of Hcy extracted per g of tissue (C). Experimental details are given in the text.

FIG. 3. Standard curve for the determination of Hcy. The graph shows the amount of radioactive [14C]adenosine incorporated into [14C]AdoHcy as a function of the amount of Hcy present in neutralized perchloric acid extract. See under "Experimental Procedures" for details.
The present report describes a method for the determination of homocysteine in tissues. This method required the use of specific enzyme inhibitors to prevent the formation of homocysteine deaminase from Aspergillus oryzae (17). Inosylhomocysteine is a substrate for AdoHcy hydrolase, albeit less effective than AdoHcy (1, 2, 28). It is, therefore, possible that high concentrations of inosylhomocysteine derived from AdoHcy accumulate in response to physiological inhibitors of AdoHcy hydrolase, like ara-A (1, 2), may serve as a Hcy donor. Treatment of tissue extract with charcoal completely removes AdoHcy. Furthermore, this step in the analytical procedure also removes adenosine, ara-A, and other nucleosides serving as inhibitors of AdoHcy hydrolase. This statement is documented by the results presented in Fig. 4.

A substantial fraction of Hcy was bound to plasma proteins, albumin, and red blood cell membranes precipitated in strong acid (14, 27). In the present work, Hcy was extracted with perchloric acid. The recovery of both exogenous Hcy added to the extract and endogenous Hcy was critically dependent on the amount of tissue extracted per ml of acid (Fig. 2). Trapping of Hcy in the protein pellet could not be avoided by immediate centrifugation, showing that Hcy binding to proteins is a rapid process. The mechanism of Hcy binding to proteins at low pH may involve formation of disulfide bonds (14). Furthermore, in acid solutions Hcy is cyclized to form homocysteine thiolactone, which readily acylates proteins (27). Our data (Fig. 2) show that the association of Hcy with the protein pellet was less pronounced when the tissue was homogenized at high dilution (Fig. 2). Thus, sufficient dilution was required to avoid artificially low values for the amount of Hcy in tissues. However, Hcy that may be tightly (covalently) bound to proteins in vivo possibly escape detection.

After the death, the amount of adenosine increased drastically, especially in brain, heart, and kidney (28, 29). Post mortem elevation of AdoHcy in several tissues has also been observed (25, 30). The increase in adenosine and AdoHcy could be avoided by freezing the organs in vivo using liquid nitrogen (25). It was conceivable that the AdoHcy accumulation following death of the animal was related to condensation of endogenous Hcy with adenosine formed in response to tissue anoxia. However, there was no statistically significant difference between the amount of Hcy in tissues frozen in vivo or isolated and frozen after the death (Table I). This finding argues against the possibility that the post mortem increase in AdoHcy (25, 30) is related to reversal of the AdoHcy hydrolase reaction. The increase in the amount of AdoHcy and related compounds may have other explanations, as indicated by the finding of a moderate elevation of the AdoMet content immediately after the death of the animal (25).

Treatment of mice with ara-A plus dCF almost totally inactivated AdoHcy hydrolase (20), the enzyme which is believed to catalyze the only pathway leading to Hcy in vertebrates (1). Therefore, we expected that ara-A plus dCF would prevent trapping of tetrahydrofolate as 5'-methyltetrahydrofolate (31).

2) Hcy in tissues is formed exclusively from AdoHcy, and
the small residual AdoHcy hydrolase activity (20, 32, 33) may account for the amount of Hcy in tissues. It has previously been demonstrated that AdoHcy hydrolase may be profoundly inhibited before accumulation of AdoHcy occurs (20), suggesting that the catalytic activity of this enzyme exceeds the metabolic demand.

3) Inactivation of AdoHcy hydrolase inhibits formation of Hcy, but the rate of the enzymatic reactions consuming Hcy (see Fig. 1) is reduced to the same extent. Thus, the turnover of Hcy is reduced, but the amount of Hcy in tissues remains essentially unaltered.

4) Cellular compartments of Hcy exist. One metabolically stable compartment may account for the main portion of Hcy in the cell, whereas a minor pool shows rapid turnover and is reduced following inactivation of AdoHcy hydrolase. Compartmentalization of Hcy has been suggested as an explanation of the finding that AdoHcy was formed from radioactive adenosine in the liver, even in the absence of exogenous Hcy (34, 35). Sequestration of Hcy through interaction with AdoHcy hydrolase should be considered in the light of the fact that tissues containing relatively large amounts of Hcy, like liver and kidney (Table 1), are rich in AdoHcy hydrolase (2, 20). We have recently reported that the other product of the AdoHcy hydrolase reaction, adenosine, forms a stable complex with AdoHcy hydrolase in intact rat hepatocytes. This complex accounts for the main part of intracellular adenosine in these cells (36).

The increase in the amount of Hcy in kidneys of mice following treatment with ara-A plus dCF (Fig. 4) is remarkable. Studies with other nucleoside analogues inactivating or inhibiting AdoHcy hydrolase (2) are required to determine whether the increase in the amount of Hcy results from inhibition of AdoHcy hydrolysis. The possibility that this metabolic effect of ara-A plus dCF is related to interference with AdoHcy metabolism is likely because a relation between accumulation of AdoHcy and elevation of Hcy content in kidneys was observed (Fig. 4). Furthermore, in some individual animals only a moderate accumulation of AdoHcy in kidneys occurred. In these animals only a slight increase in the amount of Hcy was observed (data not shown). The increase in Hcy content in kidneys may reflect effects of AdoHcy on renal handling of Hcy and possibly related amino acids (37).

It has been stated that Hcy is not normally present in plasma (37). With the present method we have demonstrated the presence of Hcy in plasma of mice. Furthermore, Hcy has recently been determined in human plasma under physiological conditions (15, 16, 18). These findings should stimulate clinical investigations on Hcy in plasma (18) of human under various pathologic conditions and of patients receiving therapy with drugs interfering with one-carbon metabolism (31).

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